Optimisation and characterisation of boar epididymal sperm retrieval, handling and preservation using computer-assisted sperm morphometry: Implications for cryopreservation and *in vitro* fertilisation

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By

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Preface

The research contained in this thesis was completed by Mamonene Angelinah Thema while based in the Discipline of Agricultural Science, Sub-Discipline of Animal Science, School of Agriculture and Science, of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg, South Africa. The research was financially supported by Department of Agriculture.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

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Declaration 2: Publications

Chapter 3

Thema, M.A., Mphaphathi, M.L., Sebopela, M.D., Ledwaba, M.R. and Mkhize, N.R., 2023. Comparison of semen retrieval methods on post-culled boar epididymal sperm motility. Poster presentation to the ARC-DALRRD Conference 2024, Roodeplaat, 12 to 14 February 2024. Presented by MA Thema.

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M.A. Thema, M.L. Mphaphathi, G. van der Horst, L. Maree, M.D Sebopela, M.R. Ledwaba and N.R Mkhize., 2025. Optimising boar epididymal sperm preservation: comparative analysis of slicing float-up vs. flushing and BTS vs. Andromed® extenders on sperm motility and morphometric traits. *American Journal of Animal Sciences*. American Journal of Animal and Veterinary Sciences, 20(4), 328-337. https://doi.org/10.3844/ajavsp.2025.328.337. I designed the experiment, collected the data, analysed the data and wrote the paper. (Published paper)

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This paper is an analysis of sperm morphometry and motility collected from boar epididymal sperm at post-mortem, cryopreserved and evaluated using computer-assisted sperm analysis system. I designed the experiment, collected the data, analysed the data and wrote the paper.

Chapter 7

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List of abbreviations

List of abbreviation	Full of meaning
A	Area
ALH	Amplitude of lateral head
APIE	Animal Production Ethics Committee
AREC	Animal Research Committee
ARC	Agricultural Research Council
BCF	Beat cross frequency
BTS	Beltsville Thawing Solution
CASA	Computer-assisted sperm analyser
DALRRD	Department of Agriculture Land Reform and Rural Development
DoA	Department of Agriculture
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic
FSH	Follicle stimulating hormone
H	Head
ICSI	Intracytoplasmic sperm injection
IVF	In vitro fertilisation
IVM	In vitro maturation
L	Length
LH	Luteinising hormone
LIN	Linearity
LSD	Least significant differences
MED	Medium
M199	Medium 199
mDPBS	modified Dulbecco's Phosphate Buffered Saline
mNCSU	modified North Carolina State University
mTris buffered media	mTBM
Nacl	Sodium Chloride
NCSU	Modified North Carolina State University
PM	Progressive motility
P	Perimeter
PFF	Porcine follicular
RAP	Rapid
STR	Straightness
SD	Standard deviation

TCM 199	Tris culture medium 199
TM	Total motility
VAP	Average pathway velocity
VCL	Curvilinear velocity
VSL	Straight line velocity
W	Width
WOB	Wobble

General abstract

The possibility of epididymal sperm recovery at post-mortem has drawn more attention due to the desire to protect endangered species. This study aimed to characterise boar epididymal sperm morphometry and motility before and post cryopreservation, using computer-assisted sperm analysis. In the first experiment, the effects of sperm retrieval techniques and extenders were compared on the epididymal sperm motility and morphometric traits. Results suggest that the slicing float-up technique is the choice to obtain viable sperm from boar epididymis. In the second experiment, the effects of time elapsed between the animal's death and sperm recovery on sperm motility and morphometry traits of boar cauda epididymides stored at 5 and 18 ℃ were investigated. It was concluded that boar epididymal sperm retrieved from the testes stored at 5 °C until 72 h post-mortem can maintain their total motility and morphometry. However, more sperm with greater motility and normal morphometry were recorded after 2 h post-mortem from the testes stored at 5 °C. In the third experiment, the effects of *in vitro* liquid semen preservation period and temperatures (5 and 18 °C) were investigated on boar epididymal sperm changes in terms of sperm motility and morphometry. It was concluded that storage at 18 °C can maintain boar epididymal sperm total motility and morphometry up to 48 h. In the fourth experiment, the effects of semen holding time at 18 °C on the quality and cryo-tolerance of boar epididymal sperm were investigated. It was concluded that holding time of 3 h at 18 °C improved the cryo-tolerance of boar epididymal sperm motility and morphometry. In the fifth experiment, the *in vitro* fertilisation potential of post-thawed boar epididymal sperm was investigated utilising pig oocytes that have undergone maturation in North Carolina State University 23 (NCSU-23) and modified NCSU-23 media. The modified NCSU-23 media was prepared with double amount of the required glucose concentration excluding sorbitol. The findings revealed that the fertilisation rate was achieved using oocytes matured with both NCSU-23 and modified NCSU-23 media. This study contributes to the advancement of reproductive biotechnology and the conservation of valuable or endangered genetic lines.

Keywords: Epididymis, sperm, retrieval, conservation, holding temperature, handling, cryobiology, protocol, extenders, in vitro, maturation, media, fertilisation.

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Chapter 1: General introduction

1.1 Rationale for research

The epididymis is the organ in charge of sperm transportation, concentration, storage, and maturation (Ali Hassan et al., 2021). Sperm functionally start to mature in the middle of the epididymal corpus and undergo additional membrane changes during ejaculation due to seminal plasma components (Joseph et al., 2010; Caballero et al., 2011). Each of the epididymal segments displays differential expression of genes and maintains distinct luminal ion concentrations, which are essential to regulate the steps of sperm maturation (Foster & Gerton, 2016).

The spread of genital disease through natural service and unexpected animal death is a constant threat in porcine and may lead to the loss of important genetic material (Chatiza et al., 2012). The recovery of epididymal sperm at post-mortem may offer the last chance to preserve genetic material of the endangered breeds which have died unexpectedly or are unable to ejaculate (Bertol, 2016). The time of animal death is unpredictable, and it can happen any time, far away from the lab where the sperm can be recovered. Therefore, the timing between the animal death and the sperm recovery is very important because sperm quality may decline in the epididymides with prolonged storage time (Silva et al., 2019). Previous studies in several species (Ram: Lone et al., 2011; bull: Strand et al 2016) have recommended storage temperature range of 4-5 °C for the transportation of epididymides while maintaining the sperm viability and fertilising capacity for several days to the lab where sperm is recovered (Thuwanut et al., 2013).

The various methods of epididymal sperm recovery have been described in many species, including humans. This includes the methods for retrieving epididymal sperm at post-mortem and in live animals (Ovics et al., 2022). At post-mortem, epididymal sperm can be extracted by either mincing, flushing, or slicing float-up (Podico & Canisso, 2022). However, according to Monaco et al. (2024), the sperm sample retrieved with mincing, may contain blood and cell debris, which may have a negative impact on the quality and freezing ability of the sperm. The flushing process has been considered as the better recovery method due to the results of less contamination in a few species, like cattle (Lamglait, 2014) and equine (Huijsmans et al., 2023). While the slicing float-up method is distinguished by its elevated efficacy, high yield and may possess negligible blood contamination (Podico & Canisso, 2022). Therefore, a quality semen extender should be used during epididymal sperm recovery to maintain sperm viability and prevent bacterial contamination (Baiee et al., 2018). Semen extenders, like Beltsville Thawing Solution (BTS), have been widely used in boar semen studies because of their composition of high concentrations of antibiotics that treat a wide range of infections, lessen, and regulate bacterial contamination during semen preservation (Thema et al., 2022). Furthermore, the use

of Andromed® as a plant-based extender has been proven to increase sperm functional motility while reducing bacterial contamination during semen preservation (El-Keraby et al. 2010).

Cryopreservation of sperm is a valuable reproductive technology for conserving genetic material, gene banking and genetic improvement (Vozaf et al., 2022). However, the use of post-thawed sperm in porcine is limited due to high susceptibility of boar sperm to cold shock, which impairs their functional competence (Daigneault et al., 2014; Thema et al., 2023). Cryopreservation may cause sperm cellular damage, oxidative stress, protein denaturation, shrinkage and irreversible membrane collapse (Khan et al., 2021; Nazari et al., 2021). Therefore, to enhance sperm cryotolerance and ease the transfer of prolonged semen from artificial insemination centres to the laboratory, a 24 h holding time has been added to the boar semen cryopreservation protocol (Wasilewska et al., 2016). Casas & Althouse (2013) reported holding time to shield boar sperm against cold shock by preserving the plasma membrane's lipid architecture. Studies by Tomás et al. (2014) and Torres et al. (2019) have recorded holding boar semen before cryopreservation to allow a better interaction between seminal plasma molecules which improves sperm motility and membrane integrity.

The sperm motility and morphology are the most critical traits for evaluating the capability of males to fertilise (van der horst et al., 2018). The sperm motility and morphology may be affected by the improper handling of samples during holding and freezing processes due to the lack of experience and knowledge of the right protocol or equipment to use (Bustani & Baiee, 2021). Therefore, to acquire reliable results about the effect of epididymides storage, holding time and cryopreservation on boar epididymal sperm morphometry and motility, the approach of computer-assisted sperm analysis (CASA) system has been employed (Yániz et al., 2012; Akhtar et al., 2022). The CASA approach is an objective method, and its use has proven to be accurate, exact, and repeatable (Esteso et al., 2015; Gatimel et al., 2017; van der Horst et al., 2018; Finelli et al., 2021). It has generated radically different analyses and dispersed empirical conclusions (Banaszewska et al., 2015; Maroto-Morales et al., 2016).

Previous studies in various animal species have described the fertilisation success of epididymal sperm (Gill et al., 2016). Limited information exists regarding boar epididymal sperm to fertilise mature oocytes *in vitro*. Due to the inadequacies, *in vitro* maturation and *in vitro* fertilisation (IVF) continue to be inefficient in the embryo production of porcine (Ledwaba et al., 2022). The North Carolina State University 23 (NCSU-23) has been widely used in the porcine *in vitro* embryo production as a maturation and culturing medium (Pyoos et al., 2018). However, improper *in vitro* oocyte maturation, often resulted, due to inadequate cytoplasmic development which limits embryo development and overall IVF success (He et al., 2024). Therefore, modification of NCSU-23 will be useful for the improvement of oocyte maturation and embryo production in porcine.

1.2 Problem statement

The unexpected animal death and spread of genital diseases through natural service is a constant threat in porcine leading to the loss of genetic material (Bertol, 2016). Keeping boar herds can be costly and may pose a risk of inbreeding to the herd (Boersma et al., 2015). Therefore, epididymal sperm recovery provides the opportunity to obtain and utilise top males' genetic material even after their death (Vernocchi et al., 2014). However, preservation of sperm is sometimes hampered by the lack of technical understanding regarding handling, transportation, and storage of the epididymides following the sudden death of an animal (Engdawork et al. 2024). Limited studies have addressed how long the boar sperm can remain viable in the epididymides during transportation until the sperm is recovered, cryopreserved and fertilised (Cunha et al., 2019). Contamination of the sperm often occurs in the farms, artificial insemination centres and during semen collection, processing and storage (Prieto-Martínez et al., 2014). Few studies have focused on the *in vitro* fertilising potential of post-thawed sperm recovered from refrigerated epididymis. While little is known about the effective retrieval methods and semen extender for boar epididymal sperm during recovery. Furthermore, following sperm recovery, little is known about the changes in boar epididymal sperm quality during liquid storage at 5 and 18 °C for up to 48 h.

Limited studies have been done on the effect of holding time on boar epididymal sperm motility and morphometry following cryopreservation. Whereas, the cryopreservation process, induces oxidative stress which affects sperm morphometry traits with a high proportion of sperm acrosome being frequently damaged (Sun et al., 2021). Vernocchi et al. (2014) recorded the sperm head to have decreased following cryopreservation than in the fresh semen. While the morphometric measurements of the head and tail of the sperm, decreased within the first 24 h of storage (Szablicka et al. 2022). The use of cryopreserved sperm is limited due to the subjectivity of the boar sperm to cryodamage which affects sperm morphometry and increases the percentage of acrosomal damage (Aydin et al., 2016). These effects include severe membrane damage, morphological changes, and morphometric alterations (Tomás et al., 2014). The dimensions of the sperm head, its length, width, area, and perimeter and tail length gradually decrease during cryopreservation as a result of cold shock (Akhtar et al., 2022).

The short lifespan of the boar sperm after thawing continues to limit the use of frozen-thawed semen in commercial farms, primarily because of the changes in sperm morphology and its functional characteristics which may affect fertilisation rate (Thema et al., 2023). Abnormalities in the sperm head have been linked to early embryonic death, decreased fertility, embryo quality, and a decreased ability to connect to the ovum (Yániz et al., 2015). Additionally, it was also reported that the increased number of acrosome-reacted sperm occurring during cryopreservation may cause changes in sperm DNA integrity (Ahmad et al., 2010). James et al. (2020) reported that boar epididymal sperm have been shown to contain a high level of abnormalities due to the unique configuration of the cell membrane. Due to

technical discrepancies among and between technicians, subjective morphological assessment of the boar sperm continues to be troublesome (Finelli et al., 2021). Wide variations in sperm morphology have resulted from subjective estimations, which have affected the utility of such morphology evaluation as a quality predictor of semen for reproduction within and across laboratories and technicians (Yániz et al., 2015). To lessen the subjective way of analysing sperm morphometry, the CASA system approach was developed (van der Horst et al., 2021). There are limited reports regarding the characterisation of boar epididymal sperm motility and morphometry using a CASA system at pre-freeze and post-thaw.

Despite sufficient motility or normal morphometry, the ability of post-thawed sperm to fertilise during IVF is infamously poor for unclear reasons in pigs. Oocyte maturation is a key determinant of developmental competence and IVF success (Lee et al., 2015). Several IVF protocol elements, including the media's composition, have a negative impact on the fertilisation outcomes in pig embryo production (Romar et al., 2016). These challenges reflect on the oocyte cytoplasmic maturation defects, due to an incomplete maturation compared to *in vivo* (Pyoos et al., 2018). Several attempts have been made to improve the IVF protocol in the past few decades. Despite extensive research on improving porcine IVF, the rates of normal fertilisation and embryonic development to the blastocyst stage have been extremely low (Ledwaba et al., 2022). Therefore, improvement of the methods for semen cryopreservation and enhancing the quality of frozen-thawed sperm is imperative to define the changes that occur in sperm/oocytes during the process of liquid preservation/cryopreservation.

1.3 Justification

The sperm in the epididymis can survive after death, and if recovered, they can either be preserved or immediately used in reproductive technologies to create new generations (Strand et al., 2016). The gametes can be used in animal biotechnologies such as artificial insemination and IVF after cryopreservation (Bertol et al., 2013). The determination of a reliable protocol to allow the conservation of boar epididymal sperm is important to enable the salvage of these gametes from genetically important individuals that may die unexpectedly. The death of an animal is unpredictable, and a processing laboratory might be distant from the place of animal death (Engdawork et al. 2024). Therefore, to determine the storage length and temperature at which a boar testis can be stored, while maintaining acceptable sperm quality following death will be useful for conservation of endangered species (Wachida et al., 2019). Additionally, determining the effective technique for boar epididymal sperm recovery, will assist in retrieving sperm of high yield and good quality.

During sperm recovery semen extenders are used as the medium for maintaining metabolic processes of the sperm, while controlling bacterial contamination (Malik et al., 2018). The BTS extender is commonly used in pig semen conservation. While Andromed® extender is a plant-based extender and

can be used as an alternative to milk or egg yolk-based extenders for semen cryopreservation in species like bovine (Kumar et al., 2015; Miguel-Jimenez et al., 2020).

Cryopreservation technique allows sperm to be distributed globally and reaches areas where liquid preserved sperm is impractical (Ugur et al., 2019). Boar semen is frequently transported to different locations in liquid form at 15 °C for cryopreservation (Kim et al., 2011). Holding time has been used to improve cryopreserved sperm in other species, as holding time allows for a prolonged interaction between sperm and seminal plasma components (Torres et al., 2019). Therefore, research into the physiological alterations that sperm undergo throughout holding time and cryopreservation may lead to the development of improved techniques for monitoring sperm viability and fertility. To my knowledge, studies examining the morphometry of sperm in particular regions of the boar epididymis during cooling and freezing are lacking. Therefore, the dimensions of sperm head, its area, and the percentage of acrosome and other factors must be precisely measured to determine the potential of epididymal sperm to fertilise (Esteso et al., 2015).

The most recent generation of CASA system fully integrates quantitative modules for measuring sperm morphometry, DNA fragmentation, vitality, acrosome intactness, and hypoosmotic swelling regularly (Naranjo et al., 2022). The CASA system has allowed for more objective, faster analysis and has been used successfully to determine the relationship between sperm shape and fertilisation ability (Yániz et al., 2015). The CASA system is user-friendly and capable of producing enormous amounts of data quickly (van der Horst et al., 2018; Naranjo et al., 2022). It was documented that there might be a 40-60% variation in sperm morphometric and genotypic outcomes when using CASA, unlike the subjective method (Maroto-Morales et al., 2016). Therefore, this study will provide current scientific illustration and applications of CASA, as well as its ability to evaluate epididymal sperm morphometry and motility in relation to fertility (Maroto-Morales et al., 2016; Soler & Cooper, 2016).

Evaluation of epididymal sperm morphometry and motility at post-thawed using CASA will be beneficial for successful IVF and artificial insemination (Jiang et al., 2013; Esteso et al., 2015; Tanga et al., 2021). Maintaining boar sperm functional status after thawing is critical for successful IVF and subsequent to embryo production (Martecikova et al., 2010). Although there have been successful *in vitro* produced embryos using ejaculated sperm, there is still limited information on the successful use of epididymal sperm recovered from epididymides at post-mortem. Nonetheless, many aspects of the IVF protocol remain unknown because there have been insufficient extensive studies evaluating the efficacy of media particularly intended for IVF in porcine using post-thawed sperm of epididymis. To address these limitations, modifications to the standard NCSU-23 media have been proposed to improve IVF protocols. Modification of the NCSU-23 maturation media will assist in enhancing *in vitro* fertilisation success in porcine. Therefore, improvement of IVF protocols is necessary to produce high-quality oocytes and improve IVF success consistently (Lin et al., 2014).

1.4 Aim

This study aimed to characterises retrieved boar epididymal sperm morphometry and motility before and post cryopreservation, using computer-assisted sperm morphometry analysis.

1.5 The specific objectives were:

a. To evaluate the effect of sperm retrieval method and semen® extenders on boar epididymal sperm motility and morphometric traits.
b. To investigate the effects of the time elapse between animal death and sperm retrieval on the sperm motility and morphometry traits of boar cauda epididymides stored between 5 and 18 °C.
c. To evaluate the effect of boar epididymal sperm storage duration and storage temperature on morphometric and motility changes.
d. To determine the effects of pre-freezing temperature and holding time on post-thaw motility and morphometry of cryopreserved boar epididymal sperm.
e. To assess in vitro fertilisation ability of pre-frozen and post-thawed boar epididymal sperm using oocytes matured in two media: North Carolina State University 23 (NCSU-23) and modified NCSU-23 (mNCSU-23).

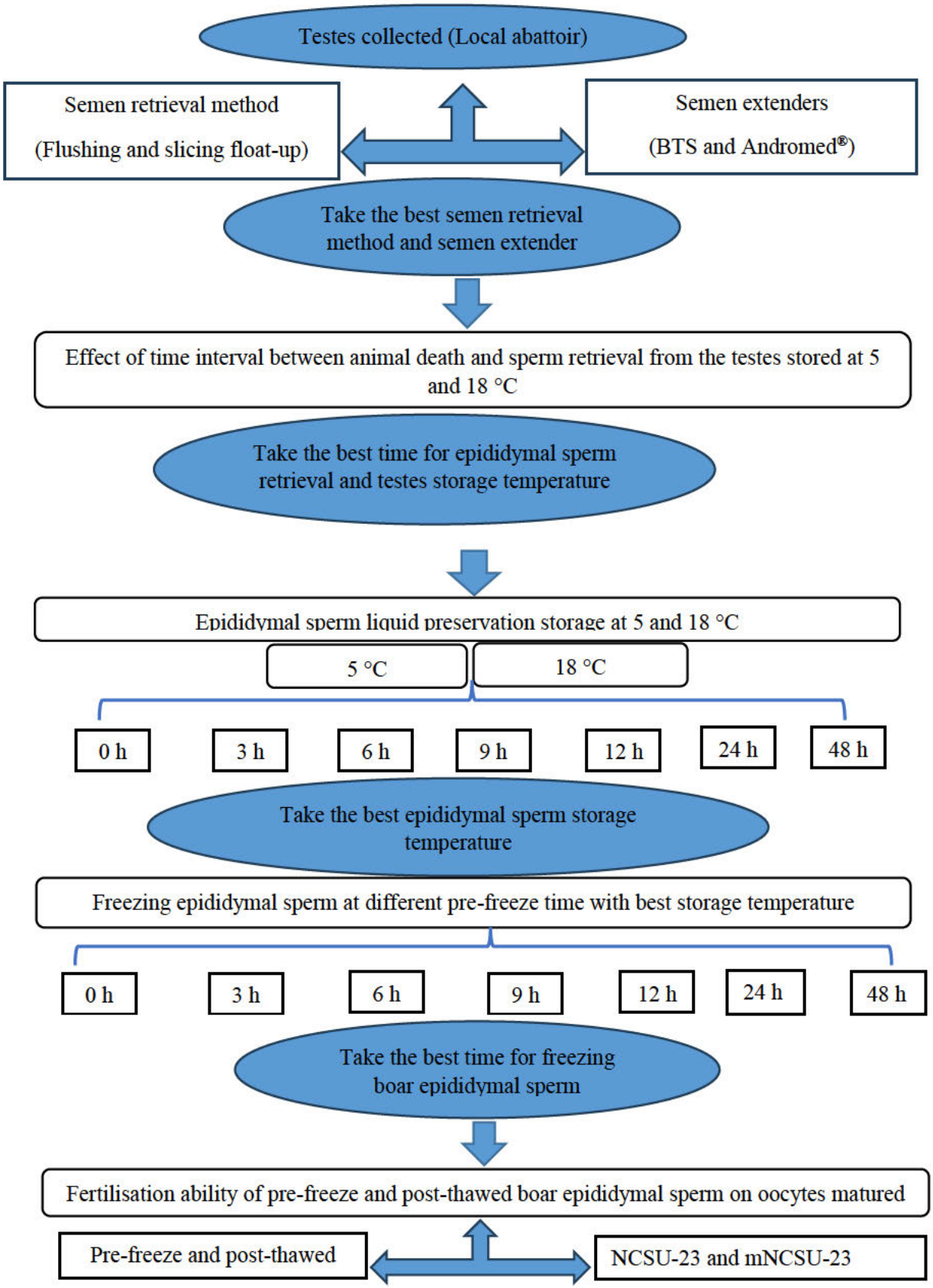

Figure 1.1: Study outline

1.6 Hypotheses

a. Sperm retrieval method (slicing float-up and flushing method) as well as the semen extenders (BTS and Andromed®) will affect the viability and quality of boar epididymal sperm motility and morphometric traits.
b. Storage of cauda epididymides for 72 h at 5 and 18 °C after boar death, significantly affect the survival, motility and morphometric characteristics of the sperm recovered.
c. Storage of boar epididymal sperm at 5 and 18 °C for different durations significantly influences sperm morphometry and motility characteristics.
d. Holding boar epididymal sperm at 18 °C for different pre-freeze durations (0, 3, 6, 9, 12, 24 and 48 h) significantly affects cryotolerance and post-thaw sperm motility and morphometric characteristics.
e. Pre-freeze and post-thawed boar epididymal sperm significantly influences in *vitro* fertilisation success when oocytes are matured in NCSU-23 and mNCSU-23 media.

1.7 References

Ahmad, L., Jalali, S., Shami, S.A., Akram, Z., Batool, S. and Kalsoom, O., 2010. Effects of cryopreservation on sperm DNA integrity in normospermic and four categories of infertile males. *Systems Biology in Reproductive Medicine*, *56*(1), pp.74-83. https://doi.org/10.3109/19396360903428352

Akhtar, M.F., Ma, Q., Li, Y., Chai, W., Zhang, Z., Li, L. and Wang, C., 2022. Effect of sperm cryopreservation in farm animals using nanotechnology. *Animals*, *12*(17), p.2277. https://doi.org/10.3390/ani12172277

Ali Hassan, H., Domain, G., Luvoni, G.C., Chaaya, R., Van Soom, A. and Wydooghe, E., 2021. Canine and feline epididymal semen—a plentiful source of gametes. *Animals*, *11*(10), p.2961. https://doi.org/10.3390/ani11102961Aydin, H., Sultana, A., Li, S., Thavalingam, A. and Lee, J.E., 2016. Molecular architecture of the human sperm IZUMO1 and egg JUNO fertilization complex. *Nature*, *534*(7608), pp.562-565. https://doi.org/10.1038/nature18595

Baiee, F., Haron, W., Yusoff, R., Ariff, O., Yimer, N., Hammadi, S., Ahmedeltayeb, T. and Kaka, A., 2018. Modification of electro-ejaculation technique to minimise discomfort during semen collection in bulls. *Pakistan Journal of Zoology*, *50*(1).

Banaszewska, D., Andraszek, K., Czubaszek, M. and Biesiada–Drzazga, B., 2015. The effect of selected staining techniques on bull sperm morphometry. *Animal Reproduction Science*, *159*, pp.17-24. https://doi.org/10.1016/j.anireprosci.2015.06.019

Bertol, M.A.F., Weiss, R.R., Thomaz-Soccol, V., Kozicki, L.E., Fujita, A.S., Abreu, R.A.D. and Green, K.T., 2013. Viability of bull spermatozoa collected from the epididymis stored at 18-20 °C. *Brazilian Archives of Biology and Technology*, *56*, pp.777-783. https://doi.org/10.1590/S1516-89132013000500008

Bertol, M.A., 2016. Cryopreservation of epididymal sperm. *Cryopreservation in eukaryotes. (Eds F Marco-Jiménez, H Akdemir)*, IntechOpen. London. pp.121-135. https://dx.doi.org/10.5772/65010Boersma, A., Olszanska, O., Walter, I. and Rülicke, T., 2015. Microsurgical and percutaneous epididymal sperm aspiration for sperm collection from live mice. *Journal of the American Association for Laboratory Animal Science*, *54*(5), pp.471-477.

Bustani, G.S. and Baiee, F.H., 2021. Semen extenders: An evaluative overview of preservative mechanisms of semen and semen extenders. *Veterinary World*, *14*(5), p.1220. https://doi.org/10.14202/vetworld.2021.1220-1233

Caballero, J., Frenette, G. and Sullivan, R., 2011. Post testicular sperm maturational changes in the bull: important role of the epididymosomes and prostasomes. *Veterinary Medicine International*, *2011*(1), p.757194. https://doi.org/10.4061/2011/757194

Casas, I. and Althouse, G.C., 2013. The protective effect of a 17 °C holding time on boar sperm plasma membrane fluidity after exposure to 5 °C. *Cryobiology*, *66*(1), pp.69-75. https://doi.org/10.1016/j.cryobiol.2012.11.006

Chatiza, F.P., Bartels, P., Nedambale, T.L. and Wagenaar, G.M., 2012. Computer assisted sperm analysis of motility patterns of postthawed epididymal spermatozoa of springbok (Antidorcas marsupialis), impala (Aepyceros melampus), and blesbok (Damaliscus dorcus phillipsi) incubated under conditions supporting domestic cattle in vitro fertilization. *Theriogenology*, *78*(2), pp.402-414. https://doi.org/10.1016/j.theriogenology.2012.02.020

Cunha, A.T., Carvalho, J.O., Guimarães, A.L., Leme, L.O., Caixeta, F.M., Viana, J.H. and Dode, M.A., 2019. Bovine epididymal spermatozoa treatment for in vitro fertilization: heparin accelerates fertilization and enables a reduction in coincubation time. *PLoS One*, *14*(1), p.e0209692. https://doi.org/10.1371/journal.pone.0209692

Daigneault, B.W., McNamara, K.A., Purdy, P.H., Krisher, R.L., Knox, R.V. and Miller, D.J., 2014. Novel and traditional traits of frozen-thawed porcine sperm related to in vitro fertilization success. *Theriogenology*, *82*(2), pp.266-273. https://doi.org/10.1016/j.theriogenology.2014.04.006

El-Keraby, F.E., Osman, K., Ganah, H.B. and El-Siefy, E.M., 2010. Soymilk-based extender for cryopreservation of bovine semen. *Journal of Animal and Poultry Production*, *1*(2), pp.61-69. https://dx.doi.org/10.21608/jappmu.2010.86093

Engdawork, A., Belayhun, T. and Aseged, T., 2024. The role of reproductive technologies and cryopreservation of genetic materials in the conservation of animal genetic resources, A review. *Ecological Genetics and Genomics*, p.100250. https://doi.org/10.1016/j.egg.2024.100250

Esteso, M.C., Rodríguez, E., Toledano-Díaz, A., Castaño, C., Pradiee, J., López-Sebastián, A. and Santiago-Moreno, J., 2015. Descriptive analysis of sperm head morphometry in Iberian ibex (Capra pyrenaica): optimum sampling procedure and staining methods using Sperm-Class Analyzer®. *Animal Reproduction Science*, *155*, pp.42-49. https://doi.org/10.1016/j.anireprosci.2015.01.014

Finelli, R., Leisegang, K., Tumallapalli, S., Henkel, R. and Agarwal, A., 2021. The validity and reliability of computer-aided semen analyzers in performing semen analysis: a systematic review. *Translational Andrology and Urology*, *10*(7), p.3069. https://doi.org/10.21037/tau-21-276

Foster, J.A. and Gerton, G.L., 2016. The acrosomal matrix. *Sperm Acrosome Biogenesis and Function During Fertilization*, pp.15-33. https://doi.org/10.1007/978-3-319-30567-7

Gatimel, N., Moreau, J., Parinaud, J. and Léandri, R.D., 2017. Sperm morphology: assessment, pathophysiology, clinical relevance, and state of the art in 2017. *Andrology*, *5*(5), pp.845-862. https://doi.org/10.1111/andr.12389

Gill, P.K. and Desai, N., 2016. Testicular sperm can effect early embryo morphokinetics. Fertility and Sterility, 105(2), p.e4. https://doi.org/10.1016/j.fertnstert.2015.12.028

He, L.N., Xu, Q., Lin, J., Liu, Y. and Chen, W., 2024. Predictive strategies for oocyte maturation in IVF cycles: from single indicators to integrated models. *Middle East Fertility Society Journal*, *29*(1), p.33. https://doi.org/10.1186/s43043-024-00193-7

Huijsmans, T.E., Hassan, H.A., Smits, K. and Van Soom, A., 2023. Post-mortem collection of gametes for the conservation of endangered mammals: A review of the current state-of-the-art. *Animals*, *13*(8), p.1360. https://doi.org/10.3390/ani13081360

James, E.R., Carrell, D.T., Aston, K.I., Jenkins, T.G., Yeste, M. and Salas-Huetos, A., 2020. The role of the epididymis and the contribution of epididymosomes to mammalian reproduction. *International Journal of Molecular Sciences*, *21*(15), p.5377. https://doi.org/10.3390/ijms21155377

Jiang, Y., Cao, Q., Zhao, X., Li, L., Li, S. and Gao, F., 2013. Percutaneous epididymal sperm aspiration and short time insemination in the treatment of men with obstructive azoospermia. *Journal of Assisted Reproduction and Genetics*, *30*, pp.1175-1179. https://doi.org/10.1007/s10815-013-0075-1

Joseph, A., Shur, B.D., Ko, C., Chambon, P. and Hess, R.A., 2010. Epididymal hypo-osmolality induces abnormal sperm morphology and function in the estrogen receptor alpha knockout mouse. *Biology of Reproduction*, *82*(5), pp.958-967. https://doi.org/10.1095/biolreprod.109.080366

Khan, I.M., Cao, Z., Liu, H., Khan, A., Rahman, S.U., Khan, M.Z., Sathanawongs, A. and Zhang, Y., 2021. Impact of cryopreservation on spermatozoa freeze-thawed traits and relevance OMICS to assess sperm cryo-tolerance in farm animals. *Frontiers in Veterinary Science*, *8*, p.609180. https://doi.org/10.3389/fvets.2021.609180

Kim, S., Lee, Y.J. and Kim, Y.J., 2011. Changes in sperm membrane and ROS following cryopreservation of liquid boar semen stored at 15 °C. *Animal Reproduction Science*, *124*(1-2), pp.118-124. https://doi.org/10.1016/j.anireprosci.2011.01.014

Kumar, P., Kumar, D., Sikka, P. and Singh, P., 2015. Sericin supplementation improves semen freezability of buffalo bulls by minimizing oxidative stress during cryopreservation. *Animal Reproduction Science*, *152*, pp.26-31. https://doi.org/10.1016/j.anireprosci.2014.11.015

Lamglait, B., 2014. Longevity of sperm cells retrieved by post-mortem epididymal aspiration in wild bovids in zoo conditions. *Journal of Zoo and Aquarium Research*, *2*(4), pp.92-100. https://doi.org/10.19227/jzar.v2i4.47

Ledwaba, M.R., Mphaphathi, M.L., Thema, M.A., Pilane, C.M. and Nedambale, T.L., 2022. Investigation of the efficacy of dithiothreitol and glutathione on in vitro fertilization of cryopreserved Large White boar semen. *Animals*, *12*(9), pp.1137. https://doi.org/10.3390/ani12091137

Lee, J., Park, J.I., Im Yun, J., Lee, Y., Yong, H., Lee, S.T., Park, C.K., Hyun, S.H., Lee, G.S. and Lee, E., 2015. Rapamycin treatment during in vitro maturation of oocytes improves embryonic development after parthenogenesis and somatic cell nuclear transfer in pigs. *Journal of Veterinary Science*, *16*(3), pp.373-380. https://doi.org/10.4142/jvs.2015.16.3.373

Lin, Z.L., Li, Y.H., Xu, Y.N., Wang, Q.L., Namgoong, S., Cui, X.S. and Kim, N.H., 2014. Effects of growth differentiation factor 9 and bone morphogenetic protein 15 on the in vitro maturation of porcine oocytes. *Reproduction in Domestic Animals*, *49*(2), pp.219-227. https://doi.org/10.1111/rda.12254

Lone, F.A., Islam, R., Khan, M.Z. and Sofi, K.A., 2011. Effect of transportation temperature on the quality of cauda epididymal spermatozoa of ram. *Animal Reproduction Science*, *123*(1-2), pp.54-59. https://doi.org/10.1016/j.anireprosci.2010.10.012

Malik, A., Jaelani, A., Widaningsih, N., Ni'Mah, G.K. and Sasongko, N., 2018. Effect of different concentration of fish oil in skim milk-egg yolk extenders on post-thawed semen qualities of Kalang swamp buffalo bull. *Asian Pacific Journal of Reproduction*, *7*(3), pp.139-142. https://doi.org/10.4103/2305-0500.233576

Maroto-Morales, A., García-Álvarez, O., Ramón, M., Martínez-Pastor, F., Fernández-Santos, M.R., Soler, A.J. and Garde, J.J., 2016. Current status and potential of morphometric sperm analysis. *Asian Journal of Andrology*, *18*(6), pp.863-870. https://doi.org/10.4103/1008-682X.187581

Martecikova, S., Hulinska, P., Reckova, Z., Pavlik, A., Jeseta, M. and Machatkova, M., 2010. Effect of acrosome reaction progress in frozen-thawed boar spermatozoa on the efficiency of in vitro oocyte fertilization. *Veterinární Medicína*, *55*(9), pp.429-437.

Miguel-Jimenez, S., Del Alamo, M.M.R., Álvarez-Rodríguez, M., Hidalgo, C.O., Peña, A.I., Muiño, R., Rodríguez-Gil, J.E. and Mogas, T., 2020. In vitro assessment of egg yolk-, soya bean lecithin-and liposome-based extenders for cryopreservation of dairy bull semen. *Animal Reproduction Science*, *215*, p.106315. https://doi.org/10.1016/j.anireprosci.2020.106315

Monaco, D., Rota, A., Carbonari, A., Lillo, E., Lacalandra, G.M. and Rizzo, A., 2024. Collection of epididymal semen in the tomcat (Felix catus) by stereomicroscope-aided retrograde flushing (SARF) improves sample quality. *Animal Reproduction Science*, *261*, p.107388. https://doi.org/10.1016/j.anireprosci.2023.107388

Naranjo, R.E., Naydenova, E., Proaño-Bolaños, C., Vizuete, K., Debut, A., Arias, M.T. and Coloma, L.A., 2022. Development of assisted reproductive technologies for the conservation of Atelopus sp.(spumarius complex). *Cryobiology*, *105*, pp.20-31. https://doi.org/ 10.1016/j.cryobiol.2021.12.005

Nazari, H., Ahmadi, E., Hosseini Fahraji, H., Afzali, A. and Davoodian, N., 2021. Cryopreservation and its effects on motility and gene expression patterns and fertilizing potential of bovine epididymal sperm. *Veterinary Medicine and Science*, *7*(1), pp.127-135. https://doi.org/10.1002/vms3.355

Ovics, S.O., Baram, S., Nothman, S., Weiss, A. and Beck-Fruchter, R., 2022. Perimortem and postmortem sperm acquisition: review of clinical data. *Journal of Assisted Reproduction and Genetics*, *39*(4), pp.977-986. https://doi.org/10.1007/s10815-022-02427-x

Prieto-Martínez, N., Bussalleu, E., Garcia-Bonavila, E., Bonet, S. and Yeste, M., 2014. Effects of Enterobacter cloacae on boar sperm quality during liquid storage at 17 °C. *Animal Reproduction Science*, *148*(1-2), pp.72-82. https://doi.org/10.1016/j.anireprosci.2014.05.008

Podico, G. and Canisso, I.F., 2022. Retrograde flushing followed by slicing float-up as an approach to optimize epididymal sperm recovery for the purpose of cryopreservation in equids. *Animals*, *12*(14), p.1802. https://doi.org/10.3390/ani12141802

Pyoos, G.M., Maqhashu, A.M., Scholtz, M.M. and Nedambale, T.L., 2018. The comparison of three media on the in vitro maturation rate of pig oocytes. South African Journal of Animal Science, 48(6). https://doi.org/10.4314/sajas.v48i6.4

Romar, R., Funahashi, H. and Coy, P., 2016. In vitro fertilization in pigs: New molecules and protocols to consider in the forthcoming years. *Theriogenology*, *85*(1), pp.125-134. https://doi.org/10.1016/j.theriogenology.2015.07.017

Silva, H.V.R., Nunes, T.G.P., Ribeiro, L.R., de Freitas, L.A., de Oliveira, M.F., de Assis Neto, A.C., Silva, A.R. and da Silva, L.D.M., 2019. Morphology, morphometry, ultrastructure, and mitochondrial activity of jaguar (Panthera onca) sperm. *Animal Reproduction Science*, *203*, pp.84-93. https://doi.org/10.1016/j.anireprosci.2019.02.011

Soler, C. and Cooper, T.G., 2016. Foreword to sperm morphometrics today and tomorrow special issue in Asian Journal of Andrology. *Asian Journal of Andrology*, *18*(6), pp.815-818. https://doi.org/10.4103/1008-682X.187582

Strand, J., Ragborg, M.M., Pedersen, H.S., Kristensen, T.N., Pertoldi, C. and Callesen, H., 2016. Effects of post-mortem storage conditions of bovine epididymides on sperm characteristics: investigating a tool for preservation of sperm from endangered species. *Conservation Physiology*, *4*(1), p.cow069. https://doi.org/10.1093/conphys/cow069

Thema, M.A., Mphaphathi, M.L., Ledwaba, M.R. and Nedambale, T.L., 2022. Investigation of the efficacy of different semen extenders and in vitro storage period on Windsnyer boar sperm quality equilibrated at 18 °C. *Reproduction in Domestic Animals*, *57*(12), pp.1644-1648. https://doi.org/10.1111/rda.14256

Thema, M.A., Mphaphathi, M.L., Ledwaba, M.R. and Nedambale, T.L., 2023. Sperm cryopreservation in Windsnyer boars; principles, technique, and updated outcomes. Animal Reproduction, 20(3), p.e20220100. https://doi.org/10.1590/1984-3143-AR2022-0100

Thuwanut, P., Srisuwatanasagul, S., Wongbandue, G., Tanpradit, N., Thongpakdee, A., Tongthainan, D., Manee-In, S. and Chatdarong, K., 2013. Sperm quality and the morphology of

cryopreserved testicular tissues recovered post-mortem from diverse wild species. *Cryobiology*, *67*(2), pp.244-247. https://doi.org/10.1016/j.cryobiol.2013.07.002

Sun, W., Jiang, S., Su, J., Zhang, J., Bao, X., Ding, R., Shi, P., Li, S., Wu, C., Zhao, G. and Cao, G., 2020. The effects of cryopreservation on the acrosome structure, enzyme activity, motility, and fertility of bovine, ovine, and goat sperm. *Animal Reproduction*, *17*(4), p.e20200219. https://doi.org/10.1590/1984-3143-ar2020-0219

Szablicka, D., Wysokińska, A., Pawlak, A. and Roman, K., 2022. Morphometry of boar spermatozoa in semen stored at 17 °C—the influence of the staining technique. *Animals*, *12*(15), p.1888. https://doi.org/10.3390/ani12151888

Tomás, C., Gómez-Fernández, J., Gómez-Izquierdo, E. and de Mercado, E., 2014. Effect of the holding time at 15 °C prior to cryopreservation, the thawing rate and the post-thaw incubation temperature on the boar sperm quality after cryopreservation. *Animal Reproduction Science*, *144*(3-4), pp.115-121. https://doi.org/10.1016/j.anireprosci.2013.12.011

Tanga, B.M., Qamar, A.Y., Raza, S., Bang, S., Fang, X., Yoon, K. and Cho, J., 2021. Semen evaluation: Methodological advancements in sperm quality-specific fertility assessment—A review. *Animal Bioscience*, *34*(8), p.1253. https://doi.org/10.5713/ab.21.0072

Torres, M.A., Monteiro, M.S., Passarelli, M.S., Papa, F.O., Dell'Aqua Jr, J.A., Alvarenga, M.A., Martins, S.M. and de Andrade, A.F., 2019. The ideal holding time for boar semen is 24 h at 17 °C prior to short-cryopreservation protocols. *Cryobiology*, *86*, pp.58-64. https://doi.org/10.1016/j.cryobiol.2018.12.004

Ugur, M.R., Saber Abdelrahman, A., Evans, H.C., Gilmore, A.A., Hitit, M., Arifiantini, R.I., Purwantara, B., Kaya, A. and Memili, E., 2019. Advances in cryopreservation of bull sperm. *Frontiers in Veterinary Science*, *6*, p.268. https://doi.org/10.3389/fvets.2019.00268

van der Horst, G., 2021. Status of sperm functionality assessment in wildlife species: from fish to primates. *Animals*, *11*(6), p.1491. https://doi.org/10.3390/ani11061491

van der Horst, G., Maree, L. and du Plessis, S.S., 2018. Current perspectives of CASA applications in diverse mammalian spermatozoa. *Reproduction, Fertility and Development*, *30*(6), pp.875-888. https://doi.org/10.1071/RD17468

Vernocchi, V., Morselli, M.G., Consiglio, A.L., Faustini, M. and Luvoni, G.C., 2014. DNA fragmentation and sperm head morphometry in cat epididymal spermatozoa. Theriogenology, 82(7), pp.982-987. https://doi.org/10.1016/j.theriogenology.2014.07.014

Vozaf, J., Svoradová, A., Baláži, A., Vašíček, J., Olexiková, L., Dujíčková, L., Makarevich, A.V., Jurčík, R., Ďúranová, H. and Chrenek, P., 2022. The cryopreserved sperm traits of various ram

breeds: towards biodiversity conservation. *Animals*, *12*(10), p.1311. https://doi.org/10.3390/ani12101311

Wachida, N., Bassey, U.E. and Dawuda, P.M., 2019. Effect of storage time on the quality of cauda epididymal spermatozoa of West African dwarf (WAD) rams. *Animal Reproduction Science*, *205*, pp.144-149. https://doi.org/10.1016/j.anireprosci.2019.05.001

Wasilewska, K., Zasiadczyk, Ł., Fraser, L., Mogielnicka-Brzozowska, M. and Kordan, W., 2016. The benefits of cooling boar semen in long-term extenders prior to cryopreservation on sperm quality characteristics. *Reproduction in Domestic Animals*, *51*(5), pp.781-788. https://doi.org/10.1111/rda.12751

Yániz, J.L., Vicente-Fiel, S., Capistrós, S., Palacín, I. and Santolaria, P., 2012. Automatic evaluation of ram sperm morphometry. *Theriogenology*, *77*(7), pp.1343-1350. https://doi.org/10.1016/j.theriogenology.2011.10.039

Yániz, J.L., Soler, C. and Santolaria, P., 2015. Computer assisted sperm morphometry in mammals: A review. *Animal Reproduction Science*, *156*, pp.1-12. https://doi.org/10.1016/j.anireprosci.2015.03.002

Chapter 2: Literature review

2.1 Introduction

Semen is usually collected through ejaculation for breeding and genetic material preservation; however, this is not feasible in all species or situations, such as in cases of unexpected death (Strand et al., 2016). The possibility of recovering viable sperm from the epididymides of deceased animals has drawn more attention due to the desire to protect endangered species (Lone et al., 2011). There has been an increasing number of studies investigating the use of frozen-thawed epididymal sperm in comparison with ejaculated sperm in other species (Bertol, 2016), intending to preserve genetic diversity in non-domestic animals that are threatened or endangered breeds (Silva et al., 2019). However, the success of epididymal sperm recovery, cryopreservation and its fertilisation are hampered by the distance from the farm where the animal dies unexpectedly to the lab where the sperm could be properly processed and stored (Engdawork et al., 2024). It has been reported in other species that sperm recovered from the cauda part of the epididymis stored at 5 °C after the animal's death, exhibit higher motility with reduced morphological defects compared to non-cooled treatments (Tamayo-Canul et al., 2011; Thuwanut et al., 2013). Studies in bulls (Chaveiro et al., 2015), stallions (Monteiro et al., 2011) and goat (Turri et al., 2014) have demonstrated fertilisation ability of the sperm recovered from epididymis.

Although cryopreservation works efficiently for long-term preservation in other species, less than 1% of frozen-thawed semen is used for artificial insemination in porcine compared to liquid preserved semen (Szablicka et al., 2022). The cryopreservation of boar semen involves well-established protocols and diluents, but challenges may persist when dealing with sperm recovered from the epididymis (Berto et al., 2013). Approximately 40-50% of the ejaculated sperm population does not survive the cryopreservation process (Maside et al., 2023). Freezing and thawing procedures subject sperm to extreme survival conditions, leading to structural and metabolic cell damage, resulting in the loss of motility and membrane integrity (Bertol, 2016). The process of cryopreservation causes oxidative damage and ice crystal formation that compromise the sperm membrane lipids, proteins, carbohydrates, and polynucleotides (Horokhovatskyi et al., 2018). Cryopreservation has a major impact on the quality of boar sperm due to its susceptibility to cold shock (Engdawork et al. 2024). Sperm's susceptibility to cold shock is correlated with the composition of the plasma membrane, which varies between species but also within individuals, according to Rodriguez-Martinez et al. (2021).

The morphometric dimensions of sperm change during storage, with a high proportion of the sperm acrosome being frequently damaged (Vernocchi et al., 2014). During cryopreservation of boar semen, sperm undergo functional and structural changes influenced by factors such as the type of extender, collection method, storage temperature, storage time, and cryopreservation method (Teixeira et al., 2015; Thema et al 2023). It is necessary to improve and uphold protocols for the cryopreservation of

boar sperm genetic material. To maintain sperm's fertilising ability following cryopreservation, the boar semen freezing protocols need to be improved (Thema et al., 2023). Improvement of boar semen freezing protocols may be crucial for the development of assisted reproductive technologies (Bezerra et al., 2023). Optimising cryopreservation procedures requires a thorough understanding of the motility and morphometric traits of swine epididymal sperm (Santiago-Moreno et al., 2023). Sperm morphology assessment not only provides information about gamete quality but also facilitates evaluation of the male reproductive system (Szablicka et al., 2022). Variations in the sizes, shapes, or nuclei of sperm may correspond to distinct subpopulations of mature sperm that directly affect fertilisation capacity.

Sperm in all species are regarded as normal if they meet the criteria for that species, which includes the dimensions and form of the head, midpiece, and tail (Czubaszek et al., 2019). Additionally, these studies emphasised morphological traits as important markers of post-thaw sperm quality, such as head shape, midpiece, and tail integrity. All these sperm morphometric traits can be reliably quantified using computer-assisted sperm analysis (CASA; van der Horst et al., 2018). The length, breadth, and tail length of sperm components are important factors in sperm function and fertility (Kerns et al., 2020; Moskovtsev et al., 2023). Reduced fertility may result from high incidences of non-progressive abnormal sperm as documented in the study by Bezerra et al. (2023) and immature oocytes (Oliveira et al., 2011).

2.2 The testes and process of sperm production

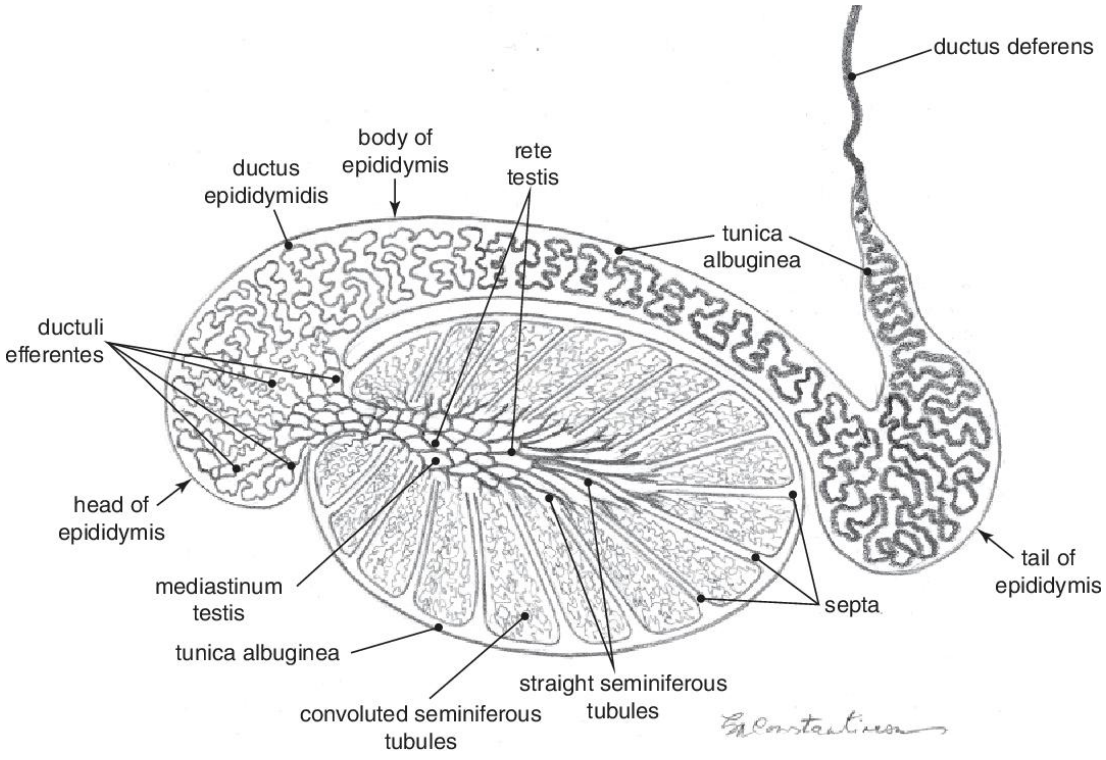

Figure 2.1: Anatomy of the testis (Constantinescu, 2017)

Testes is a primary male reproductive organ, enclosed in the scrotum, divided by partitions of the tissue and seminiferous tubules in the lobules shown on Figure 2.2 (Constantinescu, 2017). This sets of tissues expand from the tunica albuginea to the seminiferous tubules in the lobules (Houda et al., 2021). The tunica albuginea is a bilayer of fibrous connective tissue that serves as a protective and structural envelope for testis and epididymis (Federle et al., 2017). The rete testis connects head of epididymis (caput) with the mediastinum testis and seminiferous tubules being divided by the septa (Hinton & Robaire, 2015). Seminiferous tubules located within the testis and serve as a site for sperm production (Suede et al., 2023). The production of sperm begins in foetal life, when primordial germ cells form stem-cell spermatogonia within the seminiferous tubules (Bowles & Koopman, 2010; Spiller & Bowles, 2015; Spiller et al., 2017). The process at which the sperm are being produced within the seminiferous tubule located in the testes is referred to as spermatogenesis (Suede et al., 2013). Spermatogenesis is a regulated process occurring in the seminiferous tubules with the assistant of Sertoli and germ cells multiplication and differentiation which enables the continual production of sperm thereby safeguarding lifelong fertility (Zheng et al., 2022; Robertson et al., 2024). This process is initiated through the follicle-stimulating hormone interacting with Sertoli cells (provide the support and nutrition for sperm multiplication and differentiation) and luteinising hormone interacting with the Leydig cells (produce testosterone responsible for sperm production; Sharma et al., 2011; Staub et al., 2018).

The entire spermatogenesis process can be divided into three main stages, spermatogonia mitosis, spermatocyte meiosis and spermiogenesis (Abdelhamid et al., 2019). The spermatogonia stage involves mitotic cell division that allows the early cell stage to multiply; the spermatocyte step involve meiosis II, in which the haploid cells form the diploid cells, division occurs and round spermatid is formed; in the spermiogenesis stage and round spermatid form a sperm (Chandra et al., 2013; Costa et al., 2013; Fu et al., 2016). The duration of spermatogenesis process differs per species (Su et al., 2023). In boars, the entire spermatogenesis process lasts around 40-41 days with the spermatogenic cycle lasting for 9 days (Suede et al., 2023). This process is followed by the sperm maturation in the epididymis (Tiwana & Leslie, 2022).

2.3 Functions of the epididymis

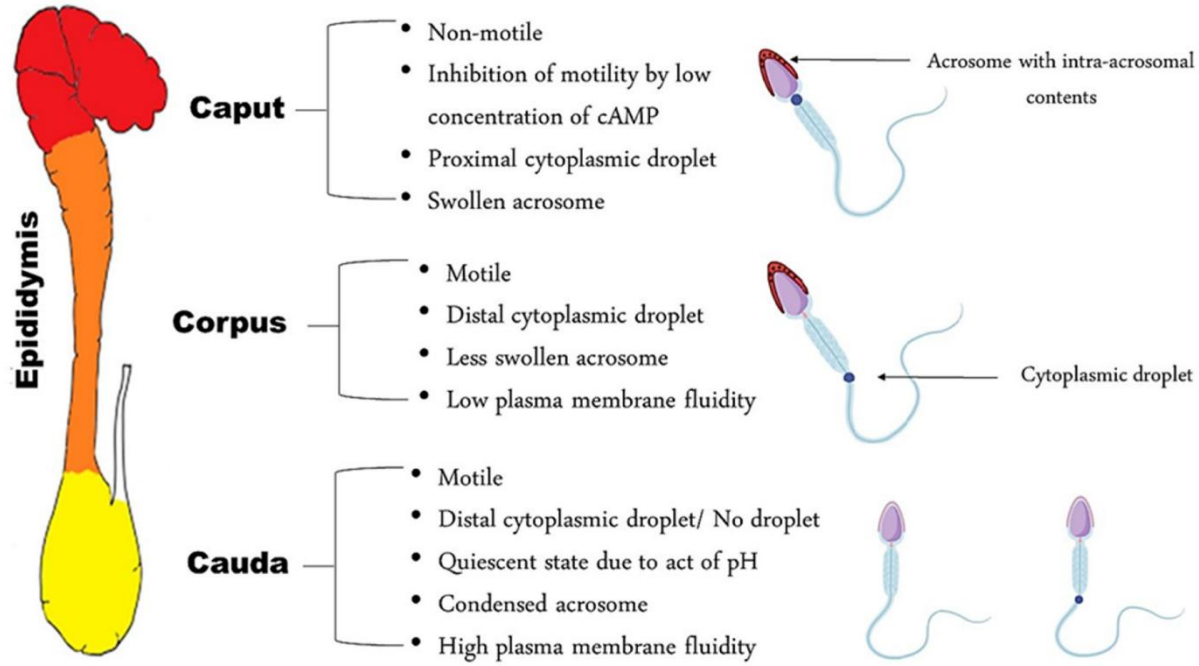

Figure 2.2: Overview of the epididymis components and their functions (Ali Hassan et al., 2021).

The epididymis is a flat and compact structure that is closely connected to one side of the testicle, with a diameter of around 4 cm (James et al., 2020; Tiwana & Leslie, 2022). Anatomically, the epididymis is divided into three segments: the caput, corpus, and cauda represented in Figure 2.2 (Ali Hassan et al., 2021). The caput and corpus consist of the sperm still in the maturation process, characterised by low concentration, motility, plasma membrane fluidity if recovered shown in Figure 2.2 (Ali Hassan et al., 2021). A cauda segment of the epididymis is considered a good source for obtaining viable, high yields, and good quality sperm that have matured (Lamglait, 2014; Leung et al., 2014). The epididymis is responsible for testicular fluid resorption, concentration, defence, transportation, maturation, and storage of mature sperm (James et al., 2020). Even though mature sperm include xeno-antigens that are unfamiliar to the immune systems of both male and female, the epididymis is responsible for providing peripheral immunological tolerance for these cells (Rodriguez-Martinez et al., 2022).

Sperm leaves the seminiferous tubules where they are produced, proceeds to the epididymis, where they mature, and then passes through the vas deferens during the ejaculation process (Dcunha et al., 2022; Eberhardt et al., 2023). These processes involve the release and absorption of liquids, ions, antioxidants, and exosomes known as epididymosomes (Trigg et al., 2019). Membrane alterations are also involved in the process of epididymal transit to allow sperm to become fertile. To avoid early activation, a billion or more sperm subsequently become retained in a quiescent metabolic stage in the cauda epididymis (Guimarães et al., 2012). Additionally, the cauda segment has been investigated in several species can support the recovery of viable sperm for preservation (Johnson, 2022) but limited in pigs.

2.4 The transportation of the epididymides to the laboratory for sperm recovery

Recovery of epididymal sperm at post-mortem is a valuable technique for obtaining germplasm from genetically endangered species (Stand et al., 2016). However, the lack of technical knowledge about the storage and transportation of the epididymides often results in tissue degeneration that prevents the recovery of viable sperm and preservation of the sperm (Thuwanut et al., 2013). Therefore, the definition of the effective epididymal transporting temperature till recovery enables the recovery of viable sperm and successful conservation of these gametes (Monteiro et al., 2011). The acceptable motility and fertilising capacity of the sperm recovered from the epididymides stored at 5 °C have been reported in bulls (Bertol et al., 2013), equine (Vidament et al., 2012), and feline (Toyonaga et al., 2011). Mir et al. (2012) preserved ram epididymides at 5 °C, and good sperm viability was recorded following 48 h postmortem; however, the *in vitro* fertility capacity declined significantly after 24 h.

Sperm in the cauda epididymis is susceptible to environmental temperature variation, while most dead animals are discovered after lying at ambient temperatures (Bergstein-Galan et al., 2017). Decrease in sperm motility was found in various studies with prolonged post-mortem length and the time of epididymal sperm recovery and storage temperature (Tittarelli et al., 2012; Bergstein-Galan et al., 2017)

2.5 Epididymal sperm recovery methods

There are efficient ways to extract sperm directly from the epididymis, either in the live animal or after death at post-mortem. Methods for retrieving sperm from live epididymis include percutaneous epididymal sperm aspiration, testicular sperm aspiration, microsurgical epididymal sperm aspiration, and open epididymal sperm retrieval (Shin & Turek, 2013). Live surgical methods are commonly utilised in human reproduction (Kamal et al., 2010), and the most commonly used method is the testicular sperm aspiration. When using testicular sperm aspiration, epididymal sperm are usually retrieved by cannulating the epididymides and using the needle to aspirate the sperm, as described by Stout (2012).

Methods of epididymal sperm recovery at post-mortem are useful for preserving the genetic material of animals that have died suddenly and when ejaculation collection is not possible for preserving endangered breeds (Strand et al., 2016). Commonly used techniques for retrieving epididymal sperm at post-mortem include mincing, flushing, and slicing. The mincing process is characterised by cutting the cauda epididymis and ductus deferens multiple times after placing them in a petri dish with a semen extender (Podico & Canisso, 2022). While the flushing method is performed by making a one cut at the top of the cauda part of the epididymis, inserting a blunted needle or cannula into the ductus deferens and pushes the extender in the opposite direction of the physiological sperm direction to the petri dish (Monoca et al., 2024). Slicing float-up involves slicing the cauda part of the epididymis with a scalpel

blade, suspending it in 5 mL of 37 °C preheated extender, and gently agitating the mixture for about 10 minutes (Falomo et al., 2016).

2.6 Semen extenders for boar sperm analysis

Experts have developed media known as extenders for sperm survival during storage and freezing because boar sperm is strongly sensitive to cold shock due to high polyunsaturated fatty (PUFAs) acid in their plasma membrane particularly long chain PUFA such as docosapentaenoic acid and docosahexaenoic acid (Baiee et al., 2018). According to Mandal et al. (2014) and Castro et al. (2025), this sensitivity of boar sperm is mostly caused by lipid phase transitions and lipid peroxidation as a result of high PUFAs in the plasma membranes. Unlike sperm of other species, boar sperm plasma membranes contain high PUFAs with low amount of cholesterol (Yeste et al., 2011). This PUFAs increases membrane flexibility, making sperm more susceptible to cold shock during sperm exposure to lower temperatures (Yeste, 2018).

Semen extenders have been developed to protect and maintain sperm viability by stabilising the plasmalemma, providing energy, and preventing harmful effects of the pH changes during preservation (Mousavi et al., 2019). Semen extenders also provide buffering capacity (Tvrdá et al., 2021), prevent clumping by thinning out the sperm concentration while increasing the metabolic activity and enhancing sperm motility. One of the requirements for semen extenders is the composition of antibiotics (gentamycin, lincomycin, penicillin, spectinomycin), which play a vital role in limiting bacterial development in the semen (Thema et al., 2022); an energy source (fructose or glucose) to sustain metabolism of the sperm and buffers (sodium citrate, bicarbonate, citric acid) that maintains semen pH (Costinar, et al., 2021). Because bacteria growth in the semen may manipulate the pH level and lead to the decrease of sperm viability (Bustani & Baiee, 2021).

Table 2.1: Chemical composition of the semen extenders

Beltsville Thawing Solution (Thema et al., 2022)	**Concentration**	**Andromed® (Auer et al., 2022)**	**Concentration**
Glucose	37 g/L	Fructose	1.25 g/L
Tri-sodium citrate	6 g/L	Citric acid	1 g/L
EDTA	1.25 g/L	Buffer	1 g/L
Sodium bicarbonate	1.25 g/L	Phospholipids	1 g/L
Spectinomycin	0.75 g/L	Spectinomycin	0.343 g/L
Penicillin	1.10 g/L	Lincomycin	0.172 g/L
Potassium chloride	1.10 g/L	Tylosin	0.57 g/L,
Gentamycin	-	Gentamicin	0.286 g/L

EDTA: Ethylenediamine tetra acetic

The semen extenders in porcine are divided into two main groups, namely: the short-term (maintains the sperm viability up to three days; Lucca et al., 2020) and long-term (maintains the sperm viability more than four days; Teixeira et al., 2015). Additionally, there are different commercial boar semen extenders available, and separate studies have compared these media, including direct comparisons between short-term (BTS, Kobidil^{+}) and long-term (x-cell, Mullbery III, Androhep-lite, Androhep plus, Acromax) extenders (Yeste, 2018). Studies compared sperm quality of the semen diluted with BTS while stored for three days with sample diluted with long-term extenders stored for five days found similar results in sperm motility, membrane, and acrosome integrities (Pinart et al., 2015).

The BTS is a short-term extender and can maintain boar sperm up to 72 h and still produce acceptable fertility results (Thema et al., 2022). With composition of potassium chloride in the BTS extender is able to maintain semen pH and sperm morphology (Vílchez et al., 2016). The present of ethylenediamine tetra acetic in the BTS extender act as an ion chelator that protect sperm against oxidative damage and lipid peroxidation during semen preservation or handling (Hussain et al., 2019). On the other hand, Andromed® is a soybean lecithin-based extender that has been used in fresh and refrigerated sperm samples from species such as alpacas, camel (Skidmore, 2019), and bulls (Miguel-Jimenez et al., 2020). Soyabean lecithin-based extender consists of phospholipids, which play a role in forming a protective film around the sperm while preventing the intracellular ice crystals formation and protecting sperm membrane against oxidative damage during preservation (Bertuzzi et al., 2020). Soybean lecithin-based extender is regarded as a suitable alternative to egg yolk for semen cryopreservation in various species (Chelucci et al., 2015).

The composition of boar semen extender especially for freezing is crucial to consist of cryoprotective substances such as glycerol and egg yolk in small concentrations to protect sperm against oxidative stress, since the boar sperm is susceptible to cold shock (Rochmi & Sofyan., 2019). Glycerol is a common permeating cryoprotectant used in mammalian semen cryopreservation protocol. It acts as a salt buffer that prevent ice crystal formation, protect sperm against cold stress and lowers freezing point by stabilising the sperm plasma membrane during cryopreservation (Anzar et al., 2019). The egg yolk form part of the standard freezing protocol for boar semen normally ranged between 15-20% with the 3% glycerol. Egg yolk play an important role on the sperm during cryopreservation by providing nutrients (low-density Lipoproteins, cholesterol, antioxidants and phospholipids), that maintains the sperm reduces sperm cold stress (Anzar et al., 2019). The composition of cholesterol in the egg yolk, strengthens and protects sperm membrane during cryopreservation by preventing structural changes (Blommaert et al., 2016). While the low-density lipoproteins composition of egg yolks, contain large amount of lipids that leads to an increase in the viscosity of the diluent (Chang et al., 2025).

2.7 Short and long-term storage of boar sperm before cryopreservation

Collected semen from the animals can be used immediately for artificial insemination/cryopreservation procedure or diluted with semen extenders and stored in a liquid form for a short duration or kept for a long term in a cryopreserved (Pantelić et al., 2018). Short term storage of boar semen refers to the lowing of boar sperm metabolism for up to 72 h at 15-18 ℃ while its quality is maintained (Szablicka et al., 2022; Thema et al., 2022). Boar semen is frequently transported to different locations in a liquid form at a regulated temperature of 15-18 ℃ for artificial insemination or cryopreservation (Kim et al., 2011). The 15-18 ℃ is considered as the boar optimum storage temperature for short duration (Schulze et al., 2013). However, courier companies offer standardized cold (4-5 ℃) transport which is cheaper and more robust to 15-18 ℃ optimum storage temperature cabinets (Waberski & Luther, 2024). Boar semen storage at 5 ℃ it has been considered innovative over 17 ℃ conventional temperature by Schulze et al. (2020).

A recently introduced step towards maintaining sperm against cold stress induced during cryopreservation or cooling to 5 ℃ is holding of the semen at 17 ℃ for 16–24 h (Luther et al., 2024). Holding semen before cryopreservation is a critical step found to address challenges faced when cryopreserving boar sperm. Holding semen at pre-freeze stage involves maintaining the semen sample at 15-18 ℃, which is one noteworthy method; it assists in lessening the susceptibility of boar sperm to cold shock while preserving sperm viability during cryopreservation (long-term storage; Prieto-Martínez et al., 2014). It has been demonstrated that holding boar sperm at pre-freeze improves its cryo-survivability (Rodriguez-Martinez et al., 2021). However, the prolonged holding of the boar epididymal sperm sample stored at 15-18 ℃ may have a negative impact on the sperm motility and morphology because the epididymal sperm lack the protection of seminal plasma (Bergstein-Galan et al., 2017).

According to Yeste et al. (2014), a 24 h holding time can increase sperm resistance to cold shock and further detailed that the prolonged holding time causes sperm capacitation processes to reverse while increases cryo-resistance. Additionally, Tomás et al. (2014) also stated that dilution of boar semen in an extender and storing it at 15 °C for 24 h should be a common practice before cryopreservation. While the storage of semen at low temperature (5 °C) during prolonged storage may have a negative impact on the sperm viability by reducing energy consumption (Schulze et al., 2020). The study by da Silva Passarelli et al. (2020) has reported the cryotolerance to have been improved when the boar semen was kept at 15-17 °C before cryopreservation.

2.8 Cryopreservation of boar semen

Cryopreservation is regarded as one of the most important reproductive biotechnologies for the establishment of conservation programs and contributes to the preservation of rare or endangered breeds (Villaverde et al., 2013; Sui et al., 2023). Cryopreservation enables the long-term storage and transportation of germplasm that can be utilised in the assisted reproductive technologies (Gangrade, 2013; Hezavehei et al., 2015). The utilisation of cryopreservation is an excellent tool to improve genetic diversity and reduce inbreeding. The role of cryobanks in preserving genetic material has expanded rapidly, and almost all semen used in reproductive industries today is cryopreserved (Dar et al., 2015). The importance of conserving genetic material, especially epididymal sperm, has increased dramatically in the field of assisted reproductive technologies in recent years (Strand et al., 2016; Comizzoli, 2015). The recovery and cryopreservation of epididymal sperm are crucial for preserving genetic material of valuable domestic or wild animals as an alternative source of gametes in cases of boar infertility, sudden death and when it is not possible to collect ejaculated semen (Bertol, 2016).

However, the analysis of cryopreserved boar sperm has largely faced poor success due to the difficulties presented in many boar semen samples. Cryopreservation processes are known to cause damage to the boar sperm, due to changes during the biotechnological process and osmotic stress caused by extracellular ice crystal formation (Nazari et al., 2021). The study by Teixeira, et al. (2022) reported sperm head rugosity to have reduced after cryopreservation as compared to fresh semen. Additionally, the cryopreservation process can cause larger alterations in the morphometric dimensions of the sperm midpiece (Gatimel et al., 2013). Furthermore, modifications to the chromatin structure and loss of sperm membrane functioning are suggested as potential reasons for the changes in the morphometric dimensions of sperm during cryopreservation (Cerdeira et al., 2020).

2.9 Ejaculated versus epididymal sperm cryotolerance

Sperm recovered from the cauda segment of the epididymis usually have higher yield, good motility, viability, normal morphology and greater ability to fertilise compared to the ejaculated (Leung et al.,

2014). This is due to that, epididymal sperm are immobile characterised with low metabolism at recovery until diluted with semen extenders or mixes with seminal plasma during ejaculation (Iolchiev et al., 2017). In addition, the cauda part of the epididymis consists of immobilin and antioxidants, which play a role in increasing epididymal fluid viscosity (James et al., 2020). Epididymal fluid compose of protein responsible for lowering sperm metabolism while maintains its quality during preservation and protecting against cryo-injury (Tomás et al., 2014).

The successful conception resulting from the use of post-thawed epididymal sperm has been achieved in many species such as goats, red deer, dogs and humans (Martins et al., 2012; Turri et al., 2014). However, the susceptibility of epididymal sperm to cold shock with, damage sperm head morphometry compared to ejaculated sperm has been reported by in Vernocchi et al., (2014) study. According to Bertol et al., 2013, poor functional quality of epididymal sperm and its susceptibility to cold shock during cryopreservation may be attributed to the absence of seminal plasma which is a component of the ejaculated semen. Sperm mix with the seminal plasma during ejaculation, which contains components/nutrients that can interact with sperm function. These interactions may alter the properties of ejaculated sperm membranes, its response to different treatments, cryopreservation and insemination as noted by Rodriguez-Martinez et al. (2021). This is because seminal plasma contains proteins that support and maintains sperm motility and morphology during preservation (Moura et al., 2018). Whereas the absence of seminal plasma protein in the epididymal sperm may affects the plasma membrane fluidity resulting in the loss of protection against cold shock (Moreira et al., 2022). Hernández et al. (2014) study showed the decline in pig sperm motility in the absence of seminal plasma protein, compared to the sample that contains atleast less concentration seminal plasma before freezing and at post-thaw. Therefore, the composition of the seminal plasma in an ejaculated semen play a critical role in stabilising the plasma membrane and delay sperm capacitation while increases sperm cryotolerance (Yánez-Ortiz et al., 2022).

2.10 Importance of sperm quality analysis

For over 70 years, clinical laboratory evaluations of male reproductive potential have relied on semen analysis as the accepted method (De Jonge, 2023). Semen quality traits provide a foundation that constitutes the traits of a typical semen sample and serve as a reference value for the fertility status of different species (van der Horst et al., 2018; Valverde et al., 2020). Analysis of the semen quality determines sperm quality, its functionality and how well the sperm can fertilise (Gaffney et al., 2011). The most common semen quality traits include macroscopic (semen volume, colour and pH) and microscopic (sperm concentration, morphometry, sperm motility, DNA integrity, acrosome integrity, membrane integrity and hyperosmotic swelling test) shown in Table 2.2 (Maside et al., 2023). Semen quality examination has frequently been regarded as the first choice for determining one's capability for conception (Nazari et al., 2021). Semen analysis is arguably the most significant clinical laboratory test

that can diagnose male infertility (Soler & Cooper, 2016). Therefore, it is imperative to make sure that the sperm quality is above standard thresholds in pig breeding (Maside et al., 2023).

Table 2.2: The most important macroscopic and microscopic traits for defining sperm quality (van der Horst, 2021).

Sperm macroscopic and microscopic traits	Methods of analysis
Semen volume	Most accurate by weighing the sample
Semen colour (white, cream, brown, grey)	Subjective visual inspection
Viscosity	Time to fill a 20 µm deep Leja chamber
Semen pH	pH meter, Panheha pH indicator strips
Sperm motility percentage	Manual or CASA
Sperm progressive motility	Manual or CASA
Sperm morphology	CASA
Sperm viability	Manual or CASA
DNA Fragmentation	Manual or CASA
Immunobead test	Manual
Mixed antiglobulin reaction test	Manual

CASA: Computer-assisted sperm morphometry analysis

2.11 Computer-assisted sperm morphometry analysis

The CASA system (Figure 2.3) is an automatic method for assessing sperm traits, including morphometry, that combines fluorescence microscopy, image analysis, and open-source software (van der Horst et al., 2010; Yániz et al., 2014; Maroto-Morales et al., 2016; van der Horst et al., 2018; van der Horst et al., 2021). This system has been widely used in domesticated animal production and reproductive toxicology laboratories (Mortimer et al., 2015). The CASA system has been effectively utilised to analyse traits, such as sperm concentration, motility, morphology, vitality and fragmentation in various animal species (Table 2.1; van der Horst, 2021). The assessment of morphology through CASA has evolved over approximately 50 years, this evolution has been driven by advances in image-capturing devices for microscopes, significant increases in computational power, and updated/expanded software algorithms (Amann & Waberski, 2014). The CASA system has been developed/established with the need to reduce subjectivity, enhance reliability, and for objective assessment of sperm morphology (Bravo et al., 2014). Additionally, CASA systems enable the analysis of a large number of sperm in a short time and provides quantitative data on kinematics and sperm morphometry (Valverde et al., 2020). The CASA plays an increasingly important role in ensuring the quality of semen marketed for artificial insemination (Mortimer et al., 2015). Sperm swim at a speed of 25 m/s at 37 °C, which translates to the length of more than five head or about half a tail length, every second (Ali Hassan et

al., 2021). The threshold speed of sperm can be estimated using the CASA system, which can objectively assess sperm velocity (Amann & Waberski, 2014).

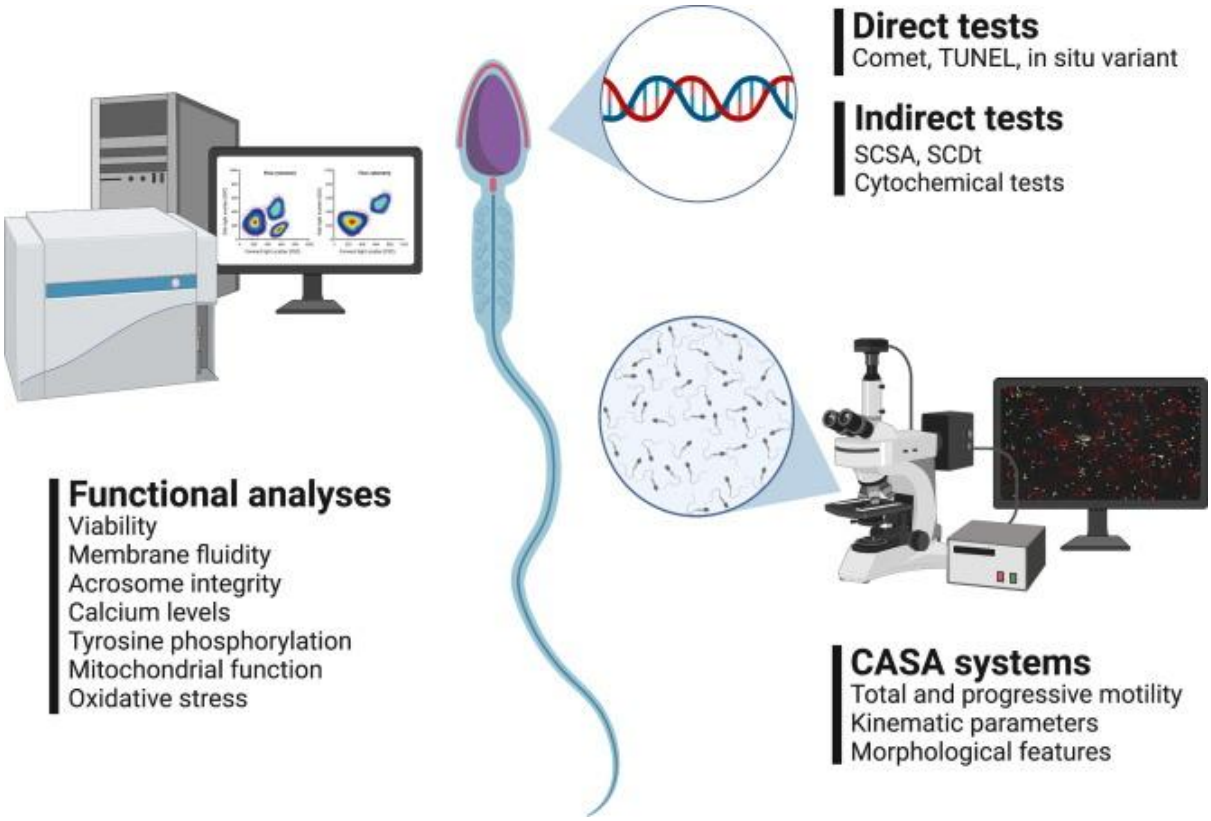

Figure 2.3: The CASA system, used to determine sperm quality and functionality traits in the farms and research laboratories (Maside et al., 2023).

2.12 Limitations in using the CASA system

There are limitations classified as biological and technical factors, which may affect CASA's ability to provide accurate results for sperm traits (Table 2.2).

Table 2.3: Advantages and limitation with the use CASA system for sperm analysis (Finelli et al., 2021).

Advantages	Limitations
Objective in a way that it saves time by allowing one to analyse large semen samples within short period of time	Typically requires trainee personnel to handle CASA
Reduce the subjectivity which may result into human error	The analysis may be challenging sometimes, with adjustment of the configuration, especially in the present of the debris and round cells in the thick sample. The CASA system may analyse the debris with the cells leading to the inaccurate results
Higher reproducibility	Analysis of sperm morphology may not be accurate without the correct configuration developed per specie.
Record keeping of data: the videos and pictures of each sample analysed can be saved.	The sperm concentration variability, for very low or high concentrated sample. Low concentrated sperm may analyse few sperm giving inaccurate result. While too concentrated sperm may sometimes display error, delays the analysis process.

CASA: computer assisted-sperm analysis

2.13 Boar epididymal sperm motility

The analysis of sperm motility is a topic of great interest in determining the infertility and potential for good animal breeding programmes (Perrett et al., 2021; Waberski et al., 2022). The term "sperm motility" describes the unique capacity of sperm to swim through the female reproductive system to fertilise an oocyte in the fallopian tubes (Dcunha et al., 2022). Analysis of sperm motility is a primary determinant of sperm fertility (Yániz et al., 2015) and may determine the success of semen preservability (Pereira et al., 2017). Contreras et al. (2020) have shown how cryopreservation may affect the motility of boar sperm. The epididymal sperm have only been reported in a few studies to have different motility patterns when compared to ejaculated sperm (Moskovtsev et al., 2023). Apart from sperm motility, other traits that constitute the sperm quality trait include the sperm motility pattern, reproductive potential, and gene expression pattern which largely connected with the rate of fertilisation (Hu et al., 2017).

2.14 Boar epididymal sperm morphometric analysis

Sperm are distinct, highly specialised cells with a variety of sizes, shapes, and forms that function at the microscopic level in a complicated environment (Banaszewska et al., 2015a; Banaszewska et al., 2015b). Another way for assessing morphology is morphometric analysis, which can be used to identify sperm abnormalities (Silva et al., 2019). The morphometry of the sperm head has been described by a variety of traits, but the most widely accepted ones are: primary traits, which give information on the dimensions of the sperm head and typically include length (L, µm), width (W, µm), area (A, μm^2), perimeter (P, µm), ellipticity = L/W, roughness = 4 πA/P2, elongation (lack of roundness) = (L − W)/(L + W) and regularity = πLW/4A (Maree et al., 2010; Valverde et al., 2020). The fertility of pig sperm has been demonstrated to be correlated with the morphometric dimensions of sperm (Kahrl et al., 2021; Szablicka et al., 2022). Sperm sizes differ among populations of the same species, within a population and among individual sperm within the same ejaculate (Yániz et al., 2015; Grønstøl et al., 2023). According to the study done by Czubaszek et al. (2019), a higher ratio of sperm head length to width and larger heads are characteristics of the infertile sperm in boars.

Sperm morphometry is an important indicator of sperm quality and its effectiveness to fertilise (Wysokińska et al., 2021). The significance of sperm morphology analysis has been recognised in the field of andrology as an indicator of reproductive disorders and the ability of the sperm to breed (Bezerra et al., 2023). Sperm morphology reflects the genetic and DNA properties of the sperm and is one of the most potent predictors of sperm capacity to fertilise *in vivo* and *vitro (*Murphy et al., 2013). According to Kondracki et al. (2017), mammalian sperm are distinguished by a unique morphological structure that allows them to transmit genetic information during fertilisation. Therefore, the morphological structure of the sperm head and its size play a crucial role in determining whether the sperm are considered normal and can fertilise (Czubaszek et al., 2019).

The CASA has been recently identified/established as a potent tool for accurately assessing motility of the sperm, including its morphological features (Amann & Waberski, 2014; Yániz et al., 2012). The phase-contrast, light, electron, fluorescence and CASA systems have all been used to study sperm morphometry in various species (Maree et al., 2010). For a correct analysis, a representative sample of the sperm population included in the analysis should atleast be 100 sperm when employing the CASA system (Bravo et al., 2014). Furthermore, the effect of cryopreservation on motility traits, including linearity, velocity, and progressiveness of the sperm, can be accurately evaluated using CASA system (Boryshpolets et al 2013). Table 2.4 shows minimum and maximum values for boar sperm morphometric traits to define normal sperm morphology.

Table 2.4: Minimum and maximum values for morphometric parameters to define normal sperm morphology in boars (van der Horst et al., 2018).

Sperm morphometry traits using CASA	Boar	
	Minimum	**Maximum**
Head Length (µm)	8.5	10.5
Head Width (µm)	4	5.5
Head Perimeter (µm)	18.5	22.6
Head area (µm)	33	44.5
Head regularity π (L*W/4*A)	0.8	1
Head ellipticity (L/W)	1.5	2.5
Acrosome cover (%)	53	61
Midpiece width (µm)	0.7	1.2
Midpiece angle (degrees)	-1	4.75

CASA: Computer-assisted sperm analysis

2.15 Pig oocyte maturation process and media

The oocyte remains arrested in prophase I from birth and does not acquire the competence to complete the meiosis II division until it has undergone fertilisation (Pan & Li, 2019; Adhikari & Liu, 2014). The acquisition of a completely developed oocyte is a gradual process that occurs in stages during oogenesis (Conti, 2018). Subsequently, the oocyte proceeds through metaphase I, releasing the first polar body, progresses to maturation (metaphase II), and then the oocyte arrests again until fertilisation occurs (Figure 2.4; Kim et al., 2023).

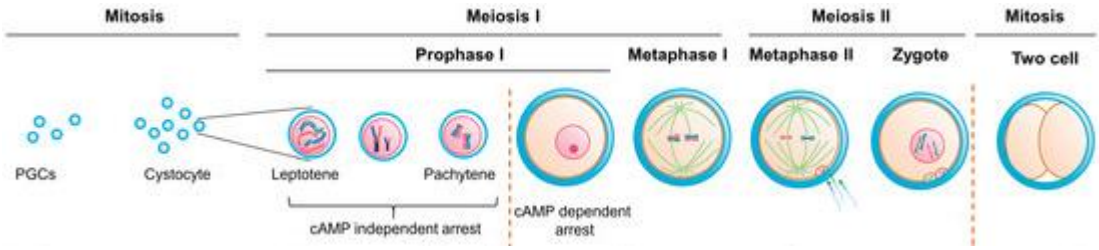

Figure 2.4: Oocyte maturation process (Kim et al., 2023)

The maturation of oocytes *in vitro* is a critical step in assisted reproductive technologies carried out in generating oocytes that are capable of being fertilised and developed into healthy offspring (Yuan & Krisher, 2011). There numerous biological processes involve such as organelle redistribution, or maternal mRNA regulation, and communications with surrounding somatic cell for both oocyte nuclear and cytoplasmic to complete maturation (Wan et al., 2023). With the current *in vitro* maturation protocols, mature oocytes are achieved after 40-44 h in terms of the oocytes arrested at the metaphase

II stage (Gil et al., 2010). However, approximately 30-40% of *in vitro* matured oocytes retrieved from the large animals have proven to be able to reach the blastocyst stage and capable of supporting embryonic development compared to the oocytes recovered from the gilts (Hamano, 2021). The majority of the studies use oocytes retrieved from the ovaries collected from the local abattoirs because they are readily available and low-cost (Grupen, 2014). The *in vitro* matured and fertilised oocytes of ovaries collected from the abattoirs have successfully developed into healthy offspring in various species. However, the developmental potential of *in vitro* matured oocytes is still lower compared to the matured *in vivo* (Bartková et al., 2024).

Therefore, availability of a large number of *in vitro* matured oocytes and developed embryos would be crucial for agricultural and biomedical purposes (Gil et al., 2010). Methods of oocyte maturation, fertilisation, and culture play a critical role in embryo, foetus, and offspring development (Redel et al., 2019). Many studies have employed different media to assist in determination of the requirements for oocyte's cytoplasmic maturation and the most common oocyte maturation media used in pigs is Tissue Culture Medium 199, NCSU- 37 and 23 (Gil et al., 2010), with NCSU-23 showing superior results in promoting oocytes' nuclear and cytoplasmic maturation that develop into embryos (Pyoos et al., 2018). The NCSU-23 maturation media contains glucose as the primary energy source (Chen et al., 2021), porcine follicular fluid and 2% antibiotic to mediate osmolality and prevent contamination (Pawlak et al., 2018; Malik et al., 2023). This defines the importance of nutritional composition in the oocyte maturation media, which may affect metabolic pathways during embryonic developmental competency (Mito et al., 2012). Although, there have been considerable efforts to improve pig oocyte maturation media in the past decade (Bartková et al., 2024), the ability to continuously modulate the composition of the oocyte's maturation media is needed to improve embryo developmental competence (Chen et al., 2021). Thus, a significant amount of information has yet to be learned regarding the oocyte maturation media environment, the ways in which maturation media can be modified to assist in improving cytoplasmic maturation and embryo developmental competency.

2.16 *In vitro* fertilisation in pigs using post-thawed sperm

The IVF is a basic technology that enables the production/development of embryos in large amounts and represents an indicator to evaluate the sperm fertilising ability (Kikuchi et al., 2016; Nguyen et al., 2021). The *in vitro* embryo production provides many advantages over *in vivo* production, including efficient selection of superior genetics (Chen et al., 2021). Retrieved sperm can be used to induce pregnancy through assisted reproductive technologies, such as intracytoplasmic sperm injection, *in vitro* or *in vivo* fertilisation process (Esteves et al, 2011). Numerous species, including camel (Badr & Abdel-Malak, 2010), ram (Pamungkas et al., 2012), equine (Vieira et al., 2013), bulls (Chaveiro et al., 2015),

and human (Hayon et al., 2021), have successfully collected epididymal sperm and consistent fertilisation rates were reported.

The use of frozen-thawed sperm in pig IVF exhibits much lower penetration (Knox, 2015) because cryopreservation presents sperm cryoinjuries which affect sperm viability and fertilisation capacity (Yeste et al., 2017). Additionally, pig *in vitro* embryo production remains a significant challenge compared to other species, mainly because of the inconsistent fertilisation rates and high incidences of polyspermy (Gil et al., 2010). Dale (2016) defined polyspermy as the penetration of more than one sperm to the zona pellucida results into multiple nuclei interaction and division. Polyspermy is a pathological phenomenon that may lead to embryo developmental failure and death in various animal species (Pamungkas et al., 2012), including human beings. Additionally excessive polyspermy in porcine IVF reduces the ability of oocytes to promote male pronuclear formation supported by Nguyen et al. (2021). The polyspermy can be caused by the membrane block and a block of the zona pellucida during penetration (Evans, 2020). Grupen, (2014), suggested that the failure of polyspermy fertilisation block may be caused by thinner zona pellucida on the traditional *in vitro* matured oocytes unlike it being thicker on the oocyte ovulated. Because naturally in a female reproductive system there are different regions/mechanisms and chemical reaction in the, which help the oocyte to protect itself from polyspermy (Yoshioka et al., 2012). Furthermore, Romar et al. (2016) study found that the pattern of cortical granule release *in vivo* and traditional *vitro* matured oocytes following penetration differs. The polyspermy situation may be connected to the quality of the oocytes, *in vitro* maturation failure and overmature cytoplasm (Xia, 2013). Oocytes with incomplete cytoplasmic maturation may lower fertilisation success and developmental competence (Wang et al., 2025). Incomplete cytoplasmic maturation is a suboptimal condition where the nuclear of the oocytes matures and extrude polar body without cytoplasm being sufficiently developed Wan et al., 2023. Whereas fertilisation success and embryo developmental competency depend on both complete nuclear and cytoplasmic maturation (Richani et al., 2021).

2.17 Summary of the literature review

Overall cryopreservation of sperm is a valuable reproductive technology for conserving genetic material, gene banking and genetic improvement (Vozaf et al., 2022). However, the use of post-thawed sperm is limited in pigs due to the high susceptibility of boar sperm to cold shock (Kim et al., 2011). Additionally, the success of epididymal sperm recovery, processing and conservation is hampered by the lack of knowledge about the testes handling after animal death, recovery methods and correct protocol for preserving epididymal sperm (Engdawork et al., 2024). To enhance proper transportation of testes or semen, handling and cryotolerance of the epididymal sperm, the evaluation of variation in the sperm quality following 24 h of holding time has been recommended in other species (Wasilewska

et al., 2016) but underutilised in pigs. Flushing technique is a recommended for bulls characterised by lower blood contamination (Turri et al., 2012). Whereas slicing technique was recommended in the few studies such as bucks to yield higher mean percentage of live and normal sperm compared to flushing (Gautane et al., 2016).

One of the requirements for semen extenders is the composition of antibiotics playing a vital role in limiting bacterial development in the semen while maintain pH and energy source to sustain metabolism of the sperm (Costinar, et al., 2021). The existence of BTS and Andromed® extenders with such composition at recovery and preservation would provide a valuable contribution to the porcine artificial insemination industry and improve the protocol for cryopreserving boar sperm. This defines the importance of nutritional composition in the oocyte's media or semen extenders, which may affect metabolic pathways during semen preservation and embryonic developmental competency (Mito et al., 2012). Although, there have been considerable efforts to improve pig oocyte maturation media in the past decade (Bartková et al., 2024), the ability to continuously modulate the composition of the oocyte's maturation media is needed to improve embryo developmental competence (Chen et al., 2021). Thus, a significant amount of information has yet to be learned regarding the oocyte maturation media environment, the ways in which maturation media can be modified to assist in improving cytoplasmic maturation and embryo developmental competency.

2.18 References

Abdelhamid, M.H.M., Walschaerts, M., Ahmad, G., Mieusset, R., Bujan, L. and Hamdi, S., 2019. Mild experimental increase in testis and epididymis temperature in men: effects on sperm morphology according to spermatogenesis stages. *Translational Andrology and Urology*, *8*(6), pp.651. https://pmc.ncbi.nlm.nih.gov/articles/PMC6987600/ Accessed 07102025

Adhikari, D. and Liu, K., 2014. The regulation of maturation promoting factor during prophase I arrest and meiotic entry in mammalian oocytes. Molecular and cellular endocrinology, 382(1), pp.480-487. https://doi.org/10.1016/j.mce.2013.07.027

Ali Hassan, H., Domain, G., Luvoni, G.C., Chaaya, R., Van Soom, A. and Wydooghe, E., 2021. Canine and feline epididymal semen—a plentiful source of gametes. *Animals*, *11*(10), p.2961. https://doi.org/10.3390/ani11102961

Amann, R.P. and Waberski, D., 2014. Computer-assisted sperm analysis (CASA): capabilities and potential developments. *Theriogenology*, *81*(1), pp.5-17. https://doi.org/10.1016/j.theriogenology.2013.09.004

Anzar, M., Rajapaksha, K. and Boswall, L., 2019. Egg yolk-free cryopreservation of bull semen. PloS one, 14(10), p.e0223977.

Badr, M.R. and Abdel-Malak, M.G., 2010. In vitro fertilization and embryo production in dromedary camel using epididymal spermatozoa. *Global Vet*, *4*(3), pp.271-276. https://www.academia.edu/download/34336977/Badr-2010.pdf accessed 26082025

Baiee, F.H., Wahid, H., Rosnina, Y., Ariff, O., Yimer, N., Jeber, Z., Salman, H., Tarig, A. and Harighi, F., 2018. Impact of Eurycoma longifolia extract on DNA integrity, lipid peroxidation, and functional parameters in chilled and cryopreserved bull sperm. *Cryobiology*, *80*, pp.43-50. https://doi.org/10.1016/j.cryobiol.2017.12.006

Banaszewska, D., Czubaszek, M., Walczak-Jędrzejowska, R. and Andraszek, K., 2015a. Morphometric dimensions of the stallion sperm head depending on the staining method used. *Journal of Veterinary Research*, *59*(2), pp.263-270. https://sciendo.com/2/v2/download/article/10.1515/bvip-2015-0039.pdf Accessed 18092025

Banaszewska, D., Andraszek, K., Czubaszek, M. and Biesiada–Drzazga, B., 2015b. The effect of selected staining techniques on bull sperm morphometry. *Animal Reproduction Science*, *159*, pp.17-24. https://doi.org/10.1016/j.anireprosci.2015.06.019

Bartková, A.R., Němcová, L., Kinterová, V., Radová, D., Strejček, F., Toralová, T., Laurinčík, J. and Procházka, R., 2024. Meiotic and developmental competence of growing pig oocytes derived from small antral follicles is enhanced in culture medium containing FGF2, LIF, and IGF1 (FLI medium). *Journal of Ovarian Research*, *17*(1), p.54. https://doi.org/10.1186/s13048-024-01360-0

Bergstein-Galan, T.G., Weiss, R.R., Bertol, M.A.F., Abreu, A.C.M., Busato, E., Kozicki, L.E. and Bicudo, S.D., 2017. Quality and fertility of frozen ovine spermatozoa from epididymides stored at room temperature (18–25 °C) for up to 48 h post-mortem. *Theriogenology*, *96*, pp.69-75. https://doi.org/10.1016/j.theriogenology.2017.04.001

Bertol, M.A.F., Weiss, R.R., Thomaz-Soccol, V., Kozicki, L.E., Fujita, A.S., Abreu, R.A.D. and Green, K.T., 2013. Viability of bull spermatozoa collected from the epididymis stored at 18-20 C. *Brazilian Archives of Biology and Technology*, *56*, pp.777-783. https://doi.org/10.1590/S1516-89132013000500008

Bertol, M.A., 2016. Cryopreservation of epididymal sperm. *Cryopreservation in eukaryotes. (Eds F Marco-Jiménez, H Akdemir)*, IntechOpen. London. pp.121-135. https://dx.doi.org/10.5772/65010

Bertuzzi, M.L., Torres, E.Y., Huanca, T., Neild, D. and Carretero, M.I., 2020. Comparison of extenders with the addition of egg yolk for cooling alpaca sperm obtained from deferent ducts. *Frontiers in Veterinary Science*, *7*, p.597954. https://doi.org/10.3389/fvets.2020.597954

Bezerra, L.G., Silva, A.M., Jurema, A.P., Dantas, M.R., Pereira, A.G., Oliveira, M.F., Comizzoli, P. and Silva, A.R., 2023. Changes in sperm morphology, morphometry, and motility from the epididymis to the vas deferens in rheas (Rhea americana, Linnaeus, 1758). *Animals*, *13*(9), p.1483. https://doi.org/10.3390/ani13091483

Blommaert, D., Franck, T., Donnay, I., Lejeune, J.P., Detilleux, J. and Serteyn, D., 2016. Substitution of egg yolk by a cyclodextrin-cholesterol complex allows a reduction of the glycerol concentration into the freezing medium of equine sperm. Cryobiology, 72(1), pp.27-32. https://doi.org/10.1016/j.cryobiol.2015.11.008

Boryshpolets, S., Kowalski, R.K., Dietrich, G.J., Dzyuba, B. and Ciereszko, A., 2013. Different computer-assisted sperm analysis (CASA) systems highly influence sperm motility parameters. *Theriogenology*, *80*(7), pp.758-765. https://doi.org/10.1016/j.theriogenology.2013.06.019

Bowles, J. and Koopman, P., 2010. Sex determination in mammalian germ cells: extrinsic versus intrinsic factors. *Reproduction*, *139*(6), pp.943-958. https://doi.org/10.1530/REP-10-0075

Bravo, J.A., Montanero, J., Calero, R. and Roy, T.J., 2014. Influence of season and reproductive management on the morphometry of ram sperm head. *Small Ruminant Research*, *119*(1-3), pp.114-119. https://doi.org/10.1016/j.smallrumres.2014.02.015

Bustani, G.S. and Baiee, F.H., 2021. Semen extenders: An evaluative overview of preservative mechanisms of semen and semen extenders. *Veterinary World*, *14*(5), p.1220. https://doi.org/10.14202/vetworld.2021.1220-1233

Conti, M. and Franciosi, F., 2018. Acquisition of oocyte competence to develop as an embryo: integrated nuclear and cytoplasmic events. Human reproduction update, 24(3), pp.245-266. https://doi.org/10.1093/humupd/dmx040

Engdawork, A., Belayhun, T. and Aseged, T., 2024. The role of reproductive technologies and cryopreservation of genetic materials in the conservation of animal genetic resources, A review. *Ecological Genetics and Genomics*, p.100250. https://doi.org/10.1016/j.egg.2024.100250

Chandra, A.K., Sengupta, P., Goswami, H. and Sarkar, M., 2013. Effects of dietary magnesium on testicular histology, steroidogenesis, spermatogenesis and oxidative stress markers in adult rats. *Indian Journal of Experimental Biology*, *51*(1), pp.37-47. https://www.academia.edu/download/30378234/IJEB_51(1)_37-47.pdf Accessed 31102025

Chang, F., Zhang, B., Liu, H., Fan, H., Xie, R., Li, J., Hu, Q. and Ruan, C., 2025. Effect of centrifuged chicken egg yolk on the cryopreservation of boar semen. Animals, 15(4), p.599. https://doi.org/10.3390/ani15040599

Chaveiro, A., Cerqueira, C., Silva, J., Franco, J. and da Silva, F.M., 2015. Evaluation of frozen thawed cauda epididymal sperms and in vitro fertilizing potential of bovine sperm collected from the cauda epididymal. *Iranian Journal of Veterinary Research*, *16*(2), p.188. https://pmc.ncbi.nlm.nih.gov/articles/PMC4827685/ accessed 26082025

Chelucci, S., Pasciu, V., Succu, S., Addis, D., Leoni, G.G., Manca, M.E., Naitana, S. and Berlinguer, F., 2015. Soybean lecithin–based extender preserves spermatozoa membrane integrity and

fertilizing potential during goat semen cryopreservation. *Theriogenology*, *83*(6), pp.1064-1074. https://doi.org/10.1016/j.theriogenology.2014.12.012

Cerdeira, J., Sánchez-Calabuig, M.J., Pérez-Gutiérrez, J.F., Hijon, M., Castaño, C. and Santiago-Moreno, J., 2020. Cryopreservation effects on canine sperm morphometric variables and ultrastructure: comparison between vitrification and conventional freezing. Cryobiology, 95, pp.164-170. https://doi.org/10.1016/j.cryobiol.2020.03.007

Chen, P.R., Redel, B.K., Kerns, K.C., Spate, L.D. and Prather, R.S., 2021. Challenges and considerations during in vitro production of porcine embryos. *Cells*, *10*(10), p.2770. https://doi.org/10.3390/cells10102770

Comizzoli, P., 2015. Biotechnologies for wildlife fertility preservation. *Animal Frontiers*, *5*(1), pp.73-78. https://doi.org/10.2527/af.2015-0011

Constantinescu. G.M., 2017. Anatomy of the reproductive system. *Animal Models and Human Reproduction, 3*, pp.1-58. https://doi.org/10.1002/9781118881286.CH1

Contreras, P., Dumorné, K., Ulloa-Rodríguez, P., Merino, O., Figueroa, E., Farías, J.G., Valdebenito, I. and Risopatrón, J., 2020. Effects of short-term storage on sperm function in fish semen: A review. *Reviews in Aquaculture, 12*(3), pp.1373-1389. https://doi.org/10.1111/raq.12387

Costa, D.S., Faria, F.J., Fernandes, C.A., Silva, J.C. and Auharek, S.A., 2013. Testis morphometry and kinetics of spermatogenesis in the feral pig (Sus scrofa). *Animal Reproduction Science*, *142*(1-2), pp.63-70. https://doi.org/10.1016/j.anireprosci.2013.09.007

Costinar, L., Herman, V., Pitoiu, E., Iancu, I., Degi, J., Hulea, A. and Pascu, C., 2021. Boar semen contamination: Identification of gram-negative bacteria and antimicrobial resistance profile. *Animals*, *12*(1), p.43. https://doi.org/10.3390/ani12010043

Czubaszek, M., Andraszek, K., Banaszewska, D. and Walczak-Jędrzejowska, R., 2019. The effect of the staining technique on morphological and morphometric parameters of boar sperm. *PloS one*, *14*(3), p.e0214243. https://doi.org/10.1371/journal.pone.0214243

Dale, B., 2016. Achieving monospermy or preventing polyspermy?. *Research and Reports in Biology*, pp.47-57. https://doi.org/10.2147/RRB.S84085

da Silva Passarelli, M., Pavaneli, A.P.P., Ravagnani, G.M., Martins, M.P., Pedrosa, A.C., Martins, S.M.M.K., de Alcântra Rocha, N.R., Rigo, V.H.B., Yasui, G., Yeste, M. and de Andrade, A.F.C., 2020. Effects of different equilibration times at 5 ° C on boar sperm cryotolerance. *Animal Reproduction Science*, *219*, p.106547. https://doi.org/10.1016/j.anireprosci.2020.106547

Dar, R.A., Ahmad, M., Kumar, S. and Reshi, M., 2015. Agriculture germplasm resources: A tool of conserving diversity. *Scientific Research and Essays, 10*(9), pp.326-338. https://doi.org/10.5897/SRE2015.6206

Dcunha, R., Hussein, R.S., Ananda, H., Kumari, S., Adiga, S.K., Kannan, N., Zhao, Y. and Kalthur, G., 2022. Current insights and latest updates in sperm motility and associated applications in

assisted reproduction. *Reproductive Sciences*, *29*(1), pp.7-25. https://doi.org/10.1007/s43032-020-00408-y

De Jonge, C.J., 2023. *Evaluating defects in sperm function*. In: Lipshultz LI, Howards SS, Niederberger CS, Lamb DJ, eds. *Infertility in the Male*. Cambridge University Press: 363-380.

Eberhardt, M., Prochowska, S., Partyka, A., Bielas, W., Van Soom, A., Olech, W. and Niżański, W., 2023. The morphology, morphometry and functionality of fresh and cryopreserved wisent (Bison bonasus) epididymal spermatozoa. *Scientific Reports*, *13*(1), p.13866. https://doi.org/10.1038/s41598-023-40798-y

Esteves, S.C., Miyaoka, R. and Agarwal, A., 2011. Sperm retrieval techniques for assisted reproduction. *International Brazilian Journal of Urology*, *37*, pp.570-583. https://doi.org/10.1590/S1677-55382011000500002

Evans, J.P., 2020. Preventing polyspermy in mammalian eggs—contributions of the membrane block and other mechanisms. *Molecular Reproduction and Development*, *87*(3), pp.341-349. https://doi.org/10.1002/mrd.23331

Falomo, M.E., Rossi, M. and Mantovani, R., 2016. Collection, storage and freezability of equine epididymal spermatozoa. *Italian Journal of Animal Science*, *15*(3), pp.386-389. https://doi.org/10.1080/1828051X.2016.1210485

Federle, M.P., Rosado-de-Christenson, M.L., Raman, S.P., Carter, B.W., Woodward, P.J. and Shaaban, A.M., 2017. Testes and scrotum**.** In *Imaging Anatomy: Chest, Abdomen (Second Edition)*, Elsevier, *Pelvis*, pp.1-1115. https://doi.org/10.1016/B978-0-323-47781-9.50043-X

Finelli, R., Leisegang, K., Tumallapalli, S., Henkel, R. and Agarwal, A., 2021. The validity and reliability of computer-aided semen analyzers in performing semen analysis: a systematic review. *Translational andrology and urology*, *10*(7), p.3069. https://doi.org/10.21037/tau-21-276

Fu, S.Y., Jiang, J.H., Yang, W.X. and Zhu, J.Q., 2016. A histological study of testis development and ultrastructural features of spermatogenesis in cultured Acrossocheilus fasciatus. *Tissue and Cell*, *48*(1), pp.49-62. https://doi.org/10.1016/j.tice.2015.10.005

Gaffney, E.A., Gadêlha, H., Smith, D.J., Blake, J.R. and Kirkman-Brown, J.C., 2011. Mammalian sperm motility: observation and theory. *Annual Review of Fluid Mechanics*, *43*(1), pp.501-528. https://doi.org/10.1146/annurev-fluid-121108-145442

Gangrade, B.K., 2013. Cryopreservation of testicular and epididymal sperm: techniques and clinical outcomes of assisted conception. *Clinics*, *68*, pp.131-140. https://doi.org/10.6061/clinics/2013(Sup01)15

Gatimel, N., Leandri, R. and Parinaud, J., 2013. Sperm vacuoles are not modified by freezing–thawing procedures. *Reproductive BioMedicine Online*, *26*(3), pp.240-246. https://doi.org/10.1016/j.rbmo.2012.11.019

Gautane, J.J., Balagan, E.J.Y., Manaois, F.I., Ocampo, M.B. and Ocampo, L.C., 2016. Characteristics of epididymal sperm recovered from slaughterhouse derived testes of nondescript/native goats in the Philippines. pp.215-228.

Gil, M.A., Cuello, C., Parrilla, I., Vazquez, J.M., Roca, J. and Martinez, E.A., 2010. Advances in swine in vitro embryo production technologies. *Reproduction in Domestic Animals*, *45*, pp.40-48. https://doi.org/10.1111/j.1439-0531.2010.01623.x

Grønstøl, G., Danielsen, M., Cramer, E.R., Johannessen, L.E., Johnsen, A., Whittington, E. and Lifjeld, J.T., 2023. Effects of fixatives and storage duration on avian sperm morphology. *Journal of Ornithology*, *164*(1), pp.171-181. https://doi.org/10.1007/s10336-022-02015-x

Grupen, C.G., 2014. The evolution of porcine embryo in vitro production. *Theriogenology*, *81*(1), pp.24-37. https://doi.org/10.1016/j.theriogenology.2013.09.022

Guimarães, T., Lopes, G., Ferreira, P., Leal, I. and Rocha, A., 2012. Characteristics of stallion epididymal spermatozoa at collection and effect of two refrigeration protocols on the quality of the frozen/thawed sperm cells. *Animal Reproduction Science*, *136*(1-2), pp.85-89. https://doi.org/10.1016/j.anireprosci.2012.10.028

Hamano, S., 2021. Production, dissemination activities, and supplying-system development for in vitro fertilized bovine embryo. *Journal of Reproduction and Development*, *67*(3), pp.167-175. https://doi.org/10.1262/jrd.2020-138

Hussain, N., Andrabi, S.M.H. and Mehmood, M.U., 2019. Effect of EDTA as chelating agent in extender on the post thaw quality of buffalo bull spermatozoa. *CryoLetters*, *40*(3), pp.159-163. https://www.cabidigitallibrary.org/doi/full/10.5555/20193475757 Accessed 28102025

Hayon, S., Moustafa, S., Boylan, C., Kohn, T.P., Peavey, M. and Coward, R.M., 2021. Surgically extracted epididymal sperm from men with obstructive azoospermia results in similar in vitro fertilization/intracytoplasmic sperm injection outcomes compared with normal ejaculated sperm. *The Journal of Urology*, *205*(2), pp.561-567. https://doi.org/10.1097/JU.0000000000001388

Hernández, C.L., Dorado, J., Hidalgo, M., Rubio, P.J. and Quintero, M.A., 2014. Effect of seminal extraction method on post-thawing viability and sperm motility in mixed-breed male goats. *Revista Científica Biológico Agropecuaria Tuxpan*, *2*(4), pp. 875-884.

Hezavehei, M., Sharafi, M., Kouchesfahani, H.M., Henkel, R., Agarwal, A., Esmaeili, V. and Shahverdi, A., 2018. Sperm cryopreservation: A review on current molecular cryobiology and advanced approaches. *Reproductive Biomedicine Online*, *37*(3), pp.327-339. https://doi.org/10.1016/j.rbmo.2018.05.012

Hinton, B.T. and Robaire, B., 2015. The epididymis. *Knobil and Neill's Physiology of Reproduction*, *1*, pp.691-771.

Horokhovatskyi, Y., Dietrich, M.A., Lebeda, I., Fedorov, P., Rodina, M. and Dzyuba, B., 2018. Cryopreservation effects on a viable sperm sterlet (Acipenser ruthenus) subpopulation obtained

by a Percoll density gradient method. *PLoS One*, *13*(8), p.e0202514. https://doi.org/10.1371/journal.pone.0202514

Houda, A., Nyaz, S., Sobhy, B.M., Bosilah, A.H., Romeo, M., Michael, J.P. and Eid, H.M., 2021. Seminiferous tubules and spermatogenesis. In *Male Reproductive Anatomy*. IntechOpen. http://dx.doi.org/10.5772/intechopen.98917.

Hu, F., Xu, K., Zhou, Y., Wu, C., Wang, S., Xiao, J., Wen, M., Zhao, R., Luo, K., Tao, M. and Duan, W., 2017. Different expression patterns of sperm motility-related genes in testis of diploid and tetraploid cyprinid fish. *Biology of Reproduction*, *96*(4), pp.907-920. https://doi.org/10.1093/biolre/iox010

Iolchiev, B.S., Abilov, A.I., Tadzhieva, A.V., Bagirov, V.A., Nasibov Sh, N., Shaidullin, I.N., Klenovitskiy, P.M., Kombarova, N.A. and Zhilinskii, M.A., 2017. Biological integrity of bison epididymal sperm under cryoconservation and long storage. *Sel'skokhozyaistvennaya Biologiya [Agricultural Biology]*, *52*(2), pp.282-290. https://doi.org/10.15389/agrobiology.2017.2.282eng

James, E.R., Carrell, D.T., Aston, K.I., Jenkins, T.G., Yeste, M. and Salas-Huetos, A., 2020. The role of the epididymis and the contribution of epididymosomes to mammalian reproduction. *International Journal of Molecular Sciences*, *21*(15), p.5377. https://doi.org/10.3390/ijms21155377

Johnson, A.K., 2022. Normal feline reproduction: *the tom*. *Journal of Feline Medicine and Surgery*, *24*(3), pp.212-220. https://doi.org/10.1177/1098612X221079

Kahrl, A.F., Snook, R.R. and Fitzpatrick, J.L., 2021. Fertilization mode drives sperm length evolution across the animal tree of life. *Nature Ecology and Evolution*, *5*(8), pp.1153-1164. https://doi.org/10.1038/s41559-021-01488-y

Kamal, A., Fahmy, I., Mansour, R., Serour, G., Aboulghar, M., Ramos, L. and Kremer, J., 2010. Does the outcome of ICSI in cases of obstructive azoospermia depend on the origin of the retrieved spermatozoa or the cause of obstruction? A comparative analysis. *Fertility and Sterility*, *94*(6), pp.2135-2140. https://doi.org/10.1016/j.fertnstert.2010.01.041

Kerns, K., Jankovitz, J., Robinson, J., Minton, A., Kuster, C. and Sutovsky, P., 2020. Relationship between the length of sperm tail mitochondrial sheath and fertility traits in boars used for artificial insemination. *Antioxidants*, *9*(11), p.1033. https://doi.org/10.3390/antiox9111033

Kikuchi, K., Kaneko, H., Nakai, M., Somfai, T., Kashiwazaki, N. and Nagai, T., 2016. Contribution of in vitro systems to preservation and utilization of porcine genetic resources. *Theriogenology*, *86*(1), pp.170-175. https://doi.org/10.1016/j.theriogenology.2016.04.029

Kim, H.M., Kang, M.K., Seong, S.Y., Jo, J.H., Kim, M.J., Shin, E.K., Lee, C.G. and Han, S.J., 2023. Meiotic cell cycle progression in mouse oocytes: role of cyclins. *International Journal of Molecular Sciences*, *24*(17), p.13659. https://doi.org/10.3390/ijms241713659

Kim, S., Lee, Y.J. and Kim, Y.J., 2011. Changes in sperm membrane and ROS following cryopreservation of liquid boar semen stored at 15 °C. *Animal Reproduction Science*, *124*(1-2), pp.118-124. https://doi.org/10.1016/j.anireprosci.2011.01.014

Knox, R.V., 2015. The fertility of frozen boar sperm when used for artificial insemination. *Reproduction in Domestic Animals*, *50*, pp.90-97. https://doi.org/10.1111/rda.12552

Kondracki, S., Wysokińska, A., Kania, M. and Górski, K., 2017. Application of two staining methods for sperm morphometric evaluation in domestic pigs. *Journal of Veterinary Research*, *61*(3), p.345. https://sciendo.com/2/v2/download/article/10.1515/jvetres-2017-0045.pdf Accessed 18082025

Lamglait, B., 2014. Longevity of sperm cells retrieved by post-mortem epididymal aspiration in wild bovids in zoo conditions. *Journal of Zoo and Aquarium Research*, *2*(4), pp.92-100. https://doi.org/10.19227/jzar.v2i4.47

Leung, A., Mira, J. and Hsiao, W., 2014. Updates on sperm retrieval techniques. *Translational Andrology and Urology*, *3*(1), p.94. https://doi.org/10.3978/j.issn.2223-4683.2014.02.03

Lone, F.A., Islam, R., Khan, M.Z. and Sofi, K.A., 2011. Effect of transportation temperature on the quality of cauda epididymal spermatozoa of ram. *Animal Reproduction Science*, *123*(1-2), pp.54-59. https://doi.org/10.1016/j.anireprosci.2010.10.012

Lucca, M.S., Ulguim, R.D.R., Bortolozzo, F.P., Wentz, I., Rocha, J.C., Souza, M.C.D.D., Escobar, R.V., Calderam, K., Quadros Filho, P.I.D. and Marcos, R.A., 2020. Reproductive performance of sows inseminated with semen doses stored for up to seven days in long-term extender in a field condition. Animal Reproduction, 17(1), p.e20190121.

Luther, A.M., Nguyen, T.Q., Verspohl, J. and Waberski, D., 2024. Update of the cooling protocol for antibiotic-free storage of boar semen at 5 C improves sperm quality and maintains low bacterial counts. *PLoS One*, *19*(6), p.e0305280. https://doi.org/10.1371/journal.pone.0305280

Malik, P., Mukherjee, S. and Mukherjee, T.K., 2023. Mammalian cell culture types and guidelines of their maintenance. In *Practical Approach to Mammalian Cell and Organ Culture* (pp. 233-259). Singapore: Springer Nature Singapore. https://doi.org/10.1007/978-981-19-1731-8_6-2

Mandal, R., Badyakar, D. and Chakrabarty, J., 2014. Role of membrane lipid fatty acids in sperm cryopreservation. *Advances in Andrology*, *2014*(1), p.190542. https://doi.org/10.1155/2014/190542

Maree, L., Du Plessis, S.S., Menkveld, R. and van der Horst, G., 2010. Morphometric dimensions of the human sperm head depend on the staining method used. *Human Reproduction*, *25*(6), pp.1369-1382. https://doi.org/10.1093/humrep/deq075

Maroto-Morales, A., García-Álvarez, O., Ramón, M., Martínez-Pastor, F., Fernández-Santos, M.R., Soler, A.J. and Garde, J.J., 2016. Current status and potential of morphometric sperm

analysis. *Asian Journal of Andrology*, *18*(6), pp.863-870. https://doi.org/10.4103/1008-682X.187581

Martins, M.I.M., Justino, R.C., Sant'Anna, M.C., Trautwein, L.G.C. and Souza, F.F., 2012. Comparison of two different extenders for cryopreservation of epididymal dog sperm. Reproduction in Domestic Animals, 47, pp.293-294. https://doi.org/10.1111/rda.12042

Maside, C., Recuero, S., Salas-Huetos, A., Ribas-Maynou, J. and Yeste, M., 2023. Animal board invited review: an update on the methods for semen quality evaluation in swine–from farm to the lab. *Animal*, *17*(3), p.100720. https://doi.org/10.1016/j.animal.2023.100720

Miguel-Jimenez, S., Del Alamo, M.M.R., Álvarez-Rodríguez, M., Hidalgo, C.O., Peña, A.I., Muiño, R., Rodríguez-Gil, J.E. and Mogas, T., 2020. In vitro assessment of egg yolk-, soya bean lecithin-and liposome-based extenders for cryopreservation of dairy bull semen. *Animal Reproduction Science*, *215*, p.106315. https://doi.org/10.1016/j.anireprosci.2020.106315

Mir, S.S., Lone, F.A., Khan, M.Z., Malik, A.A., Islam, R. and Sofi, K.A., 2012. Effect of cold storage period on the quality of ram cauda epididymal spermatozoa recovered postmortem. Turkish Journal of Veterinary & Animal Sciences, 36(6), pp.683-687. https://doi.org/10.3906/vet-1107-21

Mito, T., Yoshioka, K., Yamashita, S., Suzuki, C., Noguchi, M. and Hoshi, H., 2012. Glucose and glycine synergistically enhance the in vitro development of porcine blastocysts in a chemically defined medium. Reproduction, Fertility and Development, 24(3), pp.443-450. https://doi.org/10.1071/RD11197

Monteiro, G.A., Papa, F.O., Zahn, F.S., Dellaqua Jr, J.A., Melo, C.M., Maziero, R.R.D., Avanzi, B.R., Alvarenga, M.A. and Guasti, P.N., 2011. Cryopreservation and fertility of ejaculated and epididymal stallion sperm. *Animal Reproduction Science*, *127*(3-4), pp.197-201. https://doi.org/10.1016/j.anireprosci.2011.08.002

Monteiro, G.A., Guasti, P.N., Rocha, A.S., Martin, I., Sancler-Silva, Y.F.R., Dell'Aqua, C.P.F., Dell'Aqua Jr, J.A. and Papa, F.O., 2013. Effect of storage time and temperature of equine epididymis on the viability, motion parameters, and freezability of epididymal sperm. *Journal of Equine Veterinary Science*, *33*(3), pp.169-173. https://doi.org/10.1016/j.jevs.2012.06.010

Moreira, S.S.J., Lago, A.E.D.A., Moura, A.A.A. and Silva, A.R., 2022. Impact of seminal plasma composition on sperm freezability in wild mammals: a review. *Biopreservation and Biobanking*, *20*(1), pp.90-96. https://doi.org/10.1089/bio.2021.0026

Mortimer, S.T., van der Horst, G. and Mortimer, D., 2015. The future of computer-aided sperm analysis. *Asian Journal of Andrology*, *17*(4), pp.545-553. https://doi.org/10.4103/1008-682X.154312

Moskovtsev, S.I., Dviri, M. and Librach, C.L., 2023. Methods to select ejaculated, epididymal, and testicular spermatozoa for assisted conception. *Men's Reproductive and Sexual Health*

Throughout the Lifespan: An Integrated Approach to Fertility, Sexual Function, and Vitality, *9*, p.204.

Moura, A.A., Memili, E., Portela, A.M.R., Viana, A.G., Velho, A.L.C., Bezerra, M.J.B. and Vasconselos, F.R., 2018. Seminal plasma proteins and metabolites: Effects on sperm function and potential as fertility markers. *Animal Reproduction*, *15*(Suppl 1), p.691. https://doi.org/10.21451/1984-3143-AR2018-0029

Mousavi, S.M., Towhidi, A., Zhandi, M., Amoabediny, G., Mohammadi-Sangcheshmeh, A., Sharafi, M. and Hussaini, S.M.H., 2019. Comparison of two different antioxidants in a nano lecithin-based extender for bull sperm cryopreservation. *Animal Reproduction Science*, *209*, p.106171. https://doi.org/10.1016/j.anireprosci.2019.106171

Murphy, C., Fahey, A.G., Shafat, A. and Fair, S., 2013. Reducing sperm concentration is critical to limiting the oxidative stress challenge in liquid bull semen. *Journal of Dairy Science*, *96*(7), pp.4447-4454. https://doi.org/10.3168/jds.2012-6484

Nazari, H., Ahmadi, E., Hosseini Fahraji, H., Afzali, A. and Davoodian, N., 2021. Cryopreservation and its effects on motility and gene expression patterns and fertilizing potential of bovine epididymal sperm. *Veterinary Medicine and Science*, *7*(1), pp.127-135. https://doi.org/10.1002/vms3.355

Nguyen, H.T., Dang-Nguyen, T.Q., Somfai, T., Men, N.T., Beck-Woerner, B., Viet Linh, N., Xuan Nguyen, B., Noguchi, J., Kaneko, H. and Kikuchi, K., 2021. Excess polyspermy reduces the ability of porcine oocytes to promote male pronuclear formation after in vitro fertilization. *Animal Science Journal*, *92*(1), p.e13650. https://doi.org/10.1111/asj.13650

Oliveira, B.M., Arruda, R.P., Thomé, H.E., Maturana Filho, M., Oliveira, G., Guimarães, C., Nichi, M., Silva, L.A. and Celeghini, E.C.C., 2014. Fertility and uterine hemodynamic in cows after artificial insemination with semen assessed by fluorescent probes. *Theriogenology*, *82*(5), pp.767-772.https://doi.org/10.1016/j.theriogenology.2014.06.007

Pan, B. and Li, J., 2019. The art of oocyte meiotic arrest regulation. Reproductive biology and endocrinology, 17(1), p.8. https://doi.org/10.1186/s12958-018-0445-8

Pantelić, V., Lazarević, M., Barna, T., Petrović, V.C., Maksimović, N., Milovanović, A. and Stojanov, I., 2018. Short-term liquid storage of ram semen in various extenders. South African Journal of Animal Science, 48(4), pp.717-723.https://hdl.handle.net/10520/EJC-1348c6e367

Pamungkas, F., Setiadi, M.A. and Karja, N.W., 2012. Characteristics and in vitro fertilization ability of ram spermatozoa: comparison of epididymal and ejaculated spermatozoa. *Media Peternakan*, *35*(1), pp.38-38. https://doi.org/10.5398/medpet.2012.35.1.38

Pawlak, P., Warzych, E., Cieslak, A., Malyszka, N., Maciejewska, E., Madeja, Z.E. and Lechniak, D., 2018. The consequences of porcine IVM medium supplementation with follicular fluid become reflected in embryo quality, yield and gene expression patterns. *Scientific Reports*, *8*(1), p.15306. https://doi.org/10.1038/s41598-018-33550-4

Perrett, J., Harris, I.T., Maddock, C., Farnworth, M., Pyatt, A.Z. and Sumner, R.N., 2021. Systematic analysis of breed, methodological, and geographical impact on equine sperm progressive motility. Animals, 11(11), p.3088. https://doi.org/10.3390/ani11113088

Pereira, R., Sá, R., Barros, A. and Sousa, M., 2017. Major regulatory mechanisms involved in sperm motility. *Asian Journal of Andrology*, *19*(1), pp.5-14. https://doi.org/10.4103/1008-682X.167716

Pinart, E., Yeste, M., Prieto-Martínez, N., Reixach, J. and Bonet, S., 2015. Sperm quality and fertility of boar seminal doses after 2 days of storage: does the type of extender really matter?. *Theriogenology*, *83*(9), pp.1428-1437. https://doi.org/10.1016/j.theriogenology.2015.01.007

Podico, G. and Canisso, I.F., 2022. Retrograde flushing followed by slicing float-up as an approach to optimize epididymal sperm recovery for the purpose of cryopreservation in equids. Animals, 12(14), p.1802. https://doi.org/10.3390/ani12141802

Prieto-Martínez, N., Bussalleu, E., Garcia-Bonavila, E., Bonet, S. and Yeste, M., 2014. Effects of Enterobacter cloacae on boar sperm quality during liquid storage at 17 °C. *Animal Reproduction Science*, *148*(1-2), pp.72-82. https://doi.org/10.1016/j.anireprosci.2014.05.008

Pyoos, G.M., Maqhashu, A.M., Scholtz, M.M. and Nedambale, T.L., 2018. The comparison of three media on the in vitro maturation rate of pig oocytes. *South African Journal of Animal Science*, *48*(6). https://doi.org/10.4314/sajas.v48i6.4

Redel, B.K., Spate, L.D., Prather, R.S., 2019. *In vitro maturation, fertilization, and culture of pig oocytes and embryos*. In: Herrick, J. (eds) Comparative embryo culture. *Methods in Molecular Biology*, pp.93-103. https://doi.org/10.1007/978-1-4939-9566-0_6

Richani, D., Dunning, K.R., Thompson, J.G. and Gilchrist, R.B., 2021. Metabolic co-dependence of the oocyte and cumulus cells: essential role in determining oocyte developmental competence. *Human reproduction update*, *27*(1), pp.27-47. https://doi.org/10.1093/humupd/dmaa043

Rodriguez-Martinez, H., Martinez, E.A., Calvete, J.J., Peña Vega, F.J. and Roca, J., 2021. Seminal plasma: relevant for fertility? *International Journal of Molecular Sciences*, *22*(9), pp.4368. https://doi.org/10.3390/ijms22094368

Rodriguez-Martinez, H., Roca, J., Alvarez-Rodriguez, M. and Martinez-Serrano, C.A., 2022. How does the boar epididymis regulate the emission of fertile spermatozoa?. *Animal Reproduction Science*, *246*, p.106829. https://doi.org/10.1016/j.anireprosci.2021.106829

Robertson, M.J., Chambers, C., Spanner, E.A., de Graaf, S.P. and Rickard, J.P., 2024. The assessment of sperm DNA integrity: implications for assisted reproductive technology fertility outcomes across livestock species. *Biology*, *13*(7), pp.539. https://doi.org/10.3390/biology1307053

Rochmi, S.E. and Sofyan, M.S., 2019. A diluent containing coconut water, fructose, and chicken egg yolk increases rooster sperm quality at 5 °C. *Veterinary World*, *12*(7), p.1116. https://doi.org/10.14202/vetworld.2019.1116-1120

Romar, R., Funahashi, H. and Coy, P., 2016. In vitro fertilization in pigs: New molecules and protocols to consider in the forthcoming years. *Theriogenology*, *85*(1), pp.125-134. https://doi.org/10.1016/j.theriogenology.2015.07.017

Santiago-Moreno, J., Toledano-Díaz, A., Castaño, C., Velázquez, R., Bóveda, P., O'Brien, E., Peris-Frau, P., Pequeño, B., Martínez-Madrid, B. and Esteso, M.C., 2023. Sperm cryopreservation in wild small ruminants: morphometric, endocrine and molecular basis of cryoresistance. *Animal*, *17*, p.100741. https://doi.org/10.1016/j.animal.2023.100741

Schulze, M., Henning, H., Rüdiger, K., Wallner, U. and Waberski, D., 2013. Temperature management during semen processing: Impact on boar sperm quality under laboratory and field conditions. *Theriogenology*, *80*(9), pp.990-998. https://doi.org/10.1016/j.theriogenology.2013.07.026

Schulze, M., Nitsche-Melkus, E., Hensel, B., Jung, M. and Jakop, U., 2020. Antibiotics and their alternatives in Artificial Breeding in livestock. *Animal Reproduction Science*, *220*, p.106284. https://doi.org/10.1016/j.anireprosci.2020.106284

Sharma, R. and Agarwal, A., 2011. Spermatogenesis: an overview. *Sperm chromatin: biological and clinical applications in male infertility and assisted reproduction*, pp.19-44. https://doi.org/10.1007/978-1-4419-6857-9_2

Shin, D.H. and Turek, P.J., 2013. Sperm retrieval techniques. *Nature Reviews Urology*, *10*(12), pp.723-730. https://doi.org/10.1038/nrurol.2013.262

Silva, H.V.R., Nunes, T.G.P., Ribeiro, L.R., de Freitas, L.A., de Oliveira, M.F., de Assis Neto, A.C., Silva, A.R. and da Silva, L.D.M., 2019. Morphology, morphometry, ultrastructure, and mitochondrial activity of jaguar (Panthera onca) sperm. *Animal Reproduction Science*, *203*, pp.84-93. https://doi.org/10.1016/j.anireprosci.2019.02.011

Skidmore, J.A., 2019. The use of some assisted reproductive technologies in old world camelids. *Animal Reproduction Science*, *207*, pp.138-145. https://doi.org/10.1016/j.anireprosci.2019.06.001

Soler, C. and Cooper, T.G., 2016. Foreword to Sperm morphometrics today and tomorrow special issue in Asian Journal of Andrology. *Asian Journal of Andrology*, *18*(6), pp.815-818. https://doi.org/10.4103/1008-682X.187582

Spiller, C.M. and Bowles, J., 2015. Sex determination in mammalian germ cells. *Asian Journal of Andrology*, *17*(3), pp.427-432. https://doi.org/10.4103/1008-682X.150037

Spiller, C., Koopman, P. and Bowles, J., 2017. Sex determination in the mammalian germline. *Annual Review of Genetics*, *51*(1), pp.265-285. https://doi.org/10.1146/annurev-genet-120215-035449

Staub, C. and Johnson, L., 2018. Spermatogenesis in the bull. *Animal*, *12*, pp.s27-s35. https://doi.org/10.1017/S1751731118000435

Stout, M.A., 2012. *Comparison of epididymal and ejaculated sperm collected from the same holstein bulls*. Louisiana State University and Agricultural Mechanical College. http://dx.doi.org/10.31390/gradschool_dissertations.1160

Strand, J., Ragborg, M.M., Pedersen, H.S., Kristensen, T.N., Pertoldi, C. and Callesen, H., 2016. Effects of post-mortem storage conditions of bovine epididymides on sperm characteristics: investigating a tool for preservation of sperm from endangered species. *Conservation Physiology*, *4*(1), p.cow069.

Su, J., Yang, Y., Zhao, F., Zhang, Y., Su, H., Wang, D., Li, K., Song, Y. and Cao, G., 2023. Study of spermatogenic and Sertoli cells in the Hu sheep testes at different developmental stages. *The Federation of American Societies for Experimental Biology Journal*, *37*(8), p.e23084. https://doi.org/10.1096/fj.202300373R

Suede, S.H., Malik, A. and Sapra, A., 2023. Histology, spermatogenesis. In *StatPearls [Internet]*. StatPearls Publishing. https://www.ncbi.nlm.nih.gov/sites/books/NBK553142/ Accessed 31102025

Sui, H., Sheng, M., Luo, H., Liu, G., Meng, F., Cao, Z. and Zhang, Y., 2023. Characterization of freezability-associated metabolites in boar semen. *Theriogenology*, *196*, pp.88-96. https://doi.org/10.1016/j.theriogenology.2022.11.013

Szablicka, D., Wysokińska, A., Pawlak, A. and Roman, K., 2022. Morphometry of boar spermatozoa in semen stored at 17 °C—the influence of the staining technique. *Animals*, *12*(15), p.1888. https://doi.org/10.3390/ani12151888.

Tamayo-Canul, J., Alvarez, M., López-Urueña, E., Nicolas, M., Martinez-Pastor, F., Anel, E., Anel, L. and De Paz, P., 2011. Undiluted or extended storage of ram epididymal spermatozoa as alternatives to refrigerating the whole epididymes. *Animal Reproduction Science*, *126*(1-2), pp.76-8https://doi.org/10.1016/j.anireprosci.2011.04.011

Teixeira, S.M.P., Chaveiro, A. and Da Silva, F.M., 2015. The effects of three extenders on refrigerated boar semen. South African Journal of Animal Science, 45(1), pp.82-88. https://hdl.handle.net/10520/EJC168703 https://doi.org/10.4314/sajas.v45i1.10

Teixeira, D.O., Silva, H.V.R., Brito, B.F., Barbosa, B.D.S., Tabosa, B.E.D.A. and Silva, L.D.M.D., 2022. Sperm quality and morphometry characterization of cryopreserved canine sperm in ACP-106c or TRIS. Animal Reproduction, 19(3), p.e20210069. https://doi.org/10.1590/1984-3143-AR2021-0069

Thema, M.A., Mphaphathi, M.L., Ledwaba, M.R. and Nedambale, T.L., 2022. Investigation of the efficacy of different semen extenders and in vitro storage period on Windsnyer boar sperm

quality equilibrated at 18 °C. *Reproduction in Domestic Animals*, *57*(12), pp.1644-1648. https://doi.org/10.1111/rda.14256

Thema, M.A., Mphaphathi, M.L., Ledwaba, M.R. and Nedambale, T.L., 2023. Sperm cryopreservation in Windsnyer boars; principles, technique, and updated outcomes. *Animal Reproduction*, *20*(3), p.e20220100. https://doi.org/10.1590/1984-3143-AR2022-0100

Thuwanut, P., Srisuwatanasagul, S., Wongbandue, G., Tanpradit, N., Thongpakdee, A., Tongthainan, D., Manee-In, S. and Chatdarong, K., 2013. Sperm quality and the morphology of cryopreserved testicular tissues recovered post-mortem from diverse wild species. *Cryobiology*, *67*(2), pp.244-247. https://doi.org/10.1016/j.cryobiol.2013.07.002

Tittarelli, C.M., Jurado, S.B., Nuñez-Favre, R., Bonaura, M.C., de la Sota, R.L. and Stornelli, M.A., 2012. Effect of storage media and storage time on histological and ultrastructural changes in cat epididymal cells. *Reproduction in Domestic Animals*, *47*, pp.281-283. https://doi.org/10.1111/rda.12073

Tiwana, M.S. and Leslie, S.W., 2022. Anatomy, abdomen and pelvis, testicle. *StatPearls [Internet]. Treasure Island (FL), StatPearls Publishing*. Available from: https://www.ncbi.nlm.nih.gov/books/NBK470201/.

Tomás, C., Gómez-Fernández, J., Gómez-Izquierdo, E. and de Mercado, E., 2014. Effect of the holding time at 15 °C prior to cryopreservation, the thawing rate and the post-thaw incubation temperature on the boar sperm quality after cryopreservation. *Animal Reproduction Science*, *144*(3-4), pp.115-121. https://doi.org/10.1016/j.anireprosci.2013.12.011

Toyonaga, M., Kaihara, A. and Tsutsui, T., 2011. The quality of cryopreserved sperm collected from feline caudal epididymides stored at room temperature. *Journal of Veterinary Medical Science*, *73*(10), pp.1395-1398. https://doi.org/10.1292/jvms.11-0052

Trigg, N.A., Eamens, A.L. and Nixon, B., 2019. The contribution of epididymosomes to the sperm small RNA profile. *Reproduction*, *157*(6), pp.R209-R223. https://doi.org/10.1530/REP-18-0480

Turri, F.E.D.E.R.I.C.A., Madeddu, M., Gliozzi, T.M., Gandini, G. and Pizzi, F., 2012. Influence of recovery methods and extenders on bull epididymal spermatozoa quality. *Reproduction in Domestic Animals*, *47*(5), pp.712-717. https://doi.org/10.1111/j.1439-0531.2011.01948.x

Turri, F., Madeddu, M., Gliozzi, T.M., Gandini, G. and Pizzi, F., 2014. Effect of testicle postmortem storage on goat frozen-thawed epididymal sperm quality as a tool to improve genebanking in local breeds. *Animal*, *8*(3), pp.440-447.https://doi.org/10.1017/S1751731113002279

Tvrdá, E., Bučko, O., Rojková, K., Ďuračka, M., Kunová, S., Kováč, J., Benko, F. and Kačániová, M., 2021. The efficiency of selected extenders against bacterial contamination of boar semen in a swine breeding facility in Western Slovakia. *Animals*, *11*(11), p.3320. https://doi.org/10.3390/ani11113320

Valverde, A., Barquero, V. and Soler, C., 2020. The application of computer-assisted semen analysis (CASA) technology to optimise semen evaluation. A review. *Journal of Animal and Feed Sciences*, *29*(3), pp.189-198. https://doi.org/10.22358/jafs/127691/2020

van der Horst, G. and Maree, L., 2010. SpermBlue®: A new universal stain for human and animal sperm which is also amenable to automated sperm morphology analysis. *Biotechnic and Histochemistry*, *84*(6), pp.299-308.https://doi.org/10.3109/10520290902984274

van der Horst, G., Maree, L. and du Plessis, S.S., 2018. Current perspectives of CASA applications in diverse mammalian spermatozoa. *Reproduction, Fertility and Development*, *30*(6), pp.875-888. https://doi.org/10.1071/RD17468.

van der Horst, G., 2021. Status of sperm functionality assessment in wildlife species: from fish to primates. *Animals*, *11*(6), p.1491.https://doi.org/10.3390/ani11061491

Vieira, L.A., Gadea, J., García-Vázquez, F.A., Avilés-López, K. and Matás, C., 2013. Equine spermatozoa stored in the epididymis for up to 96 h at 4 °C can be successfully cryopreserved and maintain their fertilization capacity. *Animal Reproduction Science*, *136*(4), pp.280-288. https://doi.org/10.1016/j.anireprosci.2012.10.027

Vílchez, M.C., Morini, M., Peñaranda, D.S., Gallego, V., Asturiano, J.F. and Pérez, L., 2016. Sodium affects the sperm motility in the European eel. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 198, pp.51-58. https://doi.org/10.1016/j.cbpa.2016.04.008

Villaverde, A.I.S.B., Fioratti, E.G., Penitenti, M., Ikoma, M.R., Tsunemi, M.H., Papa, F.O. and Lopes, M.D., 2013. Cryoprotective effect of different glycerol concentrations on domestic cat spermatozoa. *Theriogenology*, *80*(7), pp.730-737. https://doi.org/10.1016/j.theriogenology.2013.06.010

Vozaf, J., Svoradová, A., Baláži, A., Vašíček, J., Olexiková, L., Dujíčková, L., Makarevich, A.V., Jurčík, R., Ďúranová, H. and Chrenek, P., 2022. The cryopreserved sperm traits of various ram breeds: towards biodiversity conservation. *Animals*, *12*(10), p.1311. https://doi.org/ 10.3390/ani12101311 Waberski, D., Suarez, S.S. and Henning, H., 2022. Assessment of sperm motility in livestock: Perspectives based on sperm swimming conditions in vivo. Animal reproduction science, 246, p.106849. https://doi.org/10.1016/j.anireprosci.2021.106849

Waberski, D. and Luther, A.M., 2024. Boar semen storage at 5 C for the reduction of antibiotic use in pig insemination: Pathways from science into practice. *Animal reproduction science*, *269*, p.107486. https://doi.org/10.1016/j.anireprosci.2024.107486

Wan, Y., Yang, S., Li, T., Cai, Y., Wu, X., Zhang, M., Muhammad, T., Huang, T., Lv, Y., Chan, W.Y. and Lu, G., 2023. <? mode longmeta?> LSM14B is essential for oocyte meiotic maturation by regulating maternal mRNA storage and clearance. *Nucleic Acids Research*, *51*(21), pp.11652-11667. https://doi.org/10.1093/nar/gkad919

Wang, Y., Zhang, Y., Li, T., Ren, Y., Zhou, P., Fu, L., Xiao, C., Huang, Z., Huang, H., Xie, W. and Luo, Y., 2025. Transcriptional insights on the incomplete cytoplasmic maturation and developmental potential of oocytes cultured without granulosa cells in mice. *BMC genomics*, *26*(1), p.270.https://doi.org/10.1186/s12864-025-11455-7

Wasilewska, K., Zasiadczyk, Ł., Fraser, L., Mogielnicka-Brzozowska, M. and Kordan, W., 2016. The benefits of cooling boar semen in long-term extenders prior to cryopreservation on sperm quality characteristics. *Reproduction in Domestic Animals*, *51*(5), pp.781-788. https://doi.org/10.1111/rda.12751Wysokińska, A., Wójcik, E. and Chłopik, A., 2021. Evaluation of the morphometry of sperm from the epididymides of dogs using different staining methods. *Animals*, *11*(1), p.227. https://doi.org/10.3390/ani11010227

Xia, P., 2013. Biology of polyspermy in IVF and its clinical indication. *Current Obstetrics and Gynecology Reports*, *2*(4), pp.226-231. https://doi.org/10.1007/s13669-013-0059-2

Yánez-Ortiz, I., Catalán, J., Rodríguez-Gil, J.E., Miró, J. and Yeste, M., 2022. Advances in sperm cryopreservation in farm animals: Cattle, horse, pig and sheep. *Animal Reproduction Science*, *246*, pp.106904 https://doi.org/10.1016/j.anireprosci.2021.106904

Yániz, J.L., Vicente-Fiel, S., Capistrós, S., Palacín, I. and Santolaria, P., 2012. Automatic evaluation of ram sperm morphometry. *Theriogenology*, *77*(7), pp.1343-1350. https://doi.org/10.1016/j.theriogenology.2011.10.039

Yániz, J.L., Capistrós, S., Vicente-Fiel, S., Soler, C., de Murga, J.N. and Santolaria, P., 2014. Study of nuclear and acrosomal sperm morphometry in ram using a computer-assisted sperm morphometry analysis fluorescence (CASA-F) method. *Theriogenology*, *82*(6), pp.921-924. https://doi.org/10.1016/j.theriogenology.2014.06.017

Yániz, J.L., Soler, C. and Santolaria, P., 2015. Computer assisted sperm morphometry in mammals: A review. *Animal Reproduction Science*, *156*, pp.1-12. https://doi.org/10.1016/j.anireprosci.2015.03.002

Yeste, M., Barrera, X., Coll, D. and Bonet, S., 2011. The effects on boar sperm quality of dietary supplementation with omega-3 polyunsaturated fatty acids differ among porcine breeds. *Theriogenology*, *76*(1), pp.184-196. https://doi.org/10.1016/j.theriogenology.2011.01.032

Yeste, M., Estrada, E., Rivera del Alamo, M.M., Bonet, S., Rigau, T. and Rodriguez-Gil, J.E., 2014. The increase in phosphorylation levels of serine residues of protein HSP70 during holding time at 17 °C is concomitant with a higher cryotolerance of boar spermatozoa. *PLoS One*, *9*(3), p.e90887. https://doi.org/10.1371/journal.pone.0090887

Yeste, M., Rodríguez-Gil, J.E. and Bonet, S., 2017. Artificial insemination with frozen-thawed boar sperm. *Molecular Reproduction and Development*, *84*(9), pp.802-813. https://doi.org/10.1002/mrd.22840

Yeste, M., 2018. State-of-the-art of boar sperm preservation in liquid and frozen state. *Animal Reproduction*, *14*(1), pp.69-81. https://doi.org/10.21451/1984-3143-AR895

Yoshioka, K., Noguchi, M. and Suzuki, C., 2012. Production of piglets from in vitro-produced embryos following non-surgical transfer. *Animal Reproduction Science*, *131*(1-2), pp.23-29. https://doi.org/10.1016/j.anireprosci.2012.01.018

Yuan, Y. and Krisher, R.L., 2011. In vitro maturation (IVM) of porcine oocytes. In *Germline Development: Methods and Protocols* (pp. 183-198). New York, NY: Springer New York. https://doi.org/10.1007/978-1-61779-436-0_14

Zheng, Y., Gao, Q., Li, T., Liu, R., Cheng, Z., Guo, M., Xiao, J., Wu, D. and Zeng, W., 2022. Sertoli cell and spermatogonial development in pigs. *Journal of Animal Science and Biotechnology*, *13*(1), pp.45. https://doi.org/10.1186/s40104-022-00687-2

Chapter 3: Optimising boar epididymal sperm preservation: comparative analysis of slicing float-up vs. flushing and BTS vs. Andromed® extenders on sperm motility and morphometric traits

M.A. Thema, M.L. Mphaphathi, G. van der Horst, L. Maree, M.D Sebopela, M.R. Ledwaba and N.R Mkhize., 2025. Optimising boar epididymal sperm preservation: comparative analysis of slicing float-up vs. flushing and BTS vs. Andromed® extenders on sperm motility and morphometric traits. American Journal of Animal and Veterinary Sciences, 20(4), 328-337. https://doi.org/10.3844/ajavsp.2025.328.337.

Abstract

This study aimed to compare slicing float-up vs. flushing and Beltsville Thawing Solution (BTS) vs. Andromed® extenders on boar epididymal sperm motility and morphometry traits. A total of 40 testes from heterogeneous boars were collected at the local abattoir and transported to the laboratory at 5 °C within 30 minutes after slaughter. Semen samples were retrieved from the cauda part of the epididymis using the slicing float-up vs flushing method and diluted with BTS vs Andromed® extender. A total of 800 sperm/treatments were analysed, for sperm morphometry traits and two fields/treatments were captured for sperm motility using computer-assisted sperm class analyser. The data were analysed using analysis of variance. Semen retrieved with a flushing method and diluted with Andromed® extender recorded the least sperm total (29.7%), progressive (4.9%), and rapid (1.24%) motilities. Highest sperm total (85.0%), progressive (57.7%) and rapid (31.8%) motility were recorded in semen retrieved with slicing float-up and diluted with BTS. There was a reduction in the sperm head dimensions, acrosome and 22.5% of pyriform sperm shape associated with Andromed® as compared to the BTS extender ($P<0.05$). The sperm diluted with Andromed® extender recorded to have undergone a premature acrosome reaction, which influenced the sperm head sizes. The current study recorded acceptable average sperm head size (length: 8.2-8.9 µm; perimeter: 18.2-19.9 µm; width: 4.1-4.6 µm and area: 34.8-38.6 μm^2), which fell within the normal range, irrespective of the semen retrieval method and extenders used. In conclusion, slicing float-up was recorded to be the better method for retrieving boar epididymal sperm using the BTS extender.

Keyword: Conservation, retrieval method, male reproduction

3.1 Introduction

Semen is commonly collected through ejaculation for breeding and preservation. However, that cannot be possible in all species or every situation, such as in instances of unexpected death or after loss of testes (Strand et al., 2016). Animals may die suddenly, far away from the laboratory where the semen sample could be handled and preserved (Turri et al., 2012). As a result, semen collection from the epididymis may be the only method available to preserve male gametes from animals with superior traits (Bertol, 2016). Therefore, determining the most effective retrieval method could help obtain the

epididymal semen of boars possessing superior traits. Several semen retrieval methods have been reported in the literature, and they differ based on the authors, species, and semen extenders used.

Semen can be retrieved post-mortem from the distal epididymis in a few different ways, including flushing, slicing float-up, or mincing (Stout, 2012). The flushing technique is commonly characterised by low blood contamination risk (Podico & Canisso, 2022) but may be challenging in obtaining a high sperm yield for procedures such as artificial insemination (Auer et al., 2022). The flushing method commonly works best, and it is used to retrieve bull epididymal semen (Monaco et al., 2024). In some cases, the cuts method might be more practical, but to improve sample condition, caution must be taken to ensure a quick extension of the sample and to prevent contamination (Bertol, 2016). Epididymal slicing float-up (Ali Hassan et al., 2023) and mincing (Podico & Canisso, 2022) have been suggested as a backup option when the flushing method is not feasible. Conversely, slicing float-up presents high sperm yield but may result in high blood contamination if not done accordingly (Falomo et al., 2016). The slicing float-up is commonly used to retrieve epididymal semen in the equidae species (Huijsmans et al., 2023). The mincing method involves cutting both the cauda epididymis and the ductus deferens several times in a petri dish to retrieve semen (Hori et al., 2015). Semen samples retrieved by mincing are often characterised by blood and cell debris, potentially causing negative effects on the quality and cryo-tolerance of the sperm (Monaco et al., 2024).

Dilution of semen extracted from the epididymides may provide the sperm with additional protection during the cooling process or preservation (Fernandez-Novo et al., 2021). Semen extenders are typically biological compounds that serve a variety of purposes, such as mitigating the effects of abrupt pH changes, preventing the sperm from oxidative stress, and damage during preservation (Bustani & Baiee, 2021). Boar semen extenders, like Beltsville Thawing Solution (BTS), have been widely used in boar semen because they contain a high concentration of antibiotics (penicillin and streptomycin) (2.20 g/L) that can treat a wide range of infections, lessen, and regulate bacterial contamination during preservation (Thema et al., 2022). The BTS extender provides the sperm with protection against oxidative damage, maintains quality and minimises the bacterial contamination during preservation (Schulze, et al., 2017). However, study by Basumatary et al. (2024) reported that, BTS extender had a negative effect on the DNA integrity of the boar sperm. Andromed® is a soya lecithin-based extender typically used as a bull semen extender and has resulted in a higher proportion of sperm with higher superoxide levels. Andromed® extender provides additional energy and nutrients that may fortify sperm against cold shock during preservation (Pérez-Durand et al., 2024). The Andromed® has been identified as a non-permeable cryoprotectant and alternative to egg yolk for the cryopreservation of bovine sperm (Akhter et al., 2012). The existence of BTS and Andromed® extenders with such characteristics would provide a valuable contribution to the porcine artificial insemination industry and improve the protocol for cryopreserving boar sperm.

Therefore, understanding the morphometric and motility traits of boar epididymal sperm after retrieval is essential for assessing their fertility potential and optimising reproductive outcomes. Sperm morphometric analysis provides insights into details of the sperm size, shape, function, and structural characteristics, which correlate with fertility potential (Yániz et al., 2015). The availability of sophisticated systems and tools, like computer-assisted sperm analyser (CASA), has made it possible to analyse sperm morphometry and motility more quickly and objectively (Amann & Waberski, 2014). The CASA system has emerged as the preferred method due to its reduced subjectivity, increased repeatability, and reliability (Yániz et al., 2015). Male sperm morphometry, motility, and fertility have all been successfully correlated using the CASA systems (Maroto-Morales, 2015; Maroto-Morales, 2016). The CASA system makes accurate sperm measurements and automatically detects the acrosome, head, and midpiece of the sperm (Mortimer et al., 2015). Dziekońska et al. (2022), reported that sperm obtained from the epididymis cauda exhibit distinct sperm motility, morphometry, membrane integrity, and DNA integrity. Sperm motility is a crucial attribute linked to the ability of sperm to fertilise, signifying their structural integrity and viability (Nagy et al., 2015). To our knowledge, this is the first study to compare the sperm motility and morphometry of boar epididymal semen retrieved with slicing float-up vs flushing and diluted with BTS vs Andromed® extenders. Only a few studies have compared these two semen retrieval methods on boar epididymal sperm quality while using the BTS and other extenders. The novelty of this study lies in a factorial approach: examining two semen retrieval methods (slicing float-up and flushing) and semen extenders (BTS and Andromed®) on boar epididymal sperm motility and morphometric features to predict reproductive success. This study aimed to compare slicing float-up vs. flushing and Beltsville Thawing Solution (BTS) vs. Andromed® extenders on boar epididymal sperm motility and morphometry traits. It was hypothesised that sperm retrieval method as well as the semen extenders will affect the viability and quality of boar epididymal sperm motility and morphometric traits.

3.2 Materials and methods

3.2.1 Study area and design

The testes were collected from the local abattoir (Renbro Abattoir, South Africa), and the study was conducted at the Germplasm Conservation and Reproductive Biotechnologies section (Agricultural Research Council), located in Pretoria, Gauteng Province. The data and study area are located at 25º 53′ 59.6″ South latitude and 28º 12′ 51.6″ longitude in Pretoria, South Africa. The entire collection took a period of 2 months. A total of 4 testes/day of heterogeneous boars were collected from the local abattoir twice a week. The collection was replicated 10 times/treatments (4x10=40) with each testes from each day of collection were randomly assigned to each treatment.

3.2.2 Statement of animal rights

The experimental procedures were performed in accordance with the ethical standards of the approved by Agricultural Research Council-Animal Production Ethics Committee [APIEC 23/05], the University of KwaZulu-Natal Research Ethics Committee [AREC/00007100/2024] and Section 20 of the Animal Diseases Act no 35 of 1984 [12/11/1/18 (6269BM)].

3.2.3 Composition and preparation of boar semen extenders

The semen extenders used in the study were prepared according to the compositions provided in Table 3.1 and stored at 5 °C.

Table 3.1: Composition of the boar semen extenders/litre

Beltsville Thawing Solution (Thema et al., 2022)	**Concentration**	**Andromed® (Auer et al., 2022)**	**Concentration**
Glucose	37 g/L	Fructose	1.25 g/L
Tri-sodium citrate	6 g/L	Citric acid	1 g/L
EDTA	1.25 g/L	Buffer	1 g/L
Sodium bicarbonate	1.25 g/L	Phospholipids	1 g/L
Spectinomycin	0.75 g/L	Spectinomycin	0.343 g/L
Penicillin	1.10 g/L	Lincomycin	0.172 g/L
Potassium chloride	1.10 g/L	Tylosin	0.57 g/L,
Gentamycin	-	Gentamicin	0.286 g/L

EDTA: Ethylenediaminetetra acetic.

3.2.4 Boar testes collection and sperm retrieval from the epididymis

A total of 40 testes of heterogeneous boars were collected (4 testes/day) from the local abattoir and transported to the laboratory at 5 °C within 30 minutes after slaughter. The testes were further stored at 5 °C for an additional 2 h before being processed. Cooling was used in this investigation as a conventional preservation procedure to limit variability related to post-mortem degeneration and to guarantee comparable sperm quality across recovered sperm samples (Nichi et al., 2017). Sperm quality is maintained by storing epididymides at 5 °C, which lowers metabolic activity while slows down the degenerative processes as supported by Monteiro et al. (2013). Following storage, the cauda epididymides were removed from the testes and cleaned with warm (37 °C) saline solution (NaCl 0.9%). To avoid blood contamination, superficial blood vessels were punctured so that most of the blood could be wiped off (Turri et al., 2012). Before semen retrieval, a total of 20 cauda epididymides were equally

allocated for flushing vs. slicing float-up and BTS vs. Andromed® extender in the separate petri dishes warmed at 37 °C. The semen was retrieved from the cauda part of the epididymis using the flushing vs slicing float-up method and diluted with BTS vs Andromed®. The flushing method involves inserting a blunted needle into the ductus deferens and pushing the extender in the opposite direction of the physiological sperm through the cauda epididymis (Esteves et al., 2011). The slicing float-up procedure involves using a scalpel blade to slice the cauda part of the epididymis to release the sperm in a petri dish, suspend it with 5 mL of a warm extender (37 °C), and gently agitate the mixture for approximately 10 minutes (Turri et al., 2012; Falomo et al., 2016).

3.2.5 Evaluations of the boar epididymal sperm motility

A total of 5µL of retrieved semen with the flushing vs. slicing float-up diluted with BTS vs. Andromed® transferred to a warmed microscope glass slide (76x26x1mm-WadmarKnittel, Germany) and covered with a warm coverslip (22x22 mm-Wadmar-Knittel, Germany) on a CASA (Microptin, Spain) phase contrast microscope (Nikon®, Japan), warm plate (Omron®, Japan) adjusted to 37 °C. Sperm motility traits [total motility (TM), progressive motility (PM), rapid (RAP), medium (MED), static] and velocity traits [curvilinear velocity (VCL), straight line velocity (VSL), average pathway velocity (VAP), linearity (LIN), straightness (STR), wobble (WOB), the amplitude of lateral head (ALH) and beat cross frequency (BCF)] were analysed under 10X magnification. Two fields with an average of 250 sperm and a final sperm concentration of approximately 34.3×10^6/mL/treatment/replication were captured at 10X magnification (Nikon®, Japan) during analysis using the CASA system.

3.2.6 Boar epididymal sperm preparation with the stain for sperm morphometry analysis

Following semen retrieval with the use of flushing and slicing float-up method, samples were transferred to 15 mL tubes as per different semen extenders, namely BTS and Andromed®. For sperm morphometry analysis, semen samples were diluted at a ratio of 1:10 with BTS or Andromed® extenders. Diluted semen samples were further fixed with 4% Formaldehyde for 10 minutes. Furthermore, a total of 10 µL of each fixed semen sample per retrieval method and extender was smeared on a separate slide and allowed to air dry overnight at room temperature. For staining, the dried sample on a slide was gently dipped in the SpermBlue® tray for 2 minutes and dipped in distilled water for 3 seconds to remove debris and ensure a clean background. A paper towel was used to wipe the back of the slide and let the slide air dry at room temperature. Following drying, the slide was mounted using Dibutylphthalate Polystyrene Xylene, covered with the coverslips and evaluated for sperm morphometry traits.

3.2.7 Boar epididymal sperm morphometry analysis

Dried slides were analysed for sperm morphometry using CASA software (Nikon, Tokyo, Japan). This software involved capturing the sperm, from a stained sperm smear using brightfield microscopy, under 40X magnification, and various morphometric traits relating to the sperm head, acrosome, and midpiece. At least a total of 80 sperm per retrieval technique/extender was analysed and a total of 3200 sperm were measured/replicated 10 times (80 x 10 x 4=3200). The sperm head area (A), head perimeter (P), head length (L), head width (W), head ellipticity (length/width), head elongation (L-W/L+W), head regularity (πx (L.W/4.A), head roughness [4π x (A/P^2)], midpiece width and the shape indices (head shape type, type of the sperm and midpiece size) were measured.

3.2.8 Statistical analysis

Sperm morphometric data were divided into two groups. The first group was the measurements of sperm head primary traits (length, L; area, A; width, W, and perimeter, P), derived traits (ellipticity, L/W; rugosity, 4πA/P2; elongation, L − W/L + W and regularity, πLW/4A). In the first sperm morphometric data group, descriptive statistics were used to establish the minimum and maximum values (threshold) using 10-90 percentiles for every morphometric trait in this group shown in Table 3.2.

Table 3.2 Minimum and maximum acceptable values for morphometric traits in order to define normal boar epididymal sperm morphometry.

	Boar	
Sperm morphometric traits	**Minimum**	**Maximum**
Length (µm)	8.20	9.70
Width (µm)	3.50	5.50
Perimeter (µm)	16.20	26.20
Area (µm)	28.49	44.00
Ellipticity	1.81	2.16
Regularity	0.70	1.00
Elongation	0.29	0.37
Roughness	0.62	1.50
Midpiece width (µm)	0.50	1.60

The data for the first sperm morphometric data group and sperm motility trait (TM, PM, RAP, MED, static, VCL, VSL, VAP, LIN, STR, WOB, ALH, and BCF) were analysed with (ANOVA) using general linear model procedure. The main independent variables being sperm retrieval methods (flushing vs. slicing float-up) and the type of extenders (Andromed® and BTS) and their interaction. The total of 40

epididymal sperm sample were collected from 40 individual testes [10 replication per treatment (each sperm retrieval method vs semen extender)]. Since each sample was exclusive to a single treatment combination, replicates were not regarded as blocking variables. The Least Significant Difference (LSD) test was used to conduct pairwise multiple comparisons across the four treatment combinations when a significant interaction or main effect was found. The LSD t-test were calculated at a 5% significance level to compare means of significant source effect of semen extenders and sperm retrieval method on the sperm morphometric first group and motility traits. In tables, statistically significant differences between groups at $P<0.05$ are shown by different superscript letters (a, b, and ab).

The second sperm morphometric data group was analysed using a Chi-square test to determine the effect of flushing vs. slicing float-up and BTS vs. Andromed® on the sperm percentage variations regarding the sperm with normal head, acrosome, midpiece and abnormal head shape (amorphous, pyriform, tapered). The ANOVA and Chi-Square analysis was performed using SAS version 9.4 statistical software.

3.3 Results

The effect of semen retrieval methods semen extender on boar epididymal sperm motility is presented in Table 3.3. There was significant interaction between sperm retrieval method and semen extender type for TM, PM, RAP, static, MED, VCL, VAP, VSL, STR, LIN, ALH, BCF ($P<0.05$), suggesting that the impact of semen extender type varied based on the retrieval method. BTS extender recorded significantly higher sperm progressive motility (57.7%) compared to the flushing with BTS extender (22.1%) while other treatment did not differ significantly. Sperm diluted with Andromed® recorded lowest sperm total (47.4 and 29.6%) and progressive (13.8 and 4.9%) motility in both slicing float-up and flushing technique, respectively. The different superscript letters in the table using the LSD test showed that these differences among treatment combinations were statistically significant ($P<0.05$).

Table 3.3: Effect of boar epididymal semen retrieval methods and extenders on sperm motility using CASA (mean ± SD).

Sperm motility trait	Slicing float-up		Flushing		Interaction P Value	Effect of retrieval method P-Value	Effect of extender media P-Value
	BTS	Andromed®	BTS	Andromed®			
TM (%)	85.0±10.3[a]	47.4±5.6[c]	60.8±5.4[b]	29.6±9.7[d]	**<0.05**	**<0.05**	**<0.05**
PM (%)	57.7±19.2[a]	13.8±8.4[bc]	22.1±9.2[b]	4.9±3.8[c]	**<0.05**	**<0.05**	**<0.05**
NPM (%)	27.3±10.4[bc]	33.6±7.7[ab]	38.7±6.6[a]	24.7±7.7[c]	**<0.05**	**>0.05**	**>0.05**
RAP (%)	31.8±16.8[a]	3.9±5.5[b]	9.3±6.3[b]	1.2±2.2[b]	**<0.05**	**<0.05**	**<0.05**
MED (%)	29.5±7.7[a]	13.9±5.4[c]	21.8±4.9[b]	7.8±5.4[d]	**<0.05**	**<0.05**	**<0.05**
SLW (%)	20.4±8.7[b]	29.6±9.1[a]	29.8±7.2[a]	20.6±5.2[b]	**<0.05**	**>0.05**	**>0.05**
Static (%)	14.9±10.3[d]	52.6±5.6[b]	39.2±5.4[c]	70.3±9.7[a]	**<0.05**	**<0.05**	**<0.05**
VAP (µm/sec)	21.1±4.5[a]	13.2±5.5[b]	15.1±4.6[b]	11.2±3.8[b]	**<0.05**	**<0.05**	**<0.05**
VCL (µm/sec)	42.1±9.3[a]	25.1±6.6[bc]	29.9±5.6[b]	23.3±4.6[c]	**<0.05**	**<0.05**	**<0.05**
VSL (µm/sec)	12.6±3.6[a]	7.7±3.7[b]	7.6±3.1[b]	4.1±1.6[c]	**<0.05**	**<0.05**	**<0.05**
STR (%)	59.0±7.6[a]	54.4±11.5[a]	50.7±8.6[a]	40.9±9.8[b]	**<0.05**	**<0.05**	**<0.05**
LIN (µm/sec)	31.6±7.5[a]	29.1±6.9[a]	26.3±8.9[a]	17.4±6.8[b]	**<0.05**	**<0.05**	**<0.05**
WOB (%)	51.6±5.6[a]	51.2±11.8[a]	51.3±9.9[a]	46.9±10.2[a]	**>0.05**	**>0.05**	**>0.05**
BCF (Hz)	6.5±1.8[a]	3.2±1.8[b]	3.6±1.1[b]	2.7±1.8[b]	**<0.05**	**<0.05**	**<0.05**
ALH (µm/sec)	1.5±0.2[a]	0.9±0.1[c]	1.1±0.1[b]	0.9±0.2[c]	**<0.05**	**<0.05**	**<0.05**

[a-d] Values with different superscripts differ significantly in the same row ($P<0.05$). TM: total motility, PM: progressive motility, RAP: rapid motility, MED: medium motility, SLW: slow motility, VCL: curvilinear velocity, VSL: straight line velocity, VAP: average pathway velocity, LIN: linearity, STR: straightness, WOB: wobble, ALH: amplitude of lateral head, BCF: beat cross frequency, BTS: Beltsville Thawing Solution.

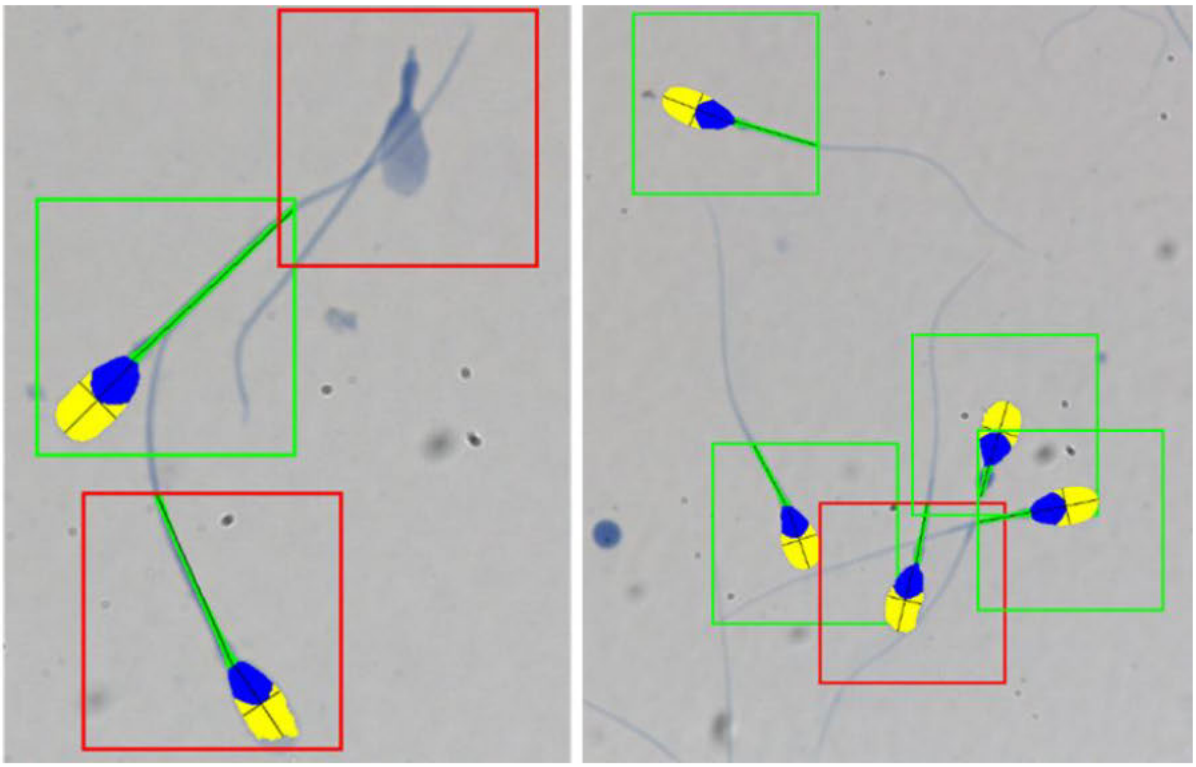

Figure 3.1: Computer-assisted sperm morphometry analysis of post-culled boar epididymal sperm morphometry using two semen retrieval methods and extenders.

The CASA system recognizes the acrosome (yellow), head (blue), and midpiece (green) of the sperm is represented in Figure 3.1. Morphologically normal sperm are enclosed in the green box, while morphologically abnormal sperm are enclosed in the red box.

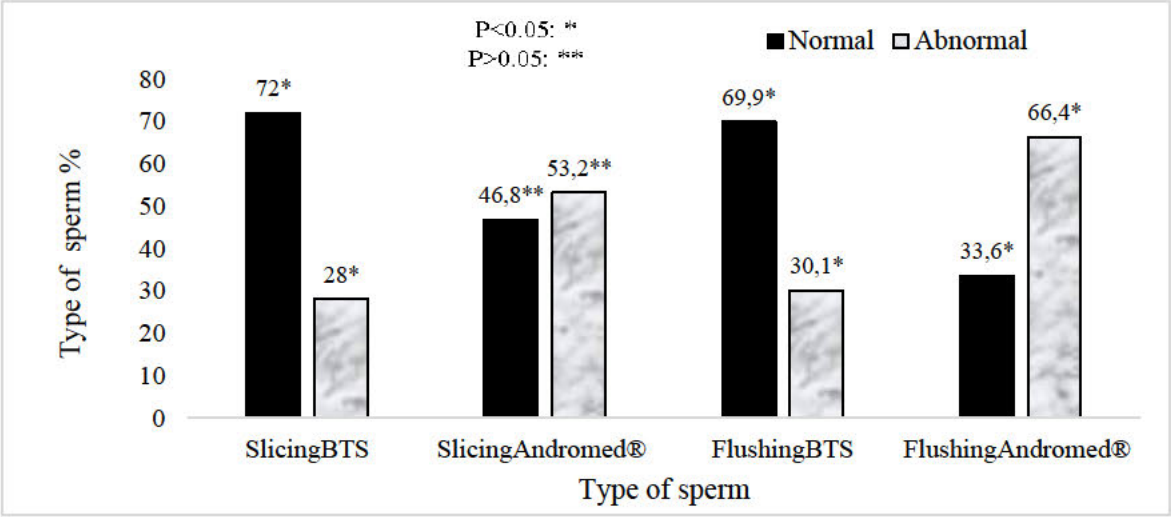

Figure 3.2: The effect of semen retrieval method and extenders on the type of sperm percentage using CASA. BTS: Beltsville Thawing Solution.

The significant difference (P<0.05) is represented by a star and non significant difference (P>0.05) is represented by two stars within the bars.

Figure 3.2 represents the effect of the semen retrieval method and extenders on the percentage of morphologically normal using CASA. The semen retrieved with the slicing float-up method and diluted with BTS extender recorded greater than 70% normal sperm compared to the Andromed® extender ($P<0.05$). An average of 31.9% abnormal sperm was recorded in the semen retrieved with the flushing method, irrespective of the type of semen extender used. The normal sperm percentage in the current study was affected by several factors, such as the shape of the sperm head, the type of sperm acrosome and the midpiece (Figure 3.3-3.4).

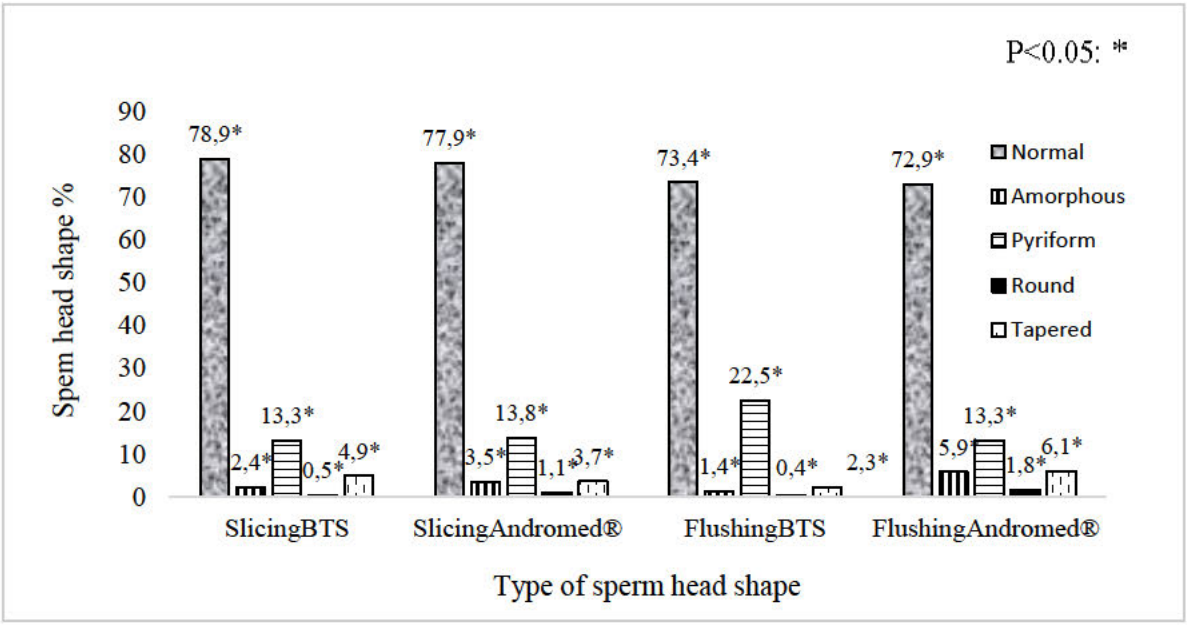

Figure 3.3: The effect of semen retrieval method and extenders on the type of sperm head shape using CASA. BTS: Beltsville Thawing Solution.
The significant difference ($P<0.05$) is represented by a star within the bars.

Figure 3.3 represents the effect of the semen retrieval method and extenders type on sperm head shape using CASA. Greater than 70% of sperm were recorded as normal shape and matured irrespective of the semen retrieval method. The results showed between 10-22.5% sperm with pyriform shape was recorded irrespective of the semen extender and retrieval method. Less than 6.0% of sperm with tapered and amorphous shapes were recorded irrespective of the semen retrieval method or extender. Moreover, an average of 0.9% of sperm with a round shape was recorded irrespective of semen retrieval method and extenders.

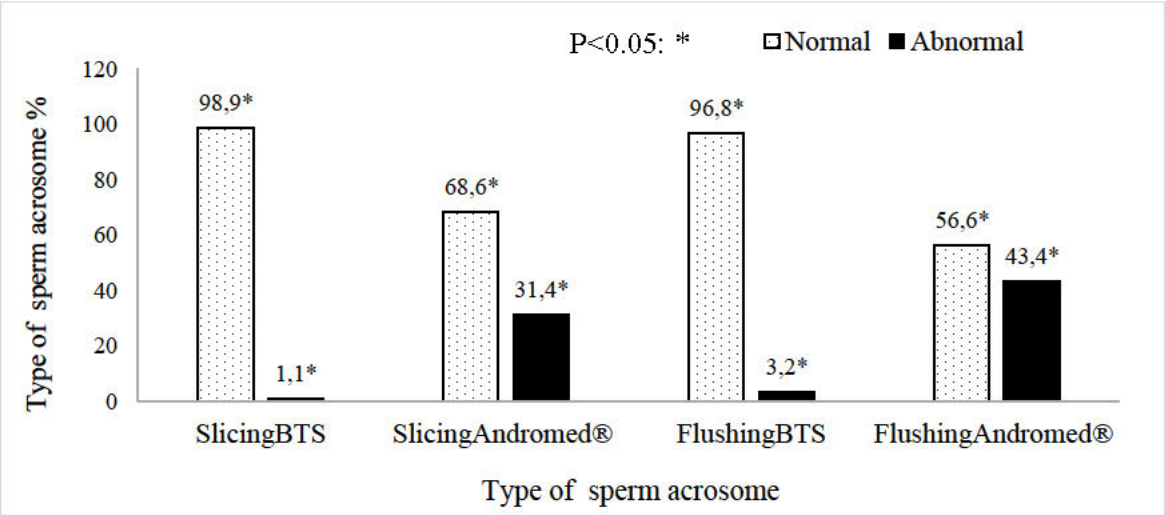

Figure 3.4: The effect of semen retrieval method and extenders on the type of sperm acrosome using CASA.

The significant difference (P<0.05) is represented by a star within the bars.

The effect of the semen retrieval method and extenders on the type of sperm acrosome using CASA is represented in Figure 3.4. There was a reduction in the sperm head dimensions and a change in the shape of the acrosome associated with the type of semen extender (P<0.05). The sperm diluted with Andromed® extender were shown to have undergone an acrosome reaction, which caused the sperm head length to be shorter than usual. The sperm diluted with Andromed® extender recorded less than 70% sperm with normal acrosome, irrespective of the semen retrieval method, compared to BTS extender treatment (P<0.05). Greater than 90% sperm with normal acrosome were recorded in the semen diluted with BTS, irrespective of the semen retrieval method, than Andromed® extender treatment (P<0.05).

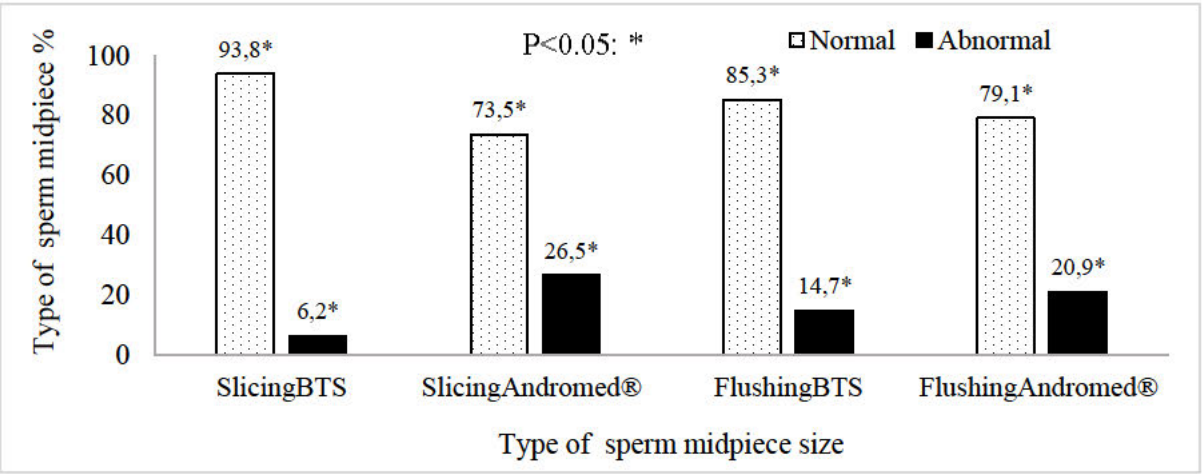

Figure 3.5: Effect of boar epididymal semen retrieval method and extenders on the sperm midpiece size using CASA.

The significant difference (P<0.05) is represented by a star within the bars.

Figure 3.5 represents the effect of boar epididymal semen retrieval method and extenders on the sperm midpiece size using CASA. Less than 30% of sperm with abnormal midpiece size were recorded irrespective of the semen retrieval method and extenders. Semen diluted with Andromed® extender recorded greater than 20% sperm with abnormal midpiece size irrespective of the semen retrieval method compared to BTS extender treatment ($P<0.05$). Greater than 80% of sperm with normal midpiece size were recorded in the semen diluted with BTS irrespective of the semen retrieval method. The more sperm undergo acrosome reaction, the higher the chances of the midpiece size being affected. Additionally, a large occurrence of cytoplasmic droplet distribution was apparent in the sperm that underwent acrosome reaction and immature sperm, which causes abnormal midpiece size.

Table 3.4 represents the effect of boar epididymal semen retrieval methods and extenders on sperm morphometry using CASA. Overall, the semen diluted with BTS and Andromed® recorded average acceptable sperm head size (length: 8.2-8.9 µm; perimeter: 18.2-19.9 µm; width: 4.1–4.6 µm and area: 34.8-38.6 μm^2), midpiece and shape indices which fell within the normal range, irrespective of the semen retrieval method.

Table 3.4: Effect of boar epididymal semen retrieval methods and extenders on sperm morphometry using CASA (mean ± SD).

Semen retrieval methods	**Slicing float-up**		**Flushing**	
Semen extenders	**BTS**	**Andromed®**	**BTS**	**Andromed®**
		Sperm head trait		
Length (µm)	8.8±1.2[a]	8.6±1.7[b]	8.9±1.9[a]	8.2±2.9[c]
Width (µm)	4.5±0.7[b]	4.4±0.9[c]	4.6±0.9[a]	4.1±1.4[d]
Area (μm^2)	37.3±6.9[b]	36.9±10.0[b]	38.6±7.5[a]	34.8±13.6[c]
Perimeter (µm)	19.9±2.8[a]	19.3±4.1[b]	19.8±3.8[a]	18.2±6.4[c]
		Sperm midpiece trait		
Width (µm)	1.1±0.4[b]	1.2±0.5[a]	1.2±0.5[a]	1.2±0.7[a]
		Sperm shape indices		
Ellipticity	2.0±0.6[a]	2.0±0.8[a]	1.9±1.1[b]	1.9±0.8[b]
Elongation	0.3±0.1[a]	0.3±0.1[a]	0.3±0.1[a]	0.3±0.1[a]
Regularity	0.8±0.1[a]	0.8±0.2[a]	0.8±0.1[a]	0.8±0.3[a]
Roughness	1.3±1.7[a]	1.2±0.3[a]	1.4±4.7[a]	1.5±6.3[a]

[a-d] Values with different superscripts in a row differ significantly ($P<0.05$). BTS: Beltsville thawing Solution.

3.4 Discussion

A comparison of two semen extenders in the current study showed an alteration in sperm motility and morphometry traits, and an effect between the two semen retrieval methods. The current study recorded

sperm motility to be higher when semen was retrieved with the slicing float-up method and diluted with a BTS extender. Similar findings were recorded in our earliest study (Thema et al., 2022), which reported that BTS performed better than Kobidil^{+} extender on the Windsnyer boar sperm motility and morphology, due to the presence of potassium chloride in its chemical composition, which plays a key role in maintaining sperm quality traits. The presence of potassium chloride in the BTS extender assists in regulating the semen intracellular pH level, calcium level and stimulates the sperm motility while supporting the acrosome reaction (Vílchez et al., 2017). The pH is one of the important requirements for extenders, due to its ability to limit bacterial development while maintaining the sperm quality (Ho et al., 2023). Furthermore, the current findings agree with the study by Kumar et al. (2023), which found the BTS extender to have maintained higher sperm motility rates in the Large White Yorkshire ejaculated semen compared to other contemporary extenders. The presence of citrate and glucose in BTS extender played a significant role in providing energy to sperm, which sustains a higher percentage of motile sperm (Kumar et al., 2023). Slicing float-up BTS treatments did not induce major changes in the proportions of motile sperm.

The current study differs from the study by Turri et al. (2012), which found that the Limousine bull sperm quality was more affected by the slicing float-up than the flushing method. According to Turri et al. (2012), utilising the slicing float-up method for sperm extraction may expose the sperm to blood that may occur during cutting. However, the blood contamination can be reduced by quickly combining the sperm with a medium or extender (Canissoa et al., 2021). The use of BTS in the current study was valuable to minimise the risk of blood contamination and improve sperm motility. The BTS extender contains 2.20 g/L amounts of antibiotics that can treat a variety of infections and reduce bacterial contamination in the retrieved semen sample (Thema et al., 2022). Moreover, to avoid blood contamination, superficial blood vessels were initially punctured with a needle, and their contents were rinsed and wiped off before semen retrieval.

The present results demonstrate that the extender and retrieval methods have a clear effect on the quality of sperm. The lowest values of sperm motility were recorded when semen was retrieved with the flushing method and diluted with Andromed®, followed by the BTS extender. Furthermore, the current study differs from the study by Podico and Canisso (2022) on the equidae, which reported a greater yield of the total motile epididymal sperm retrieved using the flushing method and recorded a lower yield with the slicing float-up method. Additionally, it was discovered that when using the flushing method, semen samples may be washed off by semen extenders and other fluids, which may limit the ability to protecting the survivability of the sperm may be limited due to the few cuts performed. However, according to Lamglait, (2014), flushing has been demonstrated to be a better method in most species due to its lower risk of contamination. Whereas, according to Nichi et al. (2017), flushing is the recommended method for retrieving bull semen.

In terms of sperm morphometry, a greater percentage of sperm with normal acrosomes, midpiece, size, and shape was recorded in the semen diluted with BTS extender, irrespective of the retrieval method. The present results agree with the study by Kumar et al. (2023) which recorded the BTS extender to have protected the sperm against acrosomal impairment. According to Govindasamy et al., (2016), the presence of tri-sodium citrate in the chemical composition of BTS extender assists in contributing a higher proportion of sperm-intact acrosomes. Overall, the semen diluted with BTS and Andromed® recorded average acceptable sperm head, midpiece and shape indices which fell within the normal range, irrespective of the semen retrieval method. However, there was a reduction in the sperm head dimensions, a change in the shape of the sperm head and acrosome associated with Andromed® was observed in the current study. Furthermore, the sperm head shrank, and the acrosome's shape changed in relation to the Andromed® extender. The sperm diluted with the Andromed®, irrespective of the semen retrieval method showed sperm to have undergone a premature acrosome reaction. The acrosome reaction observed in the Andromed® extender treatments could explain the decreasing percentage of motile, normal head, acrosome, and midpiece of the sperm recorded in the current study.

One of the key elements that influenced the retrieved sperm quality in the current study was the composition of the semen extenders used. According to Hernández et al. (2014), Andromed® extender is thought to be protein-free and contains a high phospholipid content derived from soybean lecithin. In comparison to other species, boar sperm contains a high percentage of polyunsaturated fatty acids (PUFAs) in its plasma membrane and low cholesterol content (Thema et al., 2022). This PUFAs increases membrane flexibility, making sperm more susceptible to oxidative stress and cold shock during handling and preservation (Yeste, 2015; Yeste, 2018). Futhermore, this normally causes sperm motility decrease and damaged membrane integrity when boar sperm is exposed to cooling or cryopreservation.

As a result, the interaction between the components of soya lecithin-based extenders and the plasma membrane of boar sperm may be less effective and may contribute to the lower motility and survivability of sperm during preservation (Abdel-Hafez, 2014), thus, as seen in the current investigation when epididymal sperm was diluted Andromed® during recovery. Additionally, soya lecithin-based extender alone may not be sufficient to the sperm consist of high PUFA in its membranes in preventing membrane phase transitions or reduce lipid peroxidation caused by reactive oxygen species; additionally, when cholesterol or antioxidant supplements are not present (Giaretta et al., 2015; Mangrulkar et al., 2025). Moreover, studies in ram and bull semen indicated that plant lecithin extenders may differ based on the species and technique, which may always result in different results (Üstüner et al., 2014). Whereas it is known that the absence of seminal plasma in the epididymal semen necessitates the use of a semen extender with protein content.

Therefore the composition of lipoproteins, phospholipids, and cholesterol in the egg yolk-based extenders may integrate into the sperm plasma membrane and assist in regulating lipid phase during cooling and cryopreservation (Manjunath, 2018; Perumal, 2018). Therefore, egg yolk-based extenders used in pigs' sperm preservation procedure, may enhance motility and maintain membrane integrity while reducing cold-induced membrane damage during cooling and cryopreservation (Castro et al., 2025). As a result, Andromed® extender was discovered unsuitable for use with the boar's epididymal semen in the current study. The current study differs from the reports from other studies on bull (Abdussamad et al., 2016), and buck (Quintero-Elisea et al., 2023) semen, which recorded the Andromed® extender to have a more protective effect on ejaculated sperm motility and viability. Additionally, a study by Ansari et al., (2017) reported Andromed® to be capable of protecting the buffalo bull post-thawed sperm and further reported that it can be adopted safely for routine use in replacing other extenders. Semen retrieval methods used in the current study did not affect the sperm morphometry traits. Therefore, the current study fails to reject the null hypothesis when it comes to the sperm retrieval methods' effect on sperm morphometry traits.

3.5 Conclusion

In conclusion, for post-mortem recovery of the sperm from the boar epididymis, slicing float-up would be a more recommended method than the slicing float-up method. The slicing float-up samples had higher quality sperm, probably due to the higher yield than the flushing method. Furthermore, the addition of BTS has been found to have a significant beneficial effect on protecting epididymal sperm retrieved in the slicing float-up method. The current results suggest that the use of a BTS extender can have a positive effect on the sperm in the stage of recovery using slicing float-up, before cooling and during the freezing process. Overall, the semen diluted with BTS and Andromed® recorded average acceptable sperm head, midpiece and shape indices which fell within the normal range, irrespective of the semen retrieval method. Therefore, additional research should be conducted to confirm our findings through liquid preservation, cryopreservation and fertility testing of boar. Moreover, studies should establish the framework for the regular use of these techniques (semen retrieval and extenders) for the establishment of boar semen cryobanks. With this successful protocol for epididymal sperm recovery, improvent of protocols for epididymides handling (consistent temperature and container) after death in a circumstance when boar death occurs in a distant area from the place of recovery necessary.

3.6 References

Abdel-Hafez, M.A.M., 2014. Effect of soybean lecithin-based semen extender on freezability and fertility of Rahmani ram spermatozoa. *Egyptian Journal of Sheep and Goats Sciences*, *9*(1), pp.1-8.

Abdussamad, A.M., Detterer, J., Gauly, M. and Holtz, W., 2016. Comparison of various semen extenders and addition of prostaglandin F2α on pregnancy rate in cows. *Animal*, *10*(4), pp.655-659. http://dx.doi.org/10.1017/S1751731115002256

Akhter, S., Ansari, M.S., Andrabi, S.M.H., Rakha, B.A., Ullah, N. and Khalid, M., 2012. Soya-lecithin in extender improves the freezability and fertility of buffalo (Bubalus bubalis) bull spermatozoa. *Reproduction in Domestic Animals*, *47*(5), pp.815-819. https://doi.org/10.1111/j.1439-0531.2011.01973.x

Ali Hassan, H., Banchi, P., Domain, G., El Khoury, R., Chaaya, R., Wydooghe, E., Smits, K. and Van Soom, A., 2023. A comparative study of canine epididymal sperm collection techniques and cryopreservation. *Frontiers in Veterinary Science*, *10*, p.1181054. https://doi.org/10.3389/fvets.2023.1181054

Amann, R.P. and Waberski, D., 2014. Computer-assisted sperm analysis (CASA): Capabilities and potential developments. *Theriogenology*, *81*(1), pp.5-17. https://doi.org/10.1016/j.theriogenology.2013.09.004

Ansari, M.S., Rakha, B.A. and Akhter, S., 2017. Cryopreservation of Nili-Ravi buffalo (Bubalus bubalis) semen in AndroMed® extender; in vitro and in vivo evaluation. *Reproduction in Domestic Animals*, *52*(6), pp.992-997. https://doi.org/10.1111/rda.13008

Auer, M., Wagner, H., Failing, K. and Wehrend, A., 2022. Epididymis incision as a method to collect epididymal sperm cells in alpacas. *Veterinary Medicine and Science*, *8*(1), pp.157-163. https://doi.org/10.1002/vms3.681

Bertol, M.A., 2016. Cryopreservation of epididymal sperm. *Cryopreservation in eukaryotes. (Eds F Marco-Jiménez, H Akdemir),* IntechOpen. London. pp.121-135. https://dx.doi.org/10.5772/65010

Basumatary, S., Barua, P.M., Ahmed, K., Buragohain, L., Tamuly, S., Borpujari, D. and Kashyap, B., 2024. Effect of BTS, GEPS and MODENA extender on DNA integrity and relative expression of HSP70 and Cas3 Gene in HD-K75 Boar Semen. *Indian Journal of Animal Research*, *58*(12).

Bustani, G.S. and Baiee, F.H., 2021. Semen extenders: An evaluative overview of preservative mechanisms of semen and semen extenders. *Veterinary World*, *14*(5), p.1220. https://doi.org/10.14202/vetworld.2021.1220-1233

Canissoa, I.F., Garrido, L. and Segabinazzi, T.M., 2021. Diagnosis and management strategies for urospermia in stallions. In *Anais do XXIV Congresso Brasileiro de Reprodução Animal*, *45*, pp. 443-52. https://doi.org/10.21451/1809-3000.RBRA2021.0593

Castro, M., Leal, K., Pezo, F. and Contreras, M.J., 2025. Sperm membrane: molecular implications and strategies for cryopreservation in productive species. *Animals*, *15*(12), p.1808. https://doi.org/10.3390/ani15121808

Dziekońska, A., Neuman, N.M., Burdal, K.K., Wiszniewska-Łaszczych, A. and Bogdaszewski, M., 2022. The effect of different extenders on the quality characteristics of European red deer epididymal sperm stored at 5 °C. *Animals*, *12*(19), p.2669. https://doi.org/10.3390/ani12192669

Esteves, S.C., Miyaoka, R. and Agarwal, A., 2011. Sperm retrieval techniques for assisted reproduction. *International Brazillian Journal of Urology*, *37*, pp.570-583. https://doi.org/10.1590/S1677-55382011000500002

Falomo, M.E., Rossi, M. and Mantovani, R., 2016. Collection, storage and freezability of equine epididymal spermatozoa. *Italian Journal of Animal Science*, *15*(3), pp.386-389. https://doi.org/10.1080/1828051X.2016.1210485

Fernandez-Novo, A., Santos-Lopez, S., Barrajon-Masa, C., Mozas, P., de Mercado, E., Caceres, E., Garrafa, A., Gonzalez-Martin, J.V., Perez-Villalobos, N., Oliet, A. and Astiz, S., 2021. Effects of extender type, storage time, and temperature on bull semen parameters. *Biology*, *10*(7), p.630. https://doi.org/10.3390/biology10070630

Giaretta, E., Estrada, E., Bucci, D., Spinaci, M., Rodríguez-Gil, J.E. and Yeste, M., 2015. Combining reduced glutathione and ascorbic acid has supplementary beneficial effects on boar sperm cryotolerance. *Theriogenology*, *83*(3), pp.399-407. https://doi.org/10.1016/j.theriogenology.2014.10.002

Govindasamy, K., Ponraj, P., Thulasiraman, S., Andonissamy, J., Naskar, S., Das, A., Hasin, D. and Baishya, S.K., 2016. Efficacy of different extenders on sperm characteristics and fertility in crossbred pigs of north-eastern India. *Veterinarski Arhiv*, *86*(4), pp.515-528. https://hrcak.srce.hr/165727

Hernández, C.L., Dorado, J., Hidalgo, M., Rubio, P.J. and Quintero, M.A., 2014. Effect of seminal extraction method on post-thawing viability and sperm motility in mixed-breed male goats. *Revista Científica Biológico Agropecuaria Tuxpan*, *2*(4), pp. 875-884.

Ho, T.K., Vo, C.T., Lam, T.N., Nguyen, T.K.K. and Nguyen, T.T., 2023. Effect of difference extender on extended boar semen quality in the long-term storage. *Journal of Animal Husbandry Sciences and Technic*, *9*, pp.14-23.

Hori, T., Atago, T., Kobayashi, M. and Kawakami, E., 2015. Influence of different methods of collection from the canine epididymides on post-thaw caudal epididymal sperm quality. *Journal of Veterinary Medical Science*, *77*(5), pp.625-630. https://doi.org/10.1292/jvms.14-0421

Huijsmans, T.E., Hassan, H.A., Smits, K. and Van Soom, A., 2023. Post-mortem collection of gametes for the conservation of endangered mammals: a review of the current state-of-the-art. *Animals*, *13*(8), p.1360. https://doi.org/10.3390/ani13081360

Kumar, S., Singh, A.K., Dhindsa, S.S. and Singh, P., 2023. Effect of certain extenders on semen quality of boars during preservation at 17 °C. *The Indian Journal of Animal Sciences*, *93*(8), pp.783-787. http://dx.doi.org/10.56093/ijans.v93i8.120155

Lamglait, B., 2014. Longevity of sperm cells retrieved by post-mortem epididymal aspiration in wild bovids in zoo conditions. *Journal of Zoo and Aquarium Research*, *2*(4), pp.92-100. https://doi.org/10.19227/jzar.v2i4.47

Mangrulkar, S.V., Kulkarni, S.S., Nanepag, P.V., Neje, P.S., Chaple, D.R., Taksande, B.G. and Umekar, M.J., 2025. A comprehensive review on pleiotropic effects and therapeutic potential of soy lecithin. *Advances in Traditional Medicine*, *25*(1), pp.145-164. https://doi.org/10.1007/s13596-024-00770-1

Manjunath, P., 2018. New insights into the understanding of the mechanism of sperm protection by extender components. *Animal Reproduction (AR)*, *9*(4), pp.809-815. https://www.animal-reproduction.org/journal/animreprod/article/5b5a6053f7783717068b46cc accessed 26032026

Maroto-Morales, A., Ramón, M., García-Álvarez, O., Montoro, V., Soler, A.J., Fernández-Santos, M.R., Roldán, E.R., Pérez-Guzmán, M.D. and Garde, J.J., 2015. Sperm head phenotype and male fertility in ram semen. *Theriogenology*, *84*(9), pp.1536-1541. http://dx.doi.org/10.1016/j.theriogenology.2015.07.038

Maroto-Morales, A., García-Álvarez, O., Ramón, M., Martínez-Pastor, F., Fernández-Santos, M.R., Soler, A.J. and Garde, J.J., 2016. Current status and potential of morphometric sperm analysis. *Asian Journal of Andrology*, *18*(6), pp.863-870. https://doi.org/10.4103/1008-682X.187581

Monaco, D., Rota, A., Carbonari, A., Lillo, E., Lacalandra, G.M. and Rizzo, A., 2024. Collection of epididymal semen in the tomcat (Felix catus) by stereomicroscope-aided retrograde flushing (SARF) improves sample quality. *Animal Reproduction Science*, *261*, p.107388. https://doi.org/10.1016/j.anireprosci.2023.107388

Monteiro, G.A., Guasti, P.N., Rocha, A.S., Martin, I., Sancler-Silva, Y.F.R., Dell'Aqua, C.P.F., Dell'Aqua Jr, J.A. and Papa, F.O., 2013. Effect of storage time and temperature of equine epididymis on the viability, motion parameters, and freezability of epididymal sperm. *Journal of Equine Veterinary Science*, *33*(3), pp.169-173. https://doi.org/10.1016/j.jevs.2012.06.010

Mortimer, S.T., van der Horst, G. and Mortimer, D., 2015. The future of computer-aided sperm analysis. *Asian Journal of Andrology*, *17*(4), pp.545-553. https://doi.org/10.4103/1008-682X.154312

Nagy, Á., Polichronopoulos, T., Gáspárdy, A., Solti, L. and Cseh, S., 2015. Correlation between bull fertility and sperm cell velocity parameters generated by computer-assisted semen analysis. *Acta Veterinaria Hungarica*, *63*(3), pp.370-381. https://doi.org/10.1556/004.2015.035

Nichi, M., Rijsselaere, T., Losano, J.D.A., Angrimani, D.D.S.R., Kawai, G.K.V., Goovaerts, I.G.F., Van Soom, A., Barnabe, V.H., De Clercq, J.B.P. and Bols, P.E.J., 2017. Evaluation of epididymis storage temperature and cryopreservation conditions for improved mitochondrial membrane potential, membrane integrity, sperm motility and in vitro fertilization in bovine epididymal sperm. *Reproduction in Domestic Animals*, *52*(2), pp.257-263. https://doi.org/10.1111/rda.12888

Perumal, P., 2018. Low density lipoprotein in cryopreservation of semen. *Asian Pacific Journal of Reproduction*, *7*(3), pp.103-116. https://doi.org/10.4103/2305-0500.23357

Pérez-Durand, M.G., Bustamante, C.W., Machaca, P.P., García, W., Condori, E.A., Macedo, R., Fernández, E., Manrique, Y.P., Gutiérrez-Reinoso, M.A., Perez-Guerra, U.H. and García-Herreros, M., 2024. Effect of three semen extenders on sperm quality and in vitro fertilization rates of fresh and cryopreserved sperm collected from Llama (Lama glama) vas deferens. *Animals*, *14*(11), p.1573. https://doi.org/10.3390/ani14111573

Podico, G. and Canisso, I.F., 2022. Retrograde flushing followed by slicing float-up as an approach to optimize epididymal sperm recovery for the purpose of cryopreservation in equids. *Animals*, *12*(14), p.1802.https://doi.org/10.3390/ani12141802

Quintero-Elisea, J. A., Clemente-Sánchez, F., Cortez-Romero, C., Velázquez-Morales, J. V., Olguín-Arredondo, H.A. and Orozco-Lucero, E., 2023. Increase of the motility of buck semen with Andromed® extender and the addition of HTF. https://revista-agroproductividad.org/index.php/agroproductividad/article/view/2532 Accessed 10062025

Schulze, M., Grobbel, M., Riesenbeck, A., Brüning, S., Schaefer, J., Jung, M. and Grossfeld, R., 2017. Dose rates of antimicrobial substances in boar semen preservation—time to establish new protocols. *Reproduction in Domestic Animals*, *52*(3), pp.397-402. https://doi.org/10.1111/rda.12921

Schulze, M., Nitsche-Melkus, E., Hensel, B., Jung, M. and Jakop, U., 2020. Antibiotics and their alternatives in artificial breeding in livestock. *Animal Reproduction Science*, *220*, p.106284. https://doi.org/10.1016/j.anireprosci.2020.106284

Stout, M.A., 2012. *Comparison of epididymal and ejaculated sperm collected from the same holstein bulls*. Louisiana State University and Agricultural Mechanical College. http://dx.doi.org/10.31390/gradschool_dissertations.1160

Strand, J., Ragborg, M.M., Pedersen, H.S., Kristensen, T.N., Pertoldi, C. and Callesen, H., 2016. Effects of post-mortem storage conditions of bovine epididymides on sperm characteristics: investigating a tool for preservation of sperm from endangered species. *Conservation Physiology*, *4*(1), p.cow069. http://dx.doi.org/10.1093/conphys/cow069

Thema, M.A., Mphaphathi, M.L., Ledwaba, M.R. and Nedambale, T.L., 2022. Investigation of the efficacy of different semen extenders and in vitro storage period on Windsnyer boar sperm quality equilibrated at 18 °C. *Reproduction in Domestic Animals*, *57*(12), pp.1644-1648. https://doi.org/10.1111/rda.14256

Üstüner, B., Alçay, S., Nur, Z., Sağirkaya, H. and Soylu, M.K., 2014. Effect of egg yolk and soybean lecithin on tris-based extender in post-thaw ram semen quality and in vitro fertility. *Kafkas Üniversitesi Veteriner Fakültesi Dergisi*, *20*(3). https://doi.org/10.9775/kvfd.2013.10248

Turri, F.E.D.E.R.I.C.A., Madeddu, M., Gliozzi, T.M., Gandini, G. and Pizzi, F., 2012. Influence of recovery methods and extenders on bull epididymal spermatozoa quality. *Reproduction in Domestic Animals*, *47*(5), pp.712-717.

Vílchez, M.C., Morini, M., Peñaranda, D.S., Gallego, V., Asturiano, J.F. and Pérez, L., 2017. Role of potassium and pH on the initiation of sperm motility in the European eel. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology*, *203*, pp.210-219. https://doi.org/10.1016/j.cbpa.2016.09.024

Yániz, J.L., Soler, C. and Santolaria, P., 2015. Computer assisted sperm morphometry in mammals: A review. *Animal Reproduction Science*, *156*, pp.1-12. https://doi.org/10.1016/j.anireprosci.2015.03.002

Yeste, M., 2015. Recent advances in boar sperm cryopreservation: state of the art and current perspectives. *Reproduction in domestic animals*, *50*, pp.71-79. https://doi.org/10.1111/rda.12569

Yeste, M., 2018. State-of-the-art of boar sperm preservation in liquid and frozen state. *Animal Reproduction (AR)*, *14*(1), pp.69-81. https://doi.org/10.21451/1984-3143-AR895

Chapter 4: Investigation of the effect of time interval between animal death and sperm retrieval on the sperm motility and morphometry traits of boar cauda epididymides stored at 5 and 18 °C

Abstract

This study investigated the effect of the time interval between animal death and sperm retrieval on the sperm motility and morphometry traits of boar cauda epididymides stored at 5 and 18 °C. A total of 160 testes of heterogeneous boars were collected (16 testes/day) from the local abattoir. Collected testes were divided into different storage time intervals (0, 2, 4, 8, 12, 24, 48 and 72 h) and temperature (5 and 18 °C). Sperm was retrieved from the cauda part of the epididymis using the slicing float-up method, following each testicular time interval and storage temperature. The retrieved sperm sample was diluted with Beltsville Thawing Solution extender and evaluated for sperm motility and morphometry traits using a computer computer-assisted sperm morphometry analysis system. The data were analysed using analysis of variance. Descriptive statistics assessed sperm morphometry, establishing the minimum and maximum values. Sperm retrieved following a 0 h storage time interval recorded less than 50% sperm progressive motility irrespective of the storage temperature. However, sperm retrieved following 2 h storage time recorded the highest progressive (78.9%) and rapid (68.3%) sperm motility at 5 °C in comparison with 18 °C ($P<0.05$). Sperm retrieved from the testes stored at 5 and 18 °C for up to 72 h recorded the acceptable sperm head length (8.2-9.4 µm), width (3.9-4.6 µm), area (28.2-35.7 µm2), perimeter (21.0-24.1 µm) and midpiece width (1.0-1.3 µm) which fell within the threshold. The results of this study suggest that sperm recovered from the epididymides of boars were viable after storage up to 0-72 h. Although this study showed the possibility of retrieving the epididymal from epididymides stored up to 72 h (0-72 h) at 5 °C. These results elaborate that the sperm can be best retrieved after 2-24 h storage of the epididymides at 5 °C for higher fertilisation success.

Keywords: testicular handling, storage temperature, sperm motility, morphometry

4.1 Introduction

Retrieval of semen from the cauda epididymis may be the only method available to collect superior genetic material when untimely death or severe injuries occur in valuable boars (Van Leeuwen, 2020). The epididymis is the male reproductive organ, important for the maturation and functionality of sperm. The mature sperm in the cauda epididymis exhibit an exceptional capacity to survive post-mortem for several days (Mir et al., 2012), and if retrieved, they can either be preserved or employed in assisted reproductive technologies to create new generations (Bertol et al., 2013). This is possible due to the duct system derived from the mesonephros which is the key component of this process, by comprising absorptive and secretory epithelial cells (Rodriguez-Martinez et al., 2022). To maintain sperm quality

after the animal's sudden death, the testes should be collected and be transported to the lab as soon as feasible for sperm recovery (Lone et al., 2011). However, the preservation of epididymal semen can be hampered by the distance and time from the farm where the animal dies unexpectedly to the lab where the sperm could be properly processed and stored (Stand et al., 2016); Furthermore, technical expertise in handling and processing epididymal sperm affect its viability (Engdawork et al., 2024). These factors have prompted researchers to develop standard operating procedures for: a) successfully transporting and preserving testicular and epididymal tissue; b) gathering and cryopreserving sperm; and c) successfully inseminating using the cryopreserved epididymal sperm across various animal species (Vidament et al., 2012). Although pigs are important livestock and model species for biomedical research, investigations of epididymal transportation, retrieval, and functions have been more concentrated on other species than the pig, necessitating the current study.

The testicular storage time interval and temperature play an important role in maintaining sperm motility and morphology (Faes and Goossens, 2017). This concept has recently been challenged by Chinonye and Ahemen (2021) study, demonstrating that refrigeration of epididymides at 5 °C before retrieval can slow down the process of cell degradation while increasing the chance of collecting viable sperm. However, Vidament et al. (2012) reported that epididymides stored in a refrigerator overnight had reduced sperm's ability to withstand the freeze-thaw process in cats. The temperature of 18 °C is commonly known to be the optimum temperature for storing boar semen (Thema et al., 2022). Therefore, it is critical to ascertain the right protocol for handling and transporting testes or epididymides to ensure the survivability of the sperm until they reach the lab where they can be processed appropriately and conserved (Lone et al., 2011). Little is known about the changes in boar epididymal sperm morphometry and motility during transportation as influenced by holding temperature and duration. To our knowledge, no published studies have documented the changes in boar epididymal sperm morphometry induced by different testicular storage time intervals and temperature. Therefore, it is important to determine how long the boar testes can be stored, while maintaining acceptable sperm quality, because the death of an animal is unpredictable and the processing laboratory might be distant from the place of animal death (Wachida et al., 2019).This study hypothesises that the storage of cauda epididymides for 72 h at 5 and 18 °C after boar death, significantly affect the survival, motility and morphometric characteristics of the sperm recovered. This study investigated the effect of the time interval between animal death and semen retrieval on the sperm motility and morphometry traits of boar cauda epididymides stored at 5 and 18 °C.

4.2 Materials and methods

4.2.1 Study area

The study was conducted at the Germplasm Conservation and Reproductive Biotechnologies section in the Agricultural Research Council, Irene, in Gauteng Province. The area is located at 25º 53′ 59.6″ South latitude and 28º 12′ 51.6″ East longitude in Pretoria, South Africa.

4.2.2 Statement of animal rights

The experimental procedures were performed in accordance with the ethical standards approved by the Agricultural Research Council-Animal Production Ethics Committee [APIEC 23/05], University of KwaZulu-Natal Research Ethics Committee [AREC/00007100/2024], and Section 20 of the Animal Diseases Act no 35 of 1984 [12/11/1/18 (6269BM)].

4.2.3 Preparation of boar semen extenders

The semen extender used in the experiment was Beltsville Thawing Solution (BTS; IMV Technologies, France). The semen extender was prepared a day before use. The BTS boar extender was prepared by dissolving a sachet of each commercial extender which came in a powdered form in a litre of sterile water (Sabax®, Adcock Ingram, South Africa) according to manufactures instruction and stored at 18 °C until use.

4.2.4 Retrieval of the boar epididymal sperm

A total of 160 testes of heterogeneous boars were collected (16 testes/day) from a local abattoir and divided into different testicular storage time intervals (0, 2, 4, 8, 12, 24, 48, and 72 h) and temperature (5 and 18 °C). Following each testicular storage time interval and temperature, the cauda epididymides were removed from the testes and cleaned with Sabax® saline NaCl 0.9%, solution (Adcock Ingram, South Africa). To avoid blood contamination, superficial blood vessels were punctured so that most of the blood could be wiped off (Turri et al., 2012). The semen samples were retrieved from the cauda epididymides with the aid of the slicing float-up method. The slicing float-up procedure involves using a scalpel blade to slice the cauda part of the epididymis to release the semen in a petri dish (Whitehead Scientific, South Africa), diluting with 5 mL of a warm (37 °C) BTS extender and gently agitate the mixture for approximately 10 minutes to enhance the sperm collection (Turri et al., 2012; Falomo et al., 2016). The diluted semen sample was then transferred into 15 mL tubes for further analysis.

4.2.5 Evaluations of the boar epididymal sperm motility

Sperm motility was evaluated following each testicular storage time interval (0, 2, 4, 8, 12, 24, 48 and 72 h) and temperatures (5 and 18 °C) using the computer-assisted sperm analyser (CASA; Microptic, Spain, Sperm Class Analyzer® 6.6.80). The volume of a 5 µL semen sample was transferred to a warmed (37 °C) microscope glass slide (Ducit, South Africa) and covered with a warm coverslip (Ducit, South Africa). Two fields of semen sample with an average of 250 sperm (34.3×10^6/mL/treatment/replication) were captured with the aid of a microscope (Nikon®, Japan) at 10X magnification using the CASA system.

4.2.6 Preparation of boar epididymal sperm for morphometry staining

Sperm morphometry was evaluated following each testicular storage time interval (0, 2, 4, 8, 12, 24, 48 and 72 h) and temperatures (5 and 18 °C) using the CASA system. The retrieved semen samples were first diluted with BTS extender at a ratio of 1:10. The diluted semen samples were further fixed in 4% Formaldehyde (Sigma Aldrich, United States of America) for 10 minutes. The total of 10 µL of the fixed semen sample/testicular storage temperature/time interval was smeared on a separate microscopic glass slide and allowed to air dry overnight at room temperature. During staining, the dried microscope glass slide sample was gently dipped in the SpermBlue® (Ducit, South Africa) tray for 2 minutes and in distilled water for 3 seconds (to remove debris and ensure a clean background). The paper towel was used to wipe the back of the microscope glass slide and allowed air dry at room temperature. Following drying, the slide was mounted using Dibutylphthalate Polystyrene Xylene mounting medium (Associated Chemical Enterprises, South Africa) and covered with the microscopic glass coverslip.

4.2.7 Boar epididymal sperm morphometry evaluation

Boar epididymal sperm morphometric traits were captured with the aid of a microscope (Nikon®, Japan) at 40X magnification using the CASA system. At least 100 sperm were measured at different testicular storage time intervals/temperature. The microscope glass slide was observed (the focus knob was used to focus sperm) and measured for the sperm head area (A; μm^2), head perimeter (P; µm), head length (L; µm), head width (W; µm), head ellipticity (length/width), head elongation (L-W/L+W), head regularity (πx (L.W/4.A), head roughness [4 π x (A/P^2)], midpiece width and the shape indices (head shape type, type of the sperm and midpiece size).

4.2.8 Statistical analysis

The sperm morphometric data were divided into two groups: The first group was the measurements of sperm head primary traits (length, L; area, A; width, W and perimeter, P, ellipticity, L/W; rugosity, 4 πA/P2; elongation, L−W/L+W and regularity, πLW/4 A). The second group determined the sperm head

indices (head type, normal, shape, acrosome type, and midpiece type). In the first sperm morphometric data group, descriptive statistics were used to establish the minimum and maximum values (threshold) using 10-90 percentiles for every morphometric trait shown in 4.1.

Table 4.1: Minimum and maximum acceptable values for morphometric traits in order to define normal boar epididymal sperm morphometry.

	Boar	
Sperm morphometric traits	**Minimum**	**Maximum**
Length (µm)	8.20	9.70
Width (µm)	3.50	5.50
Perimeter (µm)	16.20	26.20
Area (µm)	28.49	44.00
Ellipticity	1.81	2.16
Regularity	0.70	1.00
Elongation	0.29	0.37
Roughness	0.62	1.50
Midpiece width (µm)	0.50	1.60

The data for the first sperm morphometric data group and sperm motility trait were analysed with two-way analysis of variance. A 2 x 8 factorial design was used in this study, were testicular storage temperature (5 and 18 °C) and storage time interval (0, 2, 4, 8, 12, 24, 48, and 72 h) is the main independent factors. The main effect of independent variables (testicular storage time interval and temperature) and their interaction data were assessed with the general linear model. The interaction effects between testicular storage temperature and time interval significance were first analysed. Only when the interaction was significant, the simple effects of each testicular storage temperature x holding time intervals were analysed and were able to assign superscripts within the column (each holding temperature) to show the difference. Student's t-LSDs (Least significant differences) were calculated at a 5% significance level to compare means of significant source between the effect of testicular storage time interval and temperature on the sperm morphometric first data group and motility traits of boar cauda epididymal semen retrieved at post-mortem.

The second sperm morphometric data group was analysed using a Chi-square test to determine the effect of testicular storage time interval and temperature on the sperm morphometric percentage variations regarding the head type, normal shape, acrosome type and midpiece type of boar cauda epididymal semen retrieved at post-mortem. The scores were subjected to a 1:1 frequency table and a Chi-Square (χ^2) test was performed to test for equal proportions. Contingency RxC frequency tables were performed

for the association between treatments. The tests were performed for association (patterns) and where significant differences were found graphs were constructed to demonstrate the difference in patterns. All data analyses were performed using SAS version 9.4 statistical software.

4.3 Results

Table 4.2 and 4.3 represents the effect of testicular storage time interval and temperature on the sperm motility traits of the boar cauda epididymis. There is a distinct decline patterns between the two testicular storage temperature indicating the significant interaction between testicular storage temperature × time interval for sperm motility and kinematics trait. Semen retrieved at 0 h recorded <50% progressive motility in both testicular storage temperature used. However, semen retrieved following 2 h testicular storage at 5 °C recorded the highest sperm progressive (78.9%) and rapid (68.3%) motility compared to the other treatments ($P<0.05$). No significant difference was recorded between the total sperm motility (>70%) percentage in the sperm retrieved following 4- 24 h testicular storage at 5 °C. However, there was a drastic decrease in sperm motility recorded in the sperm retrieved from the cauda epididymides stored at 18 °C compared to 5 °C ($P<0.05$). The sperm retrieved from the cauda epididymis at 18 °C did not survive the 482-h testicular storage compared to the 5 °C testicular storage temperature. However, >60% total sperm motility was recorded from the epididymides stored at 18 °C for up to 8 h which fell within the minimum threshold. While >50% motile sperm were recorded in the sperm retrieved after 72 h testicular storage at 5 °C. This demonstrate that the effect of testicular storage time interval is dependent on the storage temperature.

Table 4.2: The effect of testicular storage time interval and temperature on the sperm motility traits of boar cauda epididymis stored at 5 °C (mean ± SD).

Time interval at 5 °C	TM (%)	PM (%)	NPM (%)	Static (%)	RAP (%)	MED (%)	SLW (%)
0 h	80.5±7.6^{b}	47.5±12.5^{c}	33.0±9.8^{b}	19.4±7.6^{b}	22.8±17.1^{d}	32.6±10.9^{a}	25.1±9.6^{b}
2 h	94.4±2.4^{a}	78.9±6.4^{a}	15.5±5.0^{d}	5.6±2.4^{c}	68.3±10.1^{a}	17.3±6.8^{c}	8.8±2.9^{d}
4 h	79.2±51.2^{b}	51.2±9.0^{c}	28.7±8.4^{bc}	20.8±6.2^{b}	36.1±9.8^{c}	23.8±5.1^{b}	19.3±6.3^{bc}
8 h	76.1±8.9^{b}	43.1±14.0^{c}	33.0±9.2^{b}	23.9±8.9^{b}	28.4±15.4^{c}	23.0±3.7^{b}	24.6±8.3^{b}
12 h	78.9±5.9^{b}	47.5±12.7^{c}	31.4±8.5^{b}	21.1±5.9^{b}	34.9±11.1^{c}	21.1±5.9^{b}	22.9±8.3^{b}
24 h	89.4±5.4^{a}	64.7±11.5^{b}	24.7±8.6^{c}	10.6±5.4^{bc}	49.0±19.3^{b}	24.9±11.7^{b}	15.4±8.6^{c}
48 h	60.4±9.5^{c}	29.0±8.6^{d}	31.4±4.9^{b}	39.6±9.5^{a}	19.7±10.0^{e}	17.7±3.0^{c}	23.0±4.6^{b}
72 h	56.6±17.1^{c}	4.5±6.3^{e}	52.1±18.9^{a}	36.7±16.5^{a}	1.8±2.9^{f}	5.1±7.0^{d}	49.7±19.4^{a}

$^{a-f}$ Values with different superscripts in a column differ significantly ($P<0.05$) for each storage temperature. TM: total motility, PM: progressive motility, RAP: rapid motility, MED: medium motility, SLW: slow motility.

Table 4.3: The effect of testicular storage time interval and temperature on the sperm motility traits of boar cauda epididymis stored at 18 °C (mean ± SD).

Time interval at 18 °C	TM (%)	PM (%)	NPM (%)	Static (%)	RAP (%)	MED (%)	SLW (%)
0 h	68.9±9.6^{b}	30.8±5.9^{b}	38.1±8.01^{a}	31.1±9.6^{c}	11.2±4.2^{b}	27.2±5.8^{a}	30.5±7.1^{a}
2 h	60.8±8.4^{c}	31.9±11.8^{b}	28.9±10.7^{ab}	39.2±8.4^{c}	19.2±8.5^{b}	19.3±7.1^{a}	22.3±11.3^{b}
4 h	80.4±3.3^{a}	49.0±7.7^{a}	31.5±6.9^{a}	19.6±3.3^{d}	35.2±12.3^{a}	22.9±6.6^{a}	22.3±7.4^{b}
8 h	83.9±3.5^{a}	46.1±21.7^{a}	37.8±21.0^{a}	16.1±3.5^{d}	34.3±18.5^{a}	25.7±7.7^{a}	23.9±12.0^{b}
12 h	50.3±3.2^{c}	15.7±3.8^{c}	34.6±4.8^{a}	49.7±3.2^{b}	10.4±2.4^{b}	11.0±2.9^{b}	28.8±4.7^{a}
24 h	25.2±11.8^{d}	3.9±4.5^{d}	21.3±8.3^{b}	74.7±11.9^{a}	0.6±1.0^{c}	4.2±5.3^{c}	20.4±8.1^{b}

$^{a-d}$ Values with different superscripts in a column differ significantly (P<0.05) for each storage temperature. TM: total motility, PM: progressive motility, RAP: rapid motility, MED: medium motility, SLW: slow motility.

Table 4.4 and 4.5 represents the effect of testicular storage time interval and temperature on the sperm kinematics traits of boar cauda epididymis. The highest number of sperm moving in curvilinear (66.3 µm/sec), average pathway (33.8 µm/sec) and straight line (18.4 µm/sec) velocity was recorded in the sperm samples retrieved following 2 h testicular storage at 5 °C compared to other treatments (P<0.05). Furthermore, the least number of sperm moving in straightness (34.2 µm/sec) and linearity (14.1 µm/sec) were recorded in the semen samples retrieved following12 h testicular storage at 18 °C.

Table 4.4: The effect of testicular storage time interval and temperature on the sperm kinematics traits of boar cauda epididymis stored at 5 °C (mean ± SD).

Time interval At 5 °C	**VCL (µm/sec)**	**VAP (µm/sec)**	**VSL (µm/sec)**	**STR (µm/sec)**	**LIN (µm/sec)**	**WOB (%)**	**ALH (µm/sec)**
0 h	40.7±11.5^{c}	19.3±5.9^{c}	10.7±3.2^{b}	52.8±4.6^{a}	25.8±5.4^{a}	46.6±5.6^{a}	1.3±0.3^{a}
2 h	66.3±12.8^{a}	33.8±5.8^{a}	18.4±2.6^{a}	53.2±4.6^{a}	28.3±4.0^{a}	51.5±3.2^{a}	1.9±0.3^{a}
4 h	47.8±7.4^{c}	23.6±3.4^{b}	13.5±3.0^{b}	54.6±4.9^{a}	27.8±4.1^{a}	50.1±4.6^{a}	1.5±1.2^{a}
8 h	46.9±10.5^{c}	22.6±6.4^{b}	10.7±3.7^{b}	45.7±7.7^{b}	21.1±4.9^{a}	46.1±5.4^{a}	1.5±0.2^{a}
12 h	49.8±11.4^{b}	23.4±4.9^{b}	11.3±2.5^{b}	47.3±7.9^{b}	22.4±5.6^{a}	46.5±4.7^{a}	1.5±0.3^{a}
24 h	55.0±12.0^{b}	27.0±5.9^{b}	14.2±2.3^{b}	51.7±5.1^{a}	25.8±3.3^{a}	49.1±3.7^{a}	1.7±0.3^{a}
48 h	42.9±8.1^{c}	20.0±4.0^{b}	9.7±1.9^{c}	45.4±4.1^{b}	21.4±4.6^{a}	46.1±7.1^{a}	1.4±0.^{2a}
72 h	22.0±7.9^{d}	9.9±3.0^{d}	3.9±1.9^{c}	42.2±5.8^{b}	17.0±2.7^{b}	45.8±4.7^{a}	0.9±0.2^{a}

$^{a-d}$ Values with different superscripts in a column differ significantly (P<0.05) for each storage temperature. VCL: curvilinear velocity, VSL: straight line velocity, VAP: average pathway velocity, LIN: linearity, STR: straightness, WOB: wobble, ALH: amplitude of lateral head.

Table 4.5: The effect of testicular storage time interval and temperature on the sperm velocity traits of boar cauda epididymis stored at 18 °C (mean ± SD).

Time interval at 18 °C	VCL (µm/sec)	VAP (µm/sec)	VSL (µm/sec)	STR (µm/sec)	LIN (µm/sec)	WOB (%)	ALH (µm/sec)
0 h	33.6±4.0^{b}	15.9±2.5^{b}	9.6±2.1^{b}	57.4±3.4^{a}	27.5±4.6^{a}	45.6±4.6^{a}	1.1±0.1^{a}
2 h	41.9±7.2^{a}	18.6±4.1^{b}	9.3±2.4^{b}	46.8±7.5^{b}	20.4±4.9^{a}	42.7±6.4^{a}	1.3±0.2^{a}
4 h	47.4±11.7^{a}	22.2±5.4^{a}	12.1±2.4^{a}	51.9±4.5^{a}	24.7±2.7^{a}	46.1±2.2^{a}	1.5±0.3^{a}
8 h	49.4±14.6^{a}	24.6±6.8^{a}	11.5±5.2^{a}	44.3±8.5^{b}	21.5±4.6^{a}	49.6±2.2^{a}	1.5±0.3^{a}
12 h	40.0±9.9^{a}	17.6±4.2^{b}	6.1±1.6^{c}	34.2±5.4^{c}	14.1±1.8^{b}	42.3±2.6^{a}	1.3±2.3^{a}
24 h	20.9±6.9^{c}	9.0±4.2^{c}	4.0±1.9^{c}	49.9±15.2^{a}	19.0±7.8^{b}	39.9±7.0^{a}	0.8±0.2^{b}

$^{a\text{-}c}$ Values with different superscripts in a column differ significantly (P<0.05) for each storage temperature. VCL: curvilinear velocity, VSL: straight line velocity, VAP: average pathway velocity, LIN: linearity, STR: straightness, WOB: wobble, ALH: amplitude of lateral head.

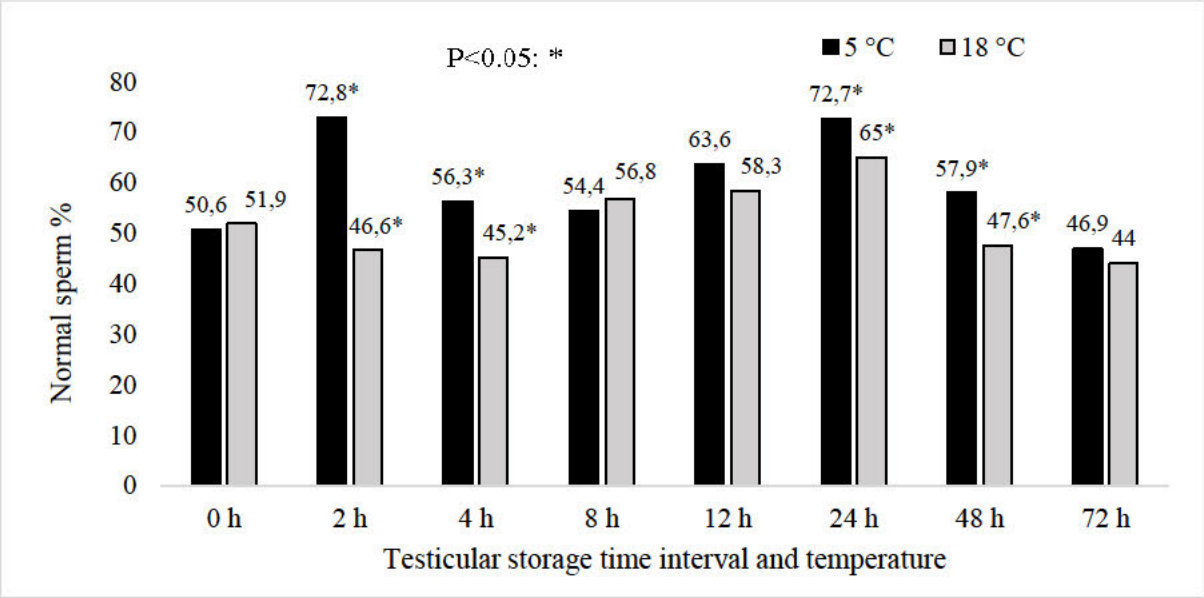

Figure 4.1: The effect of testicular storage time interval and temperature on normal sperm of the boar epididymis.

The significant difference (P<0.05) is represented by a star within the bars.

Figure 4.1 illustrates the effect of testicular storage time interval and temperature on normal sperm of boar cauda epididymis. Semen retrieved following 2 and 24 h testicular storage at 5 °C recorded >70% normal sperm as compared to 18 °C (P<0.05). While >60% normal sperm was recorded after 24 h testicular storage at 18 °C (P<0.05). There was no significant different following between the normal sperm recorded after 72 h of testicular storage at 5 and 18 °C.

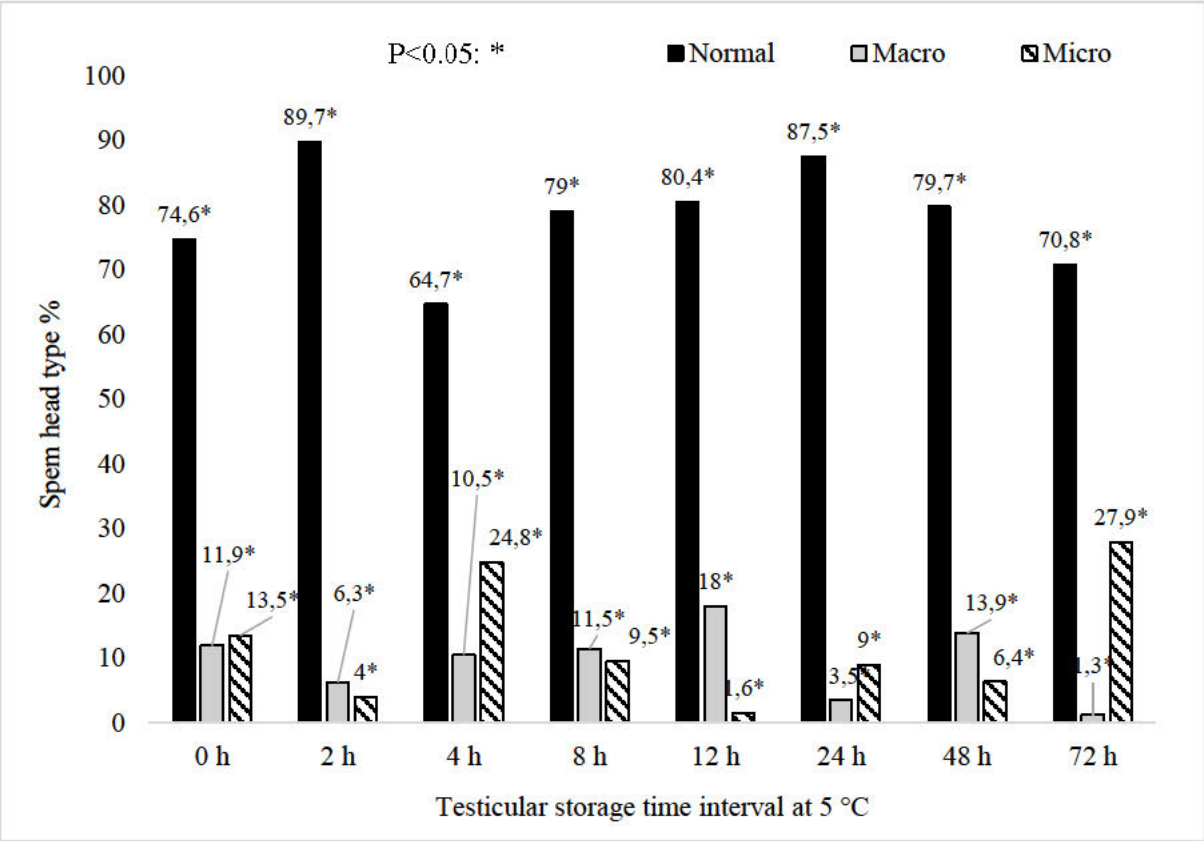

Figure 4.2: The effect of testicular storage time interval on the sperm head shape of boar cauda epididymis stored at 5 °C.

The significant difference (P<0.05) is represented by a star within the bars.

Figure 4.2 shows the effect of testicular storage time interval on the sperm head shape of boar cauda epididymis stored at 5 °C. Semen retrieved following 2 and 24 h of testicular storage at 5 °C recorded >85% sperm with normal head shape. Semen retrieved following 4 h (24.8%) and 72 h (27.9%) testicular storage at 5 °C recorded sperm with amorphous head morphology equivalent to the micro sperm percentage.

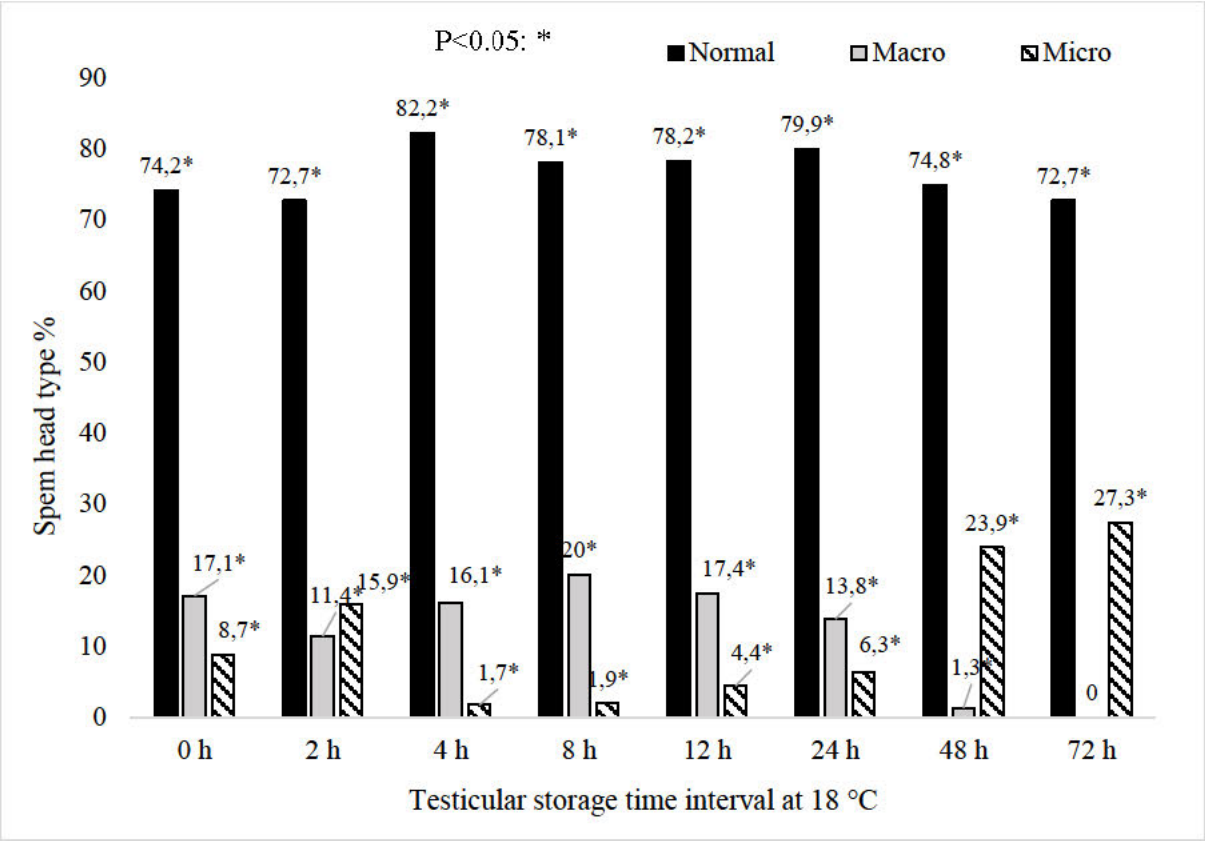

Figure 4.3: The effect of testicular storage time interval on the sperm head shape of boar cauda epididymis stored at 18 °C.

The significant difference (P<0.05) is represented by a star within the bars.

Figure 4.3 presents the effect of testicular storage time interval on the sperm head shape of boar cauda epididymis stored at 18 °C. Semen retrieved following 0-72 h testicular storage at 18 °C recorded >70% sperm with normal head shape. Sperm with the amorphous head abnormalities were recorded at higher rates after 48 h (23.9%) and 72 h (27.3%) when the testes were stored at 18 °C.

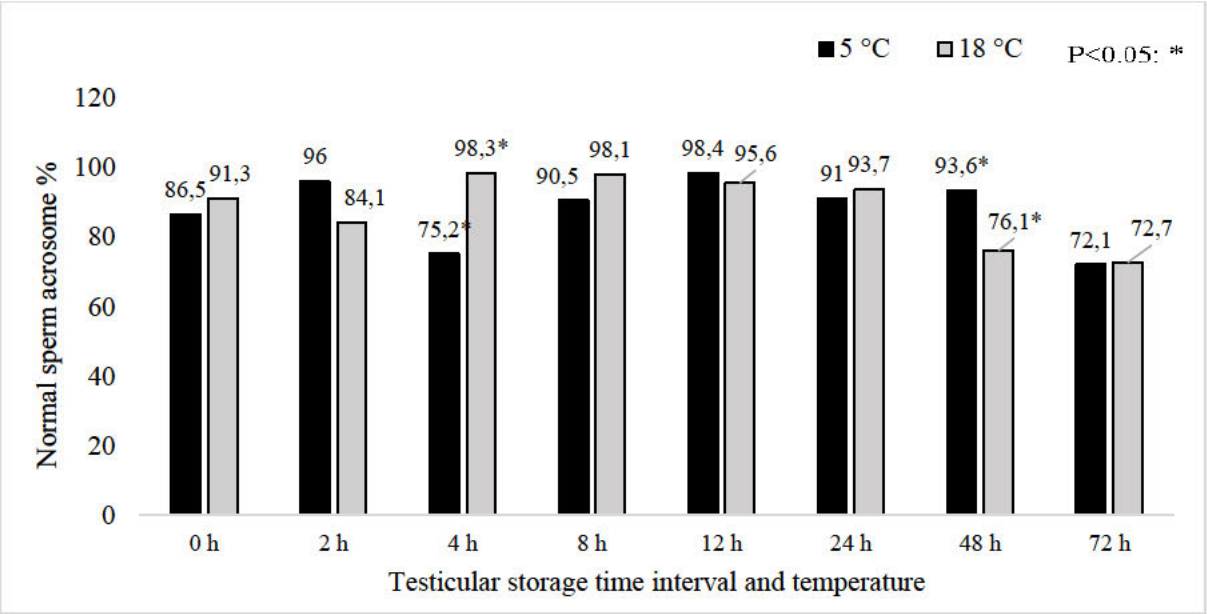

Figure 4.4: The effect of testicular storage interval and temperature on a normal sperm acrosome of the boar cauda epididymis.

The significant difference (P<0.05) is represented by a star within the bars.

Figure 4.4 shows the effect of testicular storage time interval and temperature on a normal sperm acrosome of the boar cauda epididymis. The >70% sperm with normal acrosome were recorded in the semen retrieved following 48 h testicular storage at 5 and 18 °C. The normal sperm head acrosome for both testicular storage temperature did not show any significant difference up to 24 h except at 4, 48 and 72 h duration. The 5 °C maintained acrosome head while 18 °C, significant degeneration may have begun as indicated by decreased in normal sperm acrosome percentage.

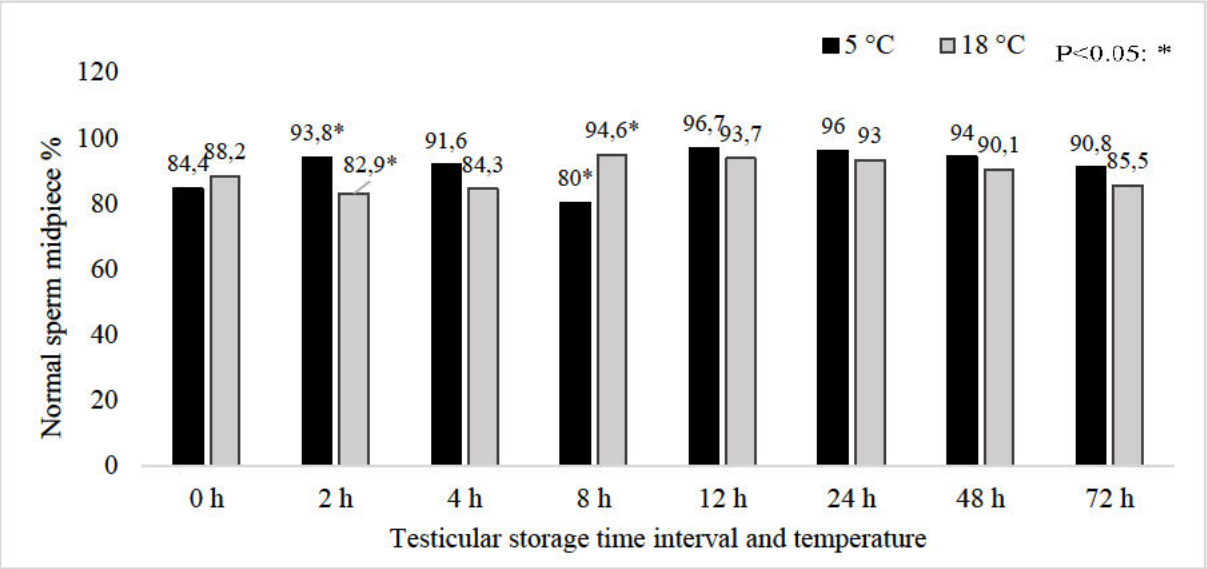

Figure 4.5: The effect of testicular storage time interval and temperature on the normal sperm midpiece of the boar cauda epididymis.

The significant difference (P<0.05) is represented by a star within the bars.

Figure 4.5 represents the effect of testicular storage time interval and temperature on a normal sperm midpiece of the boar cauda epididymis. Semen retrieved following 0-72 h testicular storage time interval recorded >80% sperm with normal midpiece irrespective of the testicular storage time interval temperature (P>0.05). However significant difference was recorded between the values recorded at 5 and 18 °C after 2 and 8 h of animal death

The effect of testicular storage time interval on the sperm morphometric trait of boar cauda epididymis stored at 5 and 18 °C is represented in Table 4.6 and 4.7. There was significant interaction between testicular storage temperature and time interval for sperm head morphometric traits. This demonstrate that the effect of testicular time interval is dependent on the storage temperature. The acceptable sperm head length (8.2-9.4 µm), width (4.0-4.6 µm), area (28.2-37.0 µm^2), perimeter (21.0-24.0 µm) and midpiece width (1.0-1.3 µm) were recorded in the sperm retrieved from the boar epididymis stored at 5 °C which fell within the threshold.

Table 4.6: The effect of testicular storage time interval on the sperm morphometric trait of boar cauda epididymis stored at 5 °C (mean ± SD).

	Testicular storage time interval at 5 °C							
	0 h	**2 h**	**4 h**	**8 h**	**12 h**	**24 h**	**48 h**	**72 h**
				Sperm head trait				
Length (µm)	8.9±0.9^b	9.0±0.8^a	8.9±1.6^b	8.2±2.0^d	9.4±0.6^a	8.8±0.8^b	8.4±0.7^c	8.8±0.7^b
Width (µm)	4.5±0.4^a	4.5±0.5^a	4.6±0.6^a	4.0±1.6^c	4.6±0.4^a	4.3±0.4^b	4.5±0.3^a	4.5±0.3^a
Area (µm^2)	35.3±6.2^b	37.0±5.6^a	34.0±5.6^b	28.2±13.4^d	35.3±3.8^b	31.2±4.1^c	31.0±3.0^c	31.7±3.0^c
Perimeter (µm)	21.4±2.2^a	21.4±2.6^b	22.5±3.1^b	21.0±5.6^b	24.0±1.6^a	22.8±1.7^b	22.2±1.5^b	22.8±0.1^b
				Sperm midpiece trait				
Width (µm)	1.3±0.3^a	1.1±0.31^a	1.1±0.4^a	1.0±0.6^a	1.0±0.2^a	1.0±0.3^a	1.1±0.3^a	1.1±0.4^a
				Sperm shape indices				
Ellipticity	1.9±0.2^a	2.0±0.5^a	1.9±0.3^a	2.6±1.2^a	2.0±0.4^a	2.0±0.2^a	2.0±0.17^a	2.0±0.2^a
Elongation	0.3±0.0^a	0.3±0.1^a	0.3±0.1^a	0.4±0.1^a	0.3±0.3^a	0.3±0.0^a	0.3±0.3^a	0.3±0.0^a
Regularity	1.0±0.1^a	0.9±0.9^a	1.0±0.1^a	1.0±0.0^a	1.0±0.0^a	1.0±0.0^a	1.0±0.0^a	1.0±0.0^a
Roughness	0.9±0.2^b	1.0±0.3^a	0.9±0.2^b	0.7±0.1^d	0.8±0.0^c	0.8±0.0^c	0.8±0.0^c	0.8±0.0^c

$^{a\text{-}c}$Values with different superscripts in a row differ significantly (P<0.05).

The semen retrieved following 72 h testicular storage at 18 °C recorded acceptable sperm head length (8.5-9.4 µm), width (4.3-4.8 µm), area (30.0-34.8 µm^2), perimeter (22.0-24.1 µm) and midpiece width (1.0-1.2 µm) which fell within the threshold.

Table 4.7: The effect of testicular storage time interval on the sperm morphometric trait of boar cauda epididymis stored at 18 °C (mean ± SD).

	Testicular storage time interval at 18 °C							
	0 h	**2 h**	**4 h**	**8 h**	**12 h**	**24 h**	**48 h**	**72 h**
				Sperm head trait				
Length (µm)	9.0±2.1^{c}	8.9±1.1^{c}	8.5±1.6^{d}	9.4±0.5^{a}	9.2±0.9^{b}	9.2±0.9^{b}	8.5±0.9^{d}	8.5±0.7^{d}
Width (µm)	4.5±0.6^{b}	4.6±0.6^{b}	4.3±0.8^{c}	4.8±0.3^{a}	4.6±0.5^{b}	4.6±0.6^{b}	4.3±0.6^{c}	4.4±0.4^{c}
Area (µm^{2})	33.4±6.0^{b}	33.3±6.0^{b}	30.6±7.0^{c}	35.7±2.8^{a}	34.8±4.9^{a}	34.2±5.2^{a}	30.0±5.1^{c}	30.4±3.8^{c}
Perimeter (µm)	23.4±2.8^{b}	23.2±2.6^{b}	22.9±4.1^{c}	24.1±1.6^{a}	23.8±2.2^{b}	23.6±2.2^{b}	22.0±2.4^{c}	22.3±1.7^{c}
				Sperm midpiece trait				
Width (µm)	1.1±0.3^{a}	1.1±0.5^{a}	1.2±0.5^{a}	1.0±0.3^{a}	1.0±0.3^{a}	1.0±0.3^{a}	1.0±0.5^{a}	1.1±0.3^{a}
				Sperm shape indices				
Ellipticity	2.0±0.3^{a}	2.0±0.2^{a}	2.0±0.6^{a}	2.0±0.1^{a}	2.0±0.4^{a}	2.0±0.4^{a}	2.1±0.7^{a}	2.0±0.2^{a}
Elongation	0.3±0.0^{a}	0.3±0.0^{a}	0.3±0.1^{a}	0.3±0.0^{a}	0.3±0.0^{a}	0.3±0.0^{a}	0.3±0.1^{a}	0.3±0.0^{a}
Regularity	0.9±0.1^{a}	0.9±0.0^{a}	0.9±0.1^{a}	0.9±0.0^{a}	0.9±0.3^{a}	0.9±0.0^{a}	0.9±0.0^{a}	0.9±0.0^{a}
Roughness	0.8±0.1^{a}	0.8±0.1^{a}	0.8±0.2^{a}	0.8±0.0^{a}	0.8±0.1^{a}	0.8±0.1^{a}	0.8±0.1^{a}	0.8±0.1^{a}

$^{a\text{-}c}$Values with different superscripts in a row differ significantly ($P<0.05$).

4.4 Discussion

Recovery of post-mortem sperm is a crucial technique for acquiring germplasm reserves from animals that are genetically valuable (García-Vázquez et al., 2015). The current study used the minimum and maximum allowable percentage of total motility: 60-70%; progressive motility: 50%; viability: 50-75%; normal morphology: 80-85% with <15% abnormality for boar sperm sample considered good enough for insemination (Basioura et al., 2025). The sperm retrieved from epididymides stored for 0-48 h at 5 °C recorded the sperm total motility percentage that falls within the maximum threshold. However, the progressive motility percentage recorded from the sperm retrieved after 2, 4 and 24 h testicular holding at 5 °C were the only once that fell within the minimum threshold. Therefore, the boar epididymal sperm can be best retrieved from the epididymides stored at 5 °C for 2 h. While the observations with epididymides stored at 5 °C following 72 h holding recorded atleast greater than 50% normal and motile sperm. Basioura et al. (2025) note that the successful fertilisation has been reported in the literature with 50% normal and motile sperm, but it is not regarded as a minimum threshold for artificial insemination in pigs because it may impose the risk of low succession rate. Although, 50% normal and motile sperm it is utilized as a bottom-line cutoff in some research to eliminate the outliers from data but is not considered as a target for a high-performance farming (Didion et al., 2013).

The reason for the survival of sperm in the epididymides stored at 5 °C seems to be consistent with the knowledge on semen preservation principles, which state that lower temperature slows down metabolic processes and lessens the pace at which slowing post-mortem tissue degradation (Monteiro et al., 2013; Aurich et al., 2020). The study by Stand et al. (2016) further stated that the sperm retrieved from the cauda contains epididymal fluid, which may play a role in providing a protective effect against cold shock to the sperm to survive up to 3 days. Furthermore, though the testicular storage temperature of 18 °C has retrieved the sperm with minimum acceptable sperm total motility following 0-8 h, there was a decline in sperm progressive motility with the prolonged testicular transportation period. The 18 °C testicular storage temperature findings might be explained by the elevated metabolic activity, which might speed up cellular deterioration and impair sperm motility in the testes (Hoang-Thi et al., 2022). These results demonstrate that the effect of testicular storage time interval is dependent on the storage temperature.

The results of this study match those observed in the previous studies on boar [48 h at 4 °C 48 h; (Kikuchi et al., 2016)], stallion [96 h at 5 °C (Monteiro et al., 2013)] and ram [<5 °C (Yang & Honaramooz, 2010)], which showed the beneficial effect of transporting epididymides at low temperature on the sperm quality. The storage of epididymis at 5 °C has proven to can maintain the sperm quality while prevent a decline in sperm deterioration in various species (Mir et al., 2012; Monteiro et al., 2013; Vidament et al., 2012). Although the 0-72 h testicular storage at 5 and 18 °C maintained acceptable sperm head traits, midpiece width and shape incidences, acrosomal damage as a result of amorphous abnormality was observed with the prolonged testicular storage at 18 °C. Similarities were observed by Lone et al. (2011), who recorded a higher percentage of acrosomal damage in the ram sperm retrieved from the epididymides stored 18 °C for 72 h. The 18 °C epididymides storage temperature and prolonged duration may impose a negative impact on the permeability and affect the distribution of the antigen in the sperm membrane (Chinonye et al., 2021). It may further contribute the oxidative stress and induces metabolic activity which may lead to lower motility, acrosomal damage leading to the abnormality in morphology of the sperm (Ostadian et al., 2022).

The evidence presented in this section suggests that the testicular storage time interval provides the most ideal condition for sperm morphometry while diminishing energy wastage and extending sperm lifespan (Vidament et al., 2012). The current result agrees with previous results of Lone et al. (2011) who recorded ram epididymides to have maintained their sperm morphology and motility with testicular transportation of 9-6 °C to the laboratory for processing and evaluations. These findings further support the idea of James et al. (2020), who stated the importance of the epididymal fluids combined with the sperm in the cauda, which play a role in improving the sperm's ability to proliferate and fertilise while also inhibiting the exocytosis of acrosomes. Therefore, sperm in the epididymis can survive for several days after death (Pleuger et al., 2020

4.5 Conclusion

Although this study showed the possibility of retrieving the epididymal from epididymides stored up to 72 h (0-72 h) at 5 °C. The results indicate that the boar epididymal sperm can be best recovered from the epididymides following 2-24 h testicular storage at 5 °C for higher fertilisation success. Further indicated that it can also be possible to retrieve the sperm from the epididymides stored at 18 °C for up to 8 h (0-8 h) when the 5 °C is not possible. The findings emphasise the importance of temperature in preserving boar testes to maintain sperm quality following animal death. These findings highlight the necessity of maintaining lower temperatures to protect not just the testes or epididymis from degrading easily, but also functional features linked to sperm motility and morphology. This study concludes that even after the death of the boar, it may be possible to recover valuable genetic material (epididymal sperm). However, with the ethical requirements of transporting the animal product such as testes, shipping of the recovered sperm in a liquid form may be the only technique practical and acceptable in many areas. Therefore, further analysis on the acceptable temperature and duration for storing epididymal sperm in a liquid form is required.

4.6 References

Aurich, J., Kuhl, J., Tichy, A. and Aurich, C., 2020. Efficiency of semen cryopreservation in stallions. *Animals*, *10*(6), p.1033. https://doi.org/10.3390/ani10061033

Basioura, A., Tsakmakidis, I.A., Morrell, J.M. and Ntallaris, T., 2025. Artificial insemination of boar semen doses prepared with a low-density colloid under field conditions. *Frontiers in Veterinary Science*, *12*, p.1611751. https://doi.org/10.3389/fvets.2025.1611751

Bertol, M.A.F., Weiss, R.R., Thomaz-Soccol, V., Kozicki, L.E., Fujita, A.S., Abreu, R.A.D. and Green, K.T., 2013. Viability of bull spermatozoa collected from the epididymis stored at 18-20 C. Brazilian Archives of Biology and Technology, 56(5), pp.777-783. https://doi.org/10.1590/S1516-89132013000500008.

Chenoweth, P.J. and McPherson, F.J., 2016. Bull breeding soundness, semen evaluation and cattle productivity. *Animal Reproduction Science*, *169*, pp.32-36. https://doi.org/10.1016/j.anireprosci.2016.03.001

Chinonye Eke, F. and Ahemen, T., 2021. Effect of cold storage on livability and progressive motility of cauda epididymal spermatozoa of red sokoto buck recovered post mortem. *ScienceOpen Preprints*. https://doi.org/10.14293/S2199-1006.1.SOR-.PPODVYP.v1

Didion, B.A., Braun, G.D. and Duggan, M.V., 2013. Field fertility of frozen boar semen: A retrospective report comprising over 2600 AI services spanning a four year period. *Animal reproduction science*, *137*(3-4), pp.189-196. https://doi.org/10.1016/j.anireprosci.2013.01.001

Engdawork, A., Belayhun, T. and Aseged, T., 2024. The role of reproductive technologies and cryopreservation of genetic materials in the conservation of animal genetic resources, A review. Ecological Genetics and Genomics, p.100250. https://doi.org/10.1016/j.egg.2024.100250

Faes, K. and Goossens, E., 2017. Short-term storage of human testicular tissue: effect of storage temperature and tissue size. Reproductive Biomedicine Online, 35(2), pp.180-188. https://doi.org/10.1016/j.rbmo.2017.04.011

Falomo, M.E., Rossi, M. and Mantovani, R., 2016. Collection, storage and freezability of equine epididymal spermatozoa. Italian Journal of Animal Science, 15(3), pp.386-389. https://doi.org/10.1080/1828051X.2016.1210485

García-Vázquez, F.A., Hernández-Caravaca, I., Matás, C., Soriano-Úbeda, C., Abril-Sánchez, S. and Izquierdo-Rico, M.J., 2015. Morphological study of boar sperm during their passage through the female genital tract. *Journal of Reproduction and Development*, *61*(5), pp.407-413. https://doi.org/10.1262/jrd.2014-170

Hoang-Thi, A.P., Dang-Thi, A.T., Phan-Van, S., Nguyen-Ba, T., Truong-Thi, P.L., Le-Minh, T., Nguyen-Vu, Q.H. and Nguyen-Thanh, T., 2022. The impact of high ambient temperature on human sperm parameters: a meta-analysis. *Iranian Journal of Public Health*, *51*(4), p.710. https://doi.org/10.18502/ijph.v51i4.9232

James, E.R., Carrell, D.T., Aston, K.I., Jenkins, T.G., Yeste, M. and Salas-Huetos, A., 2020. The role of the epididymis and the contribution of epididymosomes to mammalian reproduction. *International Journal of Molecular Sciences*, *21*(15), p.5377. https://doi.org/10.3390/ijms21155377

Kikuchi, K., Kaneko, H., Nakai, M., Somfai, T., Kashiwazaki, N. and Nagai, T., 2016. Contribution of in vitro systems to preservation and utilization of porcine genetic resources. *Theriogenology*, *86*(1), pp.170-175. https://doi.org/10.1016/j.theriogenology.2016.04.029

Lone, F.A., Islam, R., Khan, M.Z. and Sofi, K.A., 2011. Effect of transportation temperature on the quality of cauda epididymal spermatozoa of ram. *Animal Reproduction Science*, *123*(1-2), pp.54-59. https://doi.org/10.1016/j.anireprosci.2010.10.012

Mir, S.S., Lone, F.A., Khan, M.Z., Malik, A.A., Islam, R. and Sofi, K.A., 2012. Effect of cold storage period on the quality of ram cauda epididymal spermatozoa recovered postmortem. Turkish Journal of Veterinary & Animal Sciences, 36(6), pp.683-687. https://doi.org/10.3906/vet-1107-21

Monteiro, G.A., Guasti, P.N., Rocha, A.S., Martin, I., Sancler-Silva, Y.F.R., Dell'Aqua, C.P.F., Dell'Aqua Jr, J.A. and Papa, F.O., 2013. Effect of storage time and temperature of equine

epididymis on the viability, motion parameters, and freezability of epididymal sperm. *Journal of Equine Veterinary Science*, *33*(3), pp.169-173. https://doi.org/10.1016/j.jevs.2012.06.010

Ostadian, C., Mehrafza, M., Eftekhari, A., Aghajani, S., Vahabzadeh, H., Gholami, M., Raoufi, A., Samadnia, S., Hosseinzadeh, E. and Hosseini, A., 2022. The effect of prolonged incubation of sperm at testis temperature versus room temperature on semen parameters-an experimental study. *Journal of Obstetrics, Gynecology and Cancer Research*, *7*(4), pp.323-328. https://doi.org/10.30699/jogcr.7.4.323

Pleuger, C., Silva, E.J.R., Pilatz, A., Bhushan, S. and Meinhardt, A., 2020. Differential immune response to infection and acute inflammation along the epididymis. *Frontiers in Immunology*, *11*, p.599594. https://doi.org/10.3389/fimmu.2020.599594

Rodriguez-Martinez, H., Roca, J., Alvarez-Rodriguez, M. and Martinez-Serrano, C.A., 2022. How does the boar epididymis regulate the emission of fertile spermatozoa?. *Animal Reproduction Science*, *246*, p.106829. https://doi.org/10.1016/j.anireprosci.2021.106829

Thema, M.A., Mphaphathi, M.L., Ledwaba, M.R. and Nedambale, T.L., 2022. Investigation of the efficacy of different semen extenders and in vitro storage period on Windsnyer boar sperm quality equilibrated at 18 °C. *Reproduction in Domestic Animals*, *57*(12), pp.1644-1648. https://doi.org/10.1111/rda.14256

Turri, F.E.D.E.R.I.C.A., Madeddu, M., Gliozzi, T.M., Gandini, G. and Pizzi, F., 2012. Influence of recovery methods and extenders on bull epididymal spermatozoa quality. *Reproduction in Domestic Animals*, *47*(5), pp.712-717. https://doi.org/10.1111/j.1439-0531.2011.01948.x

Van Leeuwen, M.M., 2020. *The effect of processing on the short-and long-term viability of epididymal African buffalo (Syncerus caffer) spermatozoa*. Doctoral dissertation, Stellenbosch: Stellenbosch University).

Vidament, M., Magistrini, M., Le Foll, Y., Levillain, N., Yvon, J.M., Duchamp, G. and Blesbois, E., 2012. Temperatures from 4 to 15 C are suitable for preserving the fertilizing capacity of stallion semen stored for 22 h or more in INRA96 extender. Theriogenology, 78(2), pp.297-307. https://doi.org/10.1016/j.theriogenology.2012.01.018 https://scholar.sun.ac.za/handle/10019.1/108131 Accessed 27/05/2025

Wachida, N., Bassey, U.E. and Dawuda, P.M., 2019. Effect of storage time on the quality of cauda epididymal spermatozoa of West African dwarf (WAD) rams. *Animal Reproduction Science*, *205*, pp.144-149. https://doi.org/10.1016/j.anireprosci.2019.05.001

Yang, Y. and Honaramooz, A., 2010. Effects of medium and hypothermic temperatures on preservation of isolated porcine testis cells. *Reproduction, Fertility and Development*, *22*(3), pp.523-532.

Chapter 5: Comparisons of boar epididymal sperm morphometric and motility changes during semen equilibration at 5 and 18 °C

Abstract

This study aimed to evaluate boar epididymal sperm morphometric and motility traits changes during semen equilibration at 5 and 18 °C. A total of 60 testes of heterogeneous boars were collected (6 testes/day) from the local abattoir and transported to the laboratory at 5 °C within 30 minutes after slaughter. Semen was retrieved from the caudal part of the epididymis following cooling the epididymides at 5 °C for 2 h using the slicing float-up method. Retrieved samples diluted with Beltsville Thawing Solution semen extender, pooled in 50 mL centrifuge tube/6 testes/day, equally allocated to treatment groups (5 and 18 °C) and equilibrate for 48 h. Sperm motility and morphometry traits were evaluated at different equilibration time intervals (0, 3, 6, 9, 12, 24 and 48 h) using a computer-assisted sperm analyser system. The diluted sperm samples equilibrated over 48 h at 18 ° C showed a sperm progressive motility decline from 78.9-28.1%, compared to a steeper decline from 80.6-10.9 % at 5 °C ($P<0.05$). Furthermore, diluted semen samples equilibrated at 5 and 18 °C for up to 24 h recorded acceptable sperm head, midpiece, and incidences of traits that fell within the threshold range. However, the sperm head trait values recorded after 48 h at 5 °C were lower than the acceptable range, showing that sperm shrank due to the lower temperature with prolonged storage. Therefore, these findings suggest that 18 °C is a suitable storage temperature for equilibrating the boar epididymal sperm for up to 48 h. Furthermore, it is recommended that boar epididymal sperm motility and morphometry be maintained for 48 h in a diluted semen storage at 18 °C.

Keywords: Cauda, time interval, storage temperature, *in vitro*.

5.1 Introduction

Unanticipated deaths of animals with superior genetic traits and challenges of semen collection contribute to the rise in artificial reproductive technologies (Standet al. 2016). The need to save endangered species has increased interest in the potential recovery of viable sperm from the epididymis (Alvarez-Rodriguez et al., 2018; Rola et al., 2021). While extensive research has focused on ejaculated semen, studies on boar epididymal sperm preservation remain limited. Although there has been advancement of research in the creation/improvement of an efficient boar ejaculated semen cryopreservation protocols, the most common method for semen preservation is still liquid storage (Waberski et al., 2019; Wysokińska et al., 2023). Liquid preservation facilitates the shipping of semen within a limited time (Thema et al., 2022).

Among mammalian species, boar sperm are particularly susceptible to cold shock, providing an ideal model to study their sperm reaction to chilling and hypothermic storage (Bertol et al., 2013). According to Martín-Hidalgo et al. (2011), a chilling injury may induce sperm to lose motility, morphology, and membrane integrity, compromising their fertility potential. This is due to the high polyunsaturated fatty acid content and low sterol-to-phospholipid ratio in the sperm plasma membranes (Am-In et al., 2011). According to Casas and Althouse (2013), the resistance of boar sperm to chilling and damage can be increased by optimising the storage temperature and time. Sperm functionality and its tolerance to hypothermic stress are primarily influenced by semen storage time and temperature (Schulze et al., 2013). Boar sperm undergoes many modifications during storage; the storage temperature, directly related to storage time, can impair sperm morphology (Gaczarzewicz et al., 2015).

To date, approximately 99 % of artificial insemination procedures in pigs use liquid preserved semen stored at 15-18 °C (Wiebke et al., 2022). The 15-18 °C lowers sperm metabolic activity while maintaining cell membrane integrity (Gaczarzewicz et al., 2015). However, except for boars, artificial insemination in domestic animals of other species typically involves the storage of semen at 5 °C (Waberski & Luther, 2024). Storage at 5 °C is thought to improve protection against bacterial growth generally found in the ejaculates and potential drug-resistant pollutants from the environment, particularly in the absence of antibiotics in the semen extenders (Waberski et al., 2019; Schulze et al., 2020).

Sperm are highly polarized and specialised cells whose primary function is to reach the oocyte/ovum and fertilise *in vivo*/*vitro* (Teves and Roldan, 2022). The fertilisation success is dependent on sperm functionality and morphology; sperm is intimately tied to function and, if abnormal, may not be able to swim adequately and fertilise the oocyte (Teves and Roldan, 2022). Variation between ejaculated/epididymal sperm of various species in the extent to which it may be damaged by preservation has been widely reported (Esteso et al., 2015). However, to our knowledge, no published studies have documented changes in boar epididymal sperm morphometry and motility induced by liquid semen storage temperature and storage time. While this study aimed to evaluate boar epididymal sperm morphometric and motility traits changes during semen equilibration at 5 and 18 °C. It is therefore, hypothesised that the equilibration of boar epididymal sperm at 5 and 18 °C for different durations significantly influences sperm morphometry and motility traits.

5.2 Materials and methods

5.2.1 Study area and design

The study was conducted at the Germplasm Conservation and Reproductive Biotechnologies section in the Agricultural Research Council, Irene, in Gauteng Province. The area is located at 25º 53′ 59.6″ South latitude and 28º 12′ 51.6″ East longitude in Pretoria, South Africa.

5.2.2 Statement of animal rights

The experimental procedures were performed in accordance with the ethical standards approved by the Agricultural Research Council-Animal Production Ethics Committee [APIEC 23/05], University of KwaZulu-Natal Research Ethics Committee [AREC/00007100/2024], and Section 20 of the Animal Diseases Act no 35 of 1984 [12/11/1/18 (6269BM)].

5.2.3 Preparation of boar semen extenders

The semen extender used in the experiment was Beltsville Thawing Solution (BTS; IMV Technologies, France), which was prepared a day before use. The BTS extender was prepared by dissolving a sachet of each extender in a litre of sterile water (Sabax®, Adcock Ingram, South Africa) and stored at 18 °C until used.

5.2.4 Boar epididymal sperm retrieval

A total of 60 testes of heterogeneous boars were collected (6 testes/day) from the local abattoir and transported to the laboratory at 5 °C within 30 minutes after slaughter. The testes were further stored at 5 °C for 2 h before being processed. Following storage, the caudal epididymides together with and proximal ducts were removed from the testes and cleaned with saline water (NaCl 0.9%). To avoid blood contamination, superficial blood vessels were punctured so that most of the blood could be wiped off. The semen samples were retrieved from the cauda epididymides with the aid of the slicing float-up method. The slicing float-up method was performed by slicing the cauda epididymides with the blade to release the semen into the petri dish and diluting it with a 5 mL BTS semen extender. Semen samples were pooled following semen retrieval/per day, equally divided, and equilibrated at 5 and 18 °C.

5.2.5 Storage and evaluation of the boar epididymal sperm motility traits

Semen samples equilibrated at 5 and 18 °C were evaluated at different time intervals (0, 3, 6, 9, 12, 24 and 48 h) for sperm motility using a computer-assisted sperm analyser (CASA; Microptic, Sperm Class Analyzer® 6.6.80) system. The volume of 5 µL of diluted semen sample was transferred to a warmed (37 °C) microscope glass slide and covered with a warm glass coverslip. Two fields of semen samples

with an average of 250 sperm (34.3×10^6/mL/treatment/replication) were captured using a microscope (Nikon®, Japan) at 10X magnification using the CASA system.

5.2.6 Semen staining preparation for boar epididymal sperm morphometry

Semen samples equilibrated at 5 and 18 °C were evaluated at different time intervals (0, 3, 6, 9, 12, 24, and 48 h) for sperm morphometry using the CASA system. The retrieved semen samples were first diluted with BTS extender at a ratio of 1:10. The diluted semen samples were further fixed in 4% Formaldehyde for 10 minutes. Then, 10 µL of the fixed semen sample/storage temperature/time interval was smeared on a separate microscopic glass slide and allowed to air dry overnight at room temperature. During staining, the dried microscope glass slide sample was gently dipped in the SpermBlue® tray for 2 minutes and in distilled water for 3 seconds (to remove debris and ensure a clean background). The paper towel was used to wipe the back of the microscope glass slide and allowed to air-dry at room temperature. Following drying, the slide was mounted using Dibutylphthalate Polystyrene Xylene mounting medium (Associated Chemical Enterprises, South Africa) and covered with the glass coverslip.

5.2.7 Evaluation of boar epididymal sperm morphometry traits

Boar epididymal sperm morphometric traits were captured with a microscope (Nikon®, Japan) at 40X magnification using the CASA system. At least 100 sperm were measured at different time intervals/storage temperature/replication (100x10=1000 sperm). Overall, 7000 sperm at 5 °C and 7000 at 18 °C were evaluated for sperm morphometric traits at different time intervals (0,3, 6, 9, 12, 24, and 48 h). The microscope glass slide was observed (the focus knob was used to focus sperm) and measured for the sperm head area (A; μm^2), head perimeter (P; µm), head length (L; µm), head width (W; µm), head ellipticity (length/width), head elongation (L-W/L+W), head regularity (πx (L.W/4.A), head roughness [4π x (A/P^2)], midpiece width and the shape indices (head shape type, type of the sperm and midpiece size).

5.2.8 Statistical analysis

The sperm morphometric data were divided into two groups: The first group was the measurements of sperm head primary traits (length, L; area, A; width, W and perimeter, P, ellipticity, L/W; rugosity, 4 πA/P2; elongation, L − W/L + W and regularity, πLW/4A). The second group determined the sperm head indices (head type, normal, shape, acrosome type, and midpiece type). In the first sperm morphometric data group, descriptive statistics were used to establish the minimum and maximum values (thresholds) using 10-90 percentiles for every morphometric trait shown in Table 5.1.

Table 5.1: Minimum and maximum acceptable values for morphometric traits in order to define normal boar epididymal sperm morphometry.

Sperm morphometric traits	Boar Minimum	Maximum
Length (µm)	8.20	9.70
Width (µm)	3.50	5.50
Perimeter (µm)	16.20	26.20
Area (µm)	28.49	44.00
Ellipticity	1.81	2.16
Regularity	0.70	1.00
Elongation	0.29	0.37
Roughness	0.62	1.50
Midpiece width (µm)	0.50	1.60

The data for the first sperm morphometric data group and sperm motility trait were analysed with two-way repeated measures analysis of variance using general linear model. The main effect of independent variables [equilibration temperature and different time interval (repeated observation with the same sample)] and their interaction were measured based on the sperm morphometric and motility changes (dependent variables). The *Shapiro-Wilk* test was performed on standardized residuals to test for deviations from normality. Student's t-LSDs (least significant differences) were calculated at a 5% significance level to compare the means of the significant source effect of semen storage temperature on the sperm morphometric first data group and motility traits evaluated at different time intervals.

The second sperm morphometric data group was analysed using a Chi-square test to determine the effect of semen storage temperature on the sperm percentage variations regarding the head type, normal shape, acrosome type, and midpiece type evaluated at different time intervals. The scores were subjected to a 1:1 frequency table, and a Chi-Square (χ^2) test was performed to test for equal proportions. The tests were performed for association (patterns), and where significant differences were found, graphs were constructed to demonstrate the difference in patterns. All data analyses were performed using SAS version 9.4 statistical software.

5.3 Results

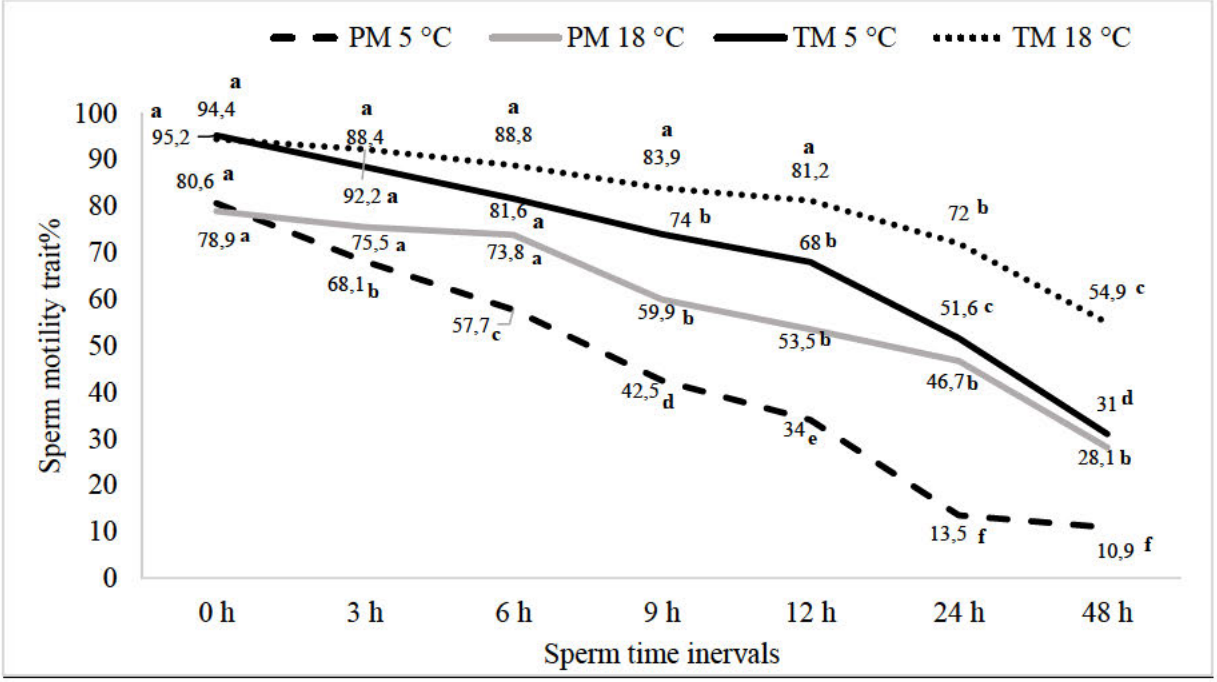

Figure 5.1: Effect of semen equilibration temperature on boar epididymal total and progressive sperm motility evaluated at different time intervals.

[a-f] Values with different superscripts in same sperm trait and storage temperature differ significantly across the bars (P<0.05). TM: Total motility, PM: Progressive motility.

The effect of liquid semen storage temperature on boar epididymal sperm total and progressive motility percentage evaluated at different time intervals is represented in Figures 5.1. The significant interaction between epididymal sperm equilibration time and temperature was observed for sperm motility and kinematics traits (P<0.05). Sperm total motility was greater than 90% immediately after retrieval (0 h) at both semen storage temperatures. The sperm total and progressive motility at 5 and 18 °C decreased with the prolonged semen storage time (P<0.05). However, sperm total motility ranged between 94.4-54.9% up to 48 h at 18 °C semen storage compared to 95.2-31.0% at 5 °C. The diluted sperm samples equilibrated over 48 h at 18 ° C showed a sperm progressive motility decline from 78.9-28.1%, compared to a steeper decline from 80.6-10.9 % at 5 °C (P<0.05). This demonstrate that the effect of epididymal sperm equilibration time is dependent on the storage temperature.

Table 5.2 and 5.3 represents the effect of liquid semen storage temperature on boar epididymal sperm velocity traits evaluated at different time intervals. Semen samples stored at 5 °C in comparison with 18 °C recorded least number of sperm moving at a curvilinear velocity [3 (66.3 vs 62.1 µm/sec), 6 (52.9 vs 64.7 µm/sec), 9 (54.3 vs 58.6 µm/sec), 12 (48.3 vs 53.2 µm/sec), 24 (47.0 vs 57.5 µm/sec) and 48 h (30.2 vs 51.8 µm/sec; P<0.05). This demonstrate that the effect of epididymal sperm equilibration time is dependent on the storage temperature.

Table 5.2: The effect of semen 5 °C equilibration temperature on boar epididymal sperm kinematics at different time intervals (mean ± SD).

Storage time interval at 5 °C	**VCL (µm/sec)**	**VAP (µm/sec)**	**VSL (µm/sec)**	**STR (%)**	**LIN (%)**	**WOB (%)**	**HA (%)**
0 h	68.4±12.0^{a}	34.7±5.5^{a}	18.8±2.6^{a}	52.5±4.2^{a}	27.6±3.7^{a}	51.2±3.2^{a}	1.5±1.6^{a}
3 h	52.9±13.3^{b}	27.9±8.9^{b}	13.7±4.3^{a}	49.0±5.0^{b}	24.0±2.1^{a}	49.0±3.0^{b}	0.3±0.6^{b}
6 h	54.3±8.9^{b}	18.4±4.2^{c}	13.5±2.4^{a}	50.3±8.4^{a}	25.1±4.6^{a}	48.9±3.7^{b}	0.2±0.2^{b}
9 h	48.3±10.1^{b}	22.6±5.2^{b}	10.8±2.2^{a}	47.4±6.6^{b}	21.7±2.5^{a}	46.0±4.0^{b}	0.4±0.8^{b}
12 h	47.0±7.3^{b}	23.5±3.8^{b}	10.9±2.1^{a}	45.9±6.1^{b}	22.8±2.6^{a}	50.0±4.8^{a}	0.2±0.4^{b}
24 h	30.2±14.2^{c}	15.6±7.2^{c}	6.9±2.8^{b}	48.4±15.3^{b}	25.3±9.2^{a}	53.2±8^{a}	0.1±0.4^{b}
48 h	37.9±11.6^{c}	18.4±4.2^{c}	7.2±2.5^{b}	40.5±6.6^{b}	18.5±4.1^{b}	48.3±7.8^{b}	0.1±0.2^{b}

$^{a\text{-}e}$Values with different superscripts differ significantly in the same column (P<0.05). VCL: curvilinear Velocity, VAP: average pathway velocity, VSL: straight line velocity, STR: straightness, LIN: linearity, WOB: Wobble, HA: hyperactivity.

Table 5.3: The effect of semen 18 °C equilibration temperature on boar epididymal sperm kinematics at different time intervals (mean ± SD).

Storage time interval at 18 °C	**VCL (µm/sec)**	**VAP (µm/sec)**	**VSL (µm/sec)**	**STR (%)**	**LIN (%)**	**WOB (%)**	**HA (%)**
0 h	66.3±12.8^{a}	33.8±5.8^{a}	18.4±2.6^{a}	53.2±4.6^{a}	28.3±4.0^{a}	51.5±3.2^{a}	1.4±1.6^{a}
3 h	62.1±17.0^{a}	31.1±8.3^{a}	15.1±3.5^{b}	48.8±6.7^{b}	24.7±3.8^{a}	50.1±2.2^{a}	0.2±0.3^{b}
6 h	64.7±15.0^{a}	32.0±7.1^{a}	18.3±2.6^{a}	56.5±6.4^{a}	28.9±4.9^{a}	49.8±2.9^{b}	0.8±0.9^{b}
9 h	58.6±12.3^{b}	28.4±6.4^{b}	12.9±3.2^{a}	45.6±9.6^{b}	21.9±5.1^{a}	48.0±3.2^{b}	0.5±0.7^{b}
12 h	53.2±13.3^{b}	25.2±7.0^{b}	11.5±2.0^{a}	47.4±8.4^{b}	21.7±3.2^{a}	46.2±3.6^{b}	0.5±0.9^{b}
24 h	57.5±20.1^{b}	27.9±8.5^{b}	12.1±2.9^{a}	44.4±11.4^{b}	22.8±7.5^{a}	49.8±2.2^{b}	1.9±2.4^{a}
48 h	51.8±26.4^{b}	22.0±11.3^{b}	9.3±4.1^{b}	44.1±8.1^{b}	18.6±5.6^{b}	42.4±6.1^{b}	0.7±1.9^{b}

$^{a\text{-}e}$Values with different superscripts differ significantly in the same column (P<0.05). VCL: curvilinear Velocity, VAP: average pathway velocity, VSL: straight line velocity, STR: straightness, LIN: linearity, WOB: Wobble, HA: hyperactivity.

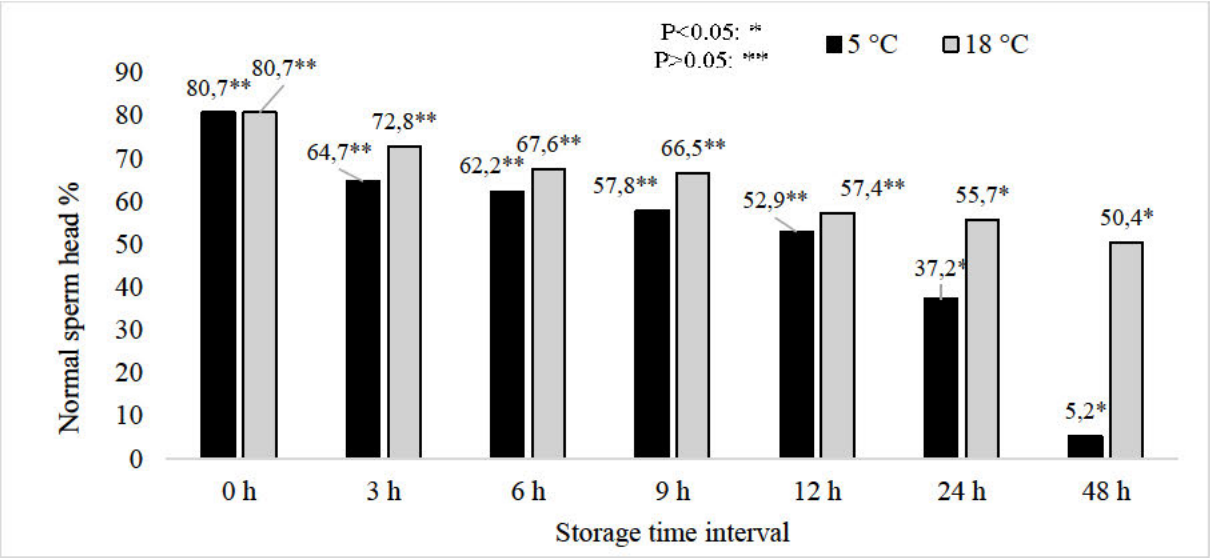

Figure 5.2: The effect of liquid semen storage temperature of boar epididymal sperm with normal heads recorded at different time intervals.

The significant difference (P<0.05) is represented by a star and non significant difference (P>0.05) is represented by two stars within the bars.

The effect of liquid semen storage temperature on boar epididymal sperm with normal heads was recorded at different time intervals (Figure 5.2). The current study recorded that the sperm with normal head percentage had numerically decreased as the time of semen storage increased, irrespective of the temperatures (P<0.05). Initially, at 0 h, the >80% sperm with normal heads were recorded in the semen stored at 5 and 18 °C. However, semen stored at 18 °C maintained >50% sperm with a normal head over 48 h of semen liquid storage, unlike 5 °C (P<0.05). In the semen samples stored at 5 °C, only >50% of those with normal sperm heads were maintained for up to 12 h of storage.

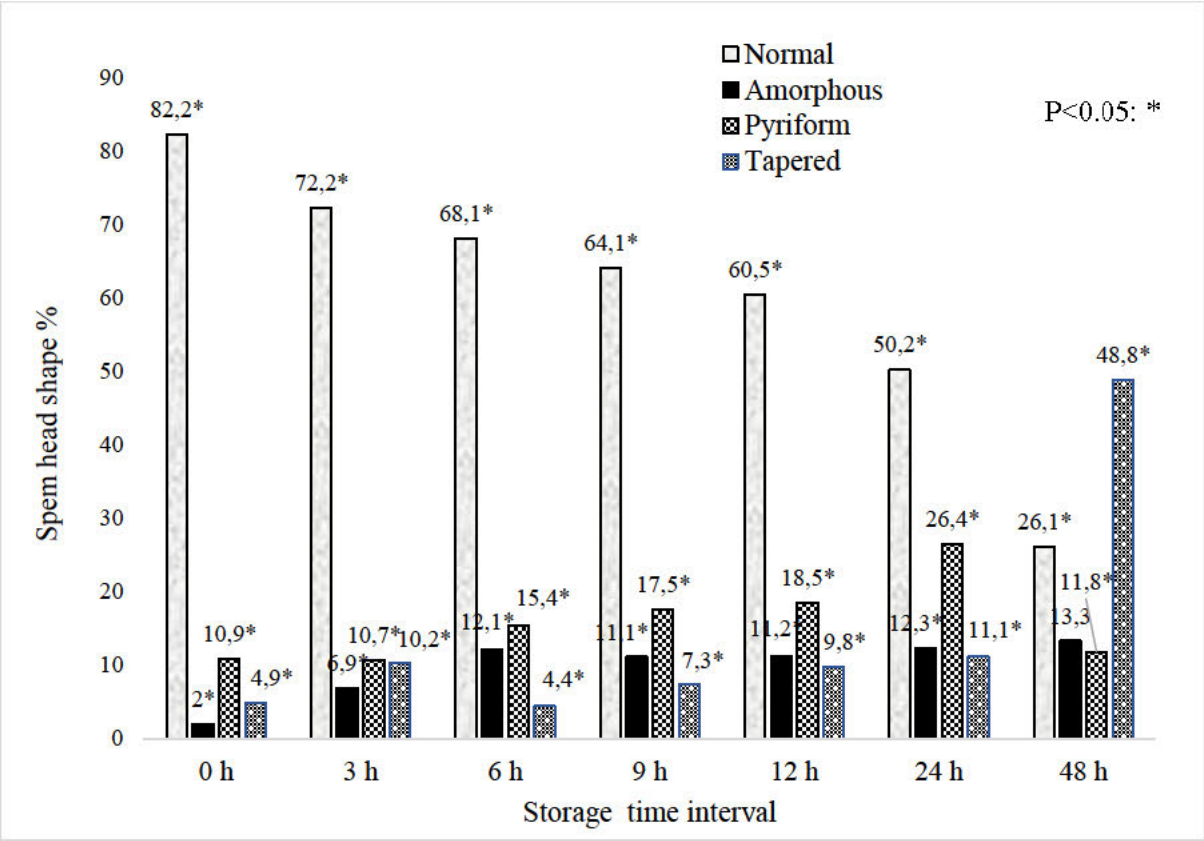

Figure 5.3: Boar epididymal sperm head shape changes during liquid semen storage at 5 °C.
The significant difference (P<0.05) is represented by a star within the bars.

Figure 5.3 represents boar epididymal sperm head shape changes during liquid semen storage at 5 °C. The percentage of sperm with normal head shape changes as the time of the semen samples increases at 5 °C. The sperm with tapered (48,8%) head shape were higher after 48 h of semen storage as compared to the other storage times, which affected the percentage of normal (26.1%) sperm (P<0.05). In the current study, the percentage of the sperm with tapered head shape recorded after 48 h of storage of semen was equivalent to the percentage of coiled-tailed sperm in the semen stored at 5 °C (P>0.05). There was >26% of sperm with a pyriform shape recorded after 24 h of storage in the semen stored at 5 °C.

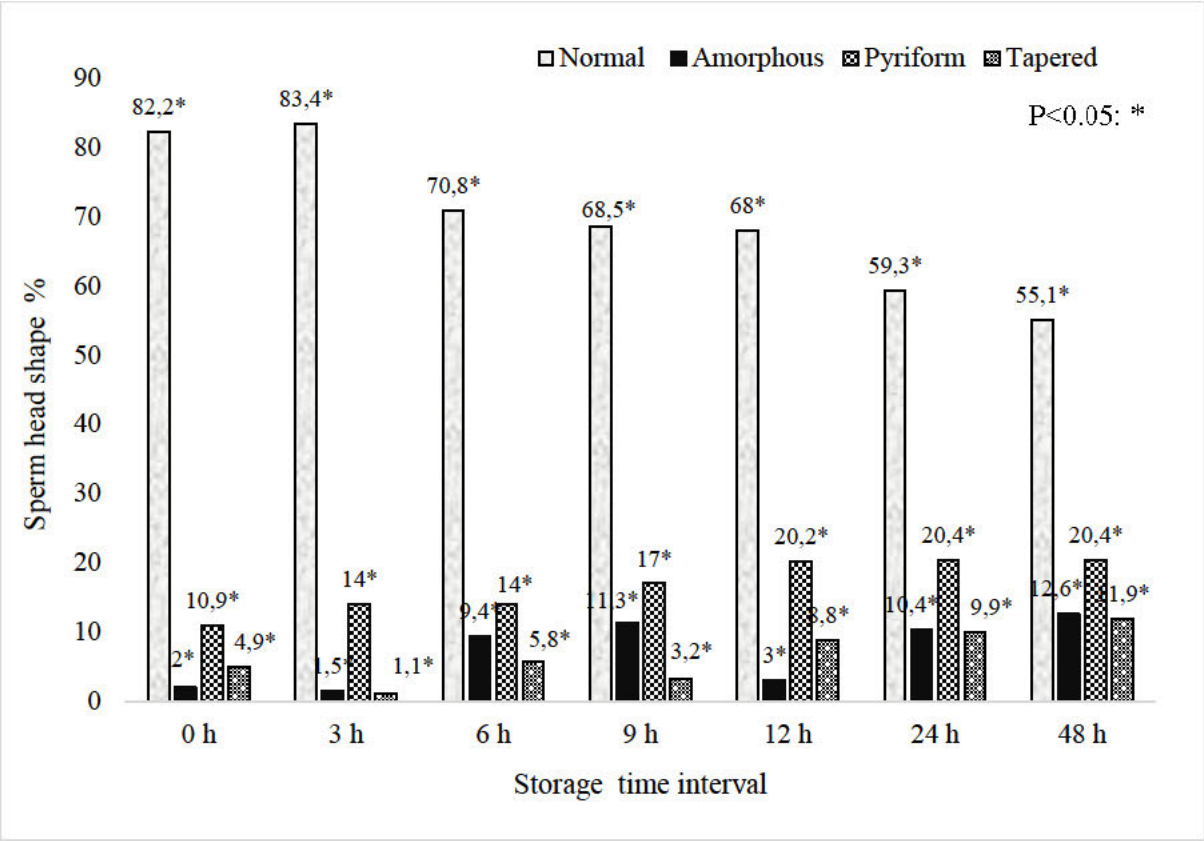

Figure 5.4: Boar epididymal sperm head shape changes during liquid semen storage at 18°C.

The significant difference (P<0.05) is represented by a star within the bars.

Figure 5.4 represents the effect of different liquid semen storage time intervals on the sperm head shape stored at 18 °C. The percentage of sperm with normal head shape changes as the increasing time storage at 18 °C. The 18 °C storage temperature maintained (82.2-55.1%) sperm with normal head shape over 48 h of semen storage. There was an increased number of sperm with pyriform (>20%) recorded after 12 h of semen storage at 18 °C compared to other abnormalities (P<0.05). However, there was only <20% of sperm with abnormal shape (amorphous, tapered, and pyriform) recorded over 48 h of semen storage at 18 °C.

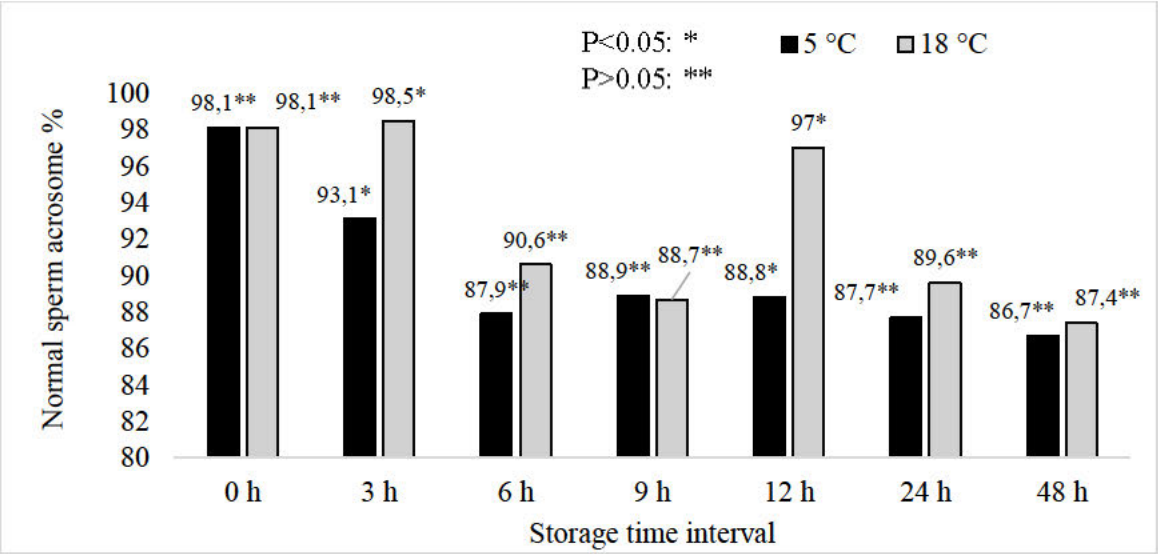

Figure 5.5: The effect of liquid storage temperature on boar epididymal sperm with normal acrosome recorded at different time intervals.

The significant difference (P<0.05) is represented by a star and non significant difference (P>0.05) is represented by two stars within the bars.

Figure 5.5 represents the effect of liquid semen storage temperature on boar epididymal sperm with normal acrosome recorded at different time intervals. There was an increasing percentage of sperm with an amorphous shape recorded in the current study, irrespective of storage temperature (P<0.05).

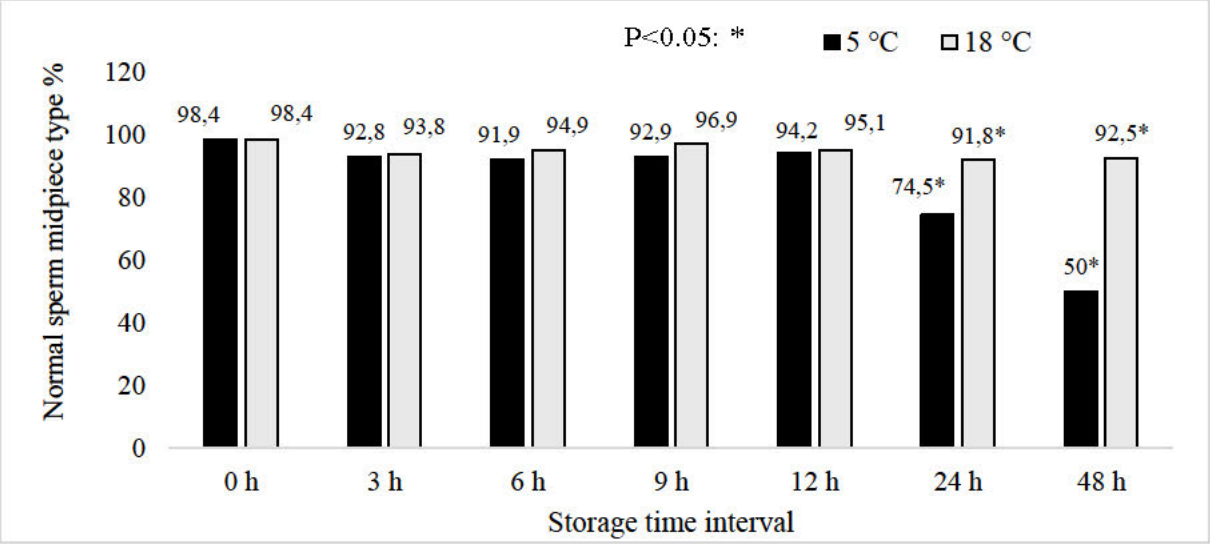

Figure 5.6: The effect of liquid semen storage temperature on boar epididymal sperm with normal midpiece recorded at different time intervals.

The significant difference (P<0.05) is represented by a star within the bars.

Figure 5.6 represents the effect of liquid storage temperature on boar epididymal sperm with normal midpieces recorded at different time intervals. The semen stored at 5 °C recorded the lowest percentage

of sperm, with normal midpiece recorded after 24-48 h storage ($P<0.05$). Furthermore, the coiled-tailed sperm recorded after 48 h in the semen stored at 5 °C influenced the sperm with a normal midpiece. Greater than 90 % sperm with normal midpieces were recorded over 48 h storage at 18 °C.

The boar epididymal sperm morphometric trait changes during liquid storage at 5 °C are presented in Table 5.4. The storage temperature of 5 °C affected the sperm morphometric measurements recorded in the current study compared to the 5 °C treatment ($P<0.05$). The results show that the sperm head length and area decreased with the increasing storage time at 5 °C compared to 18 °C treatment ($P<0.05$). Meanwhile, the sperm head width and perimeter increased after 12 h storage at 5 °C. The sperm head traits recorded up to 24 h of storage fell within an acceptable range. However, the sperm head traits recorded at 48 h were lower than the acceptable range, which shows that sperm shrank due to the lower temperature with the increasing semen storage time. No significant differences were recorded between the sperm elongation and regularity during the liquid semen storage at 5 °C.

Table 5.4: The boar epididymal sperm morphometric trait changes during liquid semen storage at 5 °C (mean ± SD).

	Liquid semen storage time interval						
	0 h	**3 h**	**6 h**	**9 h**	**12 h**	**24 h**	**48 h**
				Sperm head trait			
Length (µm)	9.0±0.8^{a}	8.9±0.8^{b}	8.8±0.6^{b}	8.8±0.7^{b}	8.9±0.6^{b}	8.7±0.8^{c}	8.1±0.6^{d}
Width (µm)	4.5±0.5^{b}	4.5±0.5^{b}	4.6±0.3^{a}	4.6±0.4^{a}	4.7±0.3^{a}	4.5±0.5^{b}	4.0±0.5^{c}
Area (μm^2)	36.6±5.7^{a}	33.5±4.9^{b}	33.4±3.6^{b}	32.8±4.0^{c}	33.6±3.3^{b}	32.0±4.4^{c}	26.6±4.0^{d}
Perimeter (µm)	21.4±2.6^{c}	23.2±2.0^{a}	23.2±1.3^{a}	23.1±1.4^{a}	23.3±1.2^{a}	22.8±1.8^{b}	21.1±2.0^{c}
				Sperm midpiece trait			
Width (µm)	1.1±0.3^{c}	2.8±1.3^{a}	2.8±1.3^{a}	2.7±1.2^{a}	2.7±1.1^{a}	2.5±1.3^{b}	2.3±1.3^{b}
				Sperm shape indices			
Ellipticity	2.0±0.4^{a}	1.9±0.2^{b}	1.9±0.2^{b}	1.9±0.2^{b}	1.9±0.2^{b}	1.9±0.2^{b}	2.0±0.2^{a}
Elongation	0.3±0.1^{a}	0.3±0.0^{a}	0.3±0.0^{a}	0.3±0.0^{a}	0.3±0.0^{a}	0.3±0.1^{a}	0.3±0.0^{a}
Regularity	0.9±0.1^{a}	0.9±0.0^{a}	0.9±0.0^{a}	0.9±0.0^{a}	0.9±0.0^{a}	0.9±0.0^{a}	0.9±0.0^{a}
Roughness	1.0±0.3^{a}	0.8±0.0^{b}	0.8±0.0^{b}	0.8±0.0^{b}	0.8±0.0^{b}	0.8±0.0^{b}	0.7±0.1^{b}

$^{a-d}$ Values with different superscripts in a row differ significantly ($P<0.05$).

The boar epididymal sperm morphometric trait changes during liquid semen storage at 18 °C are presented in Table 5.5. The 18 °C storage temperature did not affect the sperm morphometric traits over 48 h of storage. Semen stored at 18 °C recorded acceptable sperm head length (8.7-9.1 µm), width (4.5-4.8 µm), area (33.3-36.6 μm^2), perimeter (21.4-23.7 µm) and midpiece width (1.1-2.8 µm), which fell within the normal range. No significant differences were recorded between sperm elongation and regularity throughout the liquid semen storage.

Table 5.5: The boar epididymal sperm morphometric trait changes during liquid semen storage at 18 °C (mean ± SD).

	Liquid semen storage time interval						
	0 h	**3 h**	**6 h**	**9 h**	**12 h**	**24 h**	**48 h**
				Sperm head trait			
Length (µm)	9.0±0.8[a]	9.1±0.5[a]	8.9±0.8[b]	8.8±0.6[b]	8.9±0.5[b]	8.9±0.7[b]	8.7±0.6[c]
Width (µm)	4.5±0.5[b]	4.7±0.3[a]	4.7±0.4[a]	4.7±0.3[a]	4.8±0.3[a]	4.6±0.4[b]	4.7±0.3[a]
Area (μm^2)	36.6±5.7[a]	34.8±3.2[b]	34.1±4.0[b]	33.3±3.3[c]	34.6±2.9[b]	33.0±3.9[c]	33.0±3.6[c]
Perimeter (µm)	21.4±2.6[b]	23.7±1.2[a]	23.3±1.8[a]	23.0±1.3[a]	23.5±1.1[a]	23.1±1.7[a]	23.1±1.3[a]
				Sperm midpiece trait			
Width (µm)	1.1±0.3[c]	2.8±1.1[a]	2.7±1.2[a]	2.2±1.1[b]	2.6±1.0[a]	2.4±1.2[b]	2.6±1.3[a]
				Sperm shape indices			
Ellipticity	2.0±0.4[a]	1.9±0.1[b]	1.9±0.2[b]	1.9±0.2[b]	1.9±0.2[b]	1.9±0.2[b]	1.9±0.2[b]
Elongation	0.3±0.1[a]	0.3±0.0[a]	0.3±0.0[a]	0.3±0.0[a]	0.3±0.0[a]	0.3±0.0[a]	0.3±0.0[a]
Regularity	0.9±0.1[a]	0.9±0.0[a]	0.9±0.0[a]	0.9±0.0[a]	0.9±0.0[a]	0.9±0.0[a]	0.9±0.0[a]
Roughness	1.0±0.3[a]	0.8±0.0[b]	0.8±0.0[b]	0.8±0.0[b]	0.8±0.0[b]	0.8±0.0[b]	0.8±0.0[a]

[a-c] Values with different superscripts in a row differ significantly ($P<0.05$).

5.4 Discussion

The current study found that semen storage at 18 °C more effectively preserved boar epididymal sperm motility and morphometric traits over 48 h than storage at 5 °C. Furthermore, higher values of total and progressive sperm motility percentages were recorded in the semen stored at 18 °C for 48 h. These findings align with previous studies demonstrating that storage at 15-18 °C supports sperm quality, while lower temperatures increase membrane permeability and cold shock susceptibility (Guthrie & Welch, 2012; Schulze et al., 2013). Furthermore, semen storage temperature below 10 ºC is commonly known to reduce sperm enzyme activity and alternate membrane proteins (Schulze et al., 2013). The recent evidence by Schmid et al. (2013) reported chilling as the factor that led to a loss of sperm quality traits during liquid semen storage. These results agree with Yeste (2016), findings which recorded the decrease in sperm motility with the increasing storage time. The changes recorded in sperm motility over semen storage time are a common phenomenon attributed to various factors such as temperature fluctuations, oxidative stress, and changes in sperm morphology (Yeste, 2016; Guthrie & Welch, 2012).

The decrease in sperm with normal head morphology percentage with the increased semen storage time at 5 and 18 ºC was observed in this study. The findings of the current study are consistent with those of Wysokińska et al. (2023), who recorded a decrease in percentages of morphological defects in sperm with the increased storage time of the diluted ejaculated semen.

This study further recorded the changes in sperm morphometric dimensions during storage of boar epididymal semen at 5 and 18 °C. This finding agrees with the previous findings by Szablicka (2022), which reported the morphometric dimensions of sperm to have changed during storage of boar semen at 17 °C. Besides the changes recorded, in the current study, semen stored at 18 °C up to 48 h and 5 °C up to 24 h recorded acceptable sperm head, midpiece, and incidences that fell within the threshold range. However, 5 °C, in comparison with 18 °C, recorded a decrease in sperm head length, area, and midpiece with the increasing time of semen storage. The sperm head traits recorded in the semen stored for 48 h at 5 °C were recorded to be lower than the acceptable range, and the sperm were observed to have shrunk due to the lower temperature with the increasing storage time. The decrease in sperm head length, area, and midpiece recorded in the semen stored at 5 °C may be associated with the effect of cold shock on the sperm plasma membrane and mitochondria. This finding corroborates the ideas of Jäkel et al. (2021), who suggest that sperm's plasma membrane and the mitochondria are compartments that are more sensitive to react to cold shock.

These findings agree with Tomás et al. (14) findings, which showed liquid storage of boar semen at 18 °C to have decreased mitochondrial membrane potential and increased plasma membrane permeability. These findings confirm the interaction between the semen storage time and temperature on boar epididymal sperm motility and morphometry. Furthermore, this study underscores the importance of storage time and temperature of semen samples to maximize sperm motility and morphometry for assisted reproductive techniques (Guthrie & Welch, 2012).

5.5 Conclusion

The current study found that storing diluted boar epididymal semen at 18 °C better preserved sperm motility and morphometric characteristics over a 48 h storage compared to the liquid storage at 5 °C. While both storage temperatures allowed for acceptable sperm quality up to 24 h, semen stored at 18 °C exhibited significantly higher total and progressive motility at 48 h and maintained more stable morphometric traits. These findings highlight the importance of optimising semen storage temperatures to prevent sperm quality deterioration, particularly when using epididymal sperm, which may be more vulnerable than ejaculated sperm. For long-term storage and conservation of threatened/endangered species, cryopreservation may the valuable technique used, making it easier for genetic material to be transported and exchanged over long distances. However, boar sperm cells are sensitive to cold shock due to high polyunsaturated fatty acid. Therefore, future research should focus on evaluating the potential of sperm stored under liquid preservation conditions to validate its viability during preservation.

5.6 References

Alvarez-Rodriguez, M., Álvarez, M., Anel-López, L., Guerra, C., Chamorro, C.A., Anel, L., de Paz, P. and Martínez-Pastor, F., 2018. Effect of length of time post-mortem on quality and freezing capacity of Cantabric chamois (Rupicapra pyrenaica parva) epididymal spermatozoa. *Animal Reproduction Science, 198*, pp.184-192. https://doi.org/10.1016/j.anireprosci.2018.09.018

Am-In, N., Kirkwood, R.N., Techakumphu, M. and Tantasuparuk, W., 2011. Lipid profiles of sperm and seminal plasma from boars having normal or low sperm motility. *Theriogenology, 75*(5), pp.897-903. https://doi.org/10.1016/j.theriogenology.2010.10.032

Bertol, M.A.F., Weiss, R.R., Thomaz-Soccol, V., Kozicki, L.E., Fujita, A.S., Abreu, R.A.D. and Green, K.T., 2013. Viability of bull spermatozoa collected from the epididymis stored at 18-20 C. Brazilian Archives of Biology and Technology, 56(5), pp.777-783. https://doi.org/10.1590/S1516-89132013000500008

Casas, I. and Althouse, G.C., 2013. The protective effect of a 17 °C holding time on boar sperm plasma membrane fluidity after exposure to 5 °C. *Cryobiology, 66*(1), pp.69-75. https://doi.org/10.1016/j.cryobiol.2012.11.006

Demir, K., Öztürk, G.B., Cİrİt, Ü., Bozkurt, H.H., Aktaş, A., Birler, S., Ak, K. and Pabuccuoğlu, S., 2014. Effects of cooling rate on membrane integrity and motility parameters of cryopreserved ram spermatozoa. https://doi.org/10.9775/kvfd.2014.11726

Esteso, M.C., Rodríguez, E., Toledano-Díaz, A., Castaño, C., Pradiee, J., López-Sebastián, A. and Santiago-Moreno, J., 2015. Descriptive analysis of sperm head morphometry in Iberian ibex (Capra pyrenaica): optimum sampling procedure and staining methods using Sperm-Class Analyzer®. *Animal Reproduction Science*, *155*, pp.42-49. https://doi.org/10.1016/j.anireprosci.2015.01.014

Jäkel, H., Henning, H., Luther, A.M., Rohn, K. and Waberski, D., 2021. Assessment of chilling injury in hypothermic stored boar spermatozoa by multicolor flow cytometry. *Cytometry Part A*, *99*(10), pp.1033-1041.https://doi.org/10.1002/cyto.a.24301

Gaczarzewicz, D., Udala, J., Piasecka, M., Blaszczyk, B. and Stankiewicz, T., 2015. Storage temperature of boar semen and its relationship to changes in sperm plasma membrane integrity, mitochondrial membrane potential, and oxidoreductive capability. *Turkish Journal of Biology*, *39*(4), pp.582-594. https://doi.org/10.3906/biy-1412-76

Guthrie, H. D. and Welch, G. R.,2012. Effects of reactive oxygen species on sperm function. *Theriogenology*, *78*(8), 1700-1708. https://doi.org/10.1016/j.theriogenology.2012.05.002

Martín-Hidalgo, D., Barón, F.J., Bragado, M.J., Carmona, P., Robina, A., García-Marín, L.J. and Gil, M.C., 2011. The effect of melatonin on the quality of extended boar semen after long-term storage at 17 °C. *Theriogenology*, *75*(8), pp.1550-1560. https://doi.org/10.1016/j.theriogenology.2010.12.021

Rola, L.D., Buzanskas, M.E., Melo, L.M., Chaves, M.S., Freitas, V.J.F. and Duarte, J.M.B., 2021. Assisted reproductive technology in neotropical deer: a model approach to preserving genetic diversity. *Animals*, *11*(7), p.1961. https://doi.org/10.3390/ani11071961

Schmid, S., Henning, H., Oldenhof, H., Wolkers, W.F., Petrunkina, A.M. and Waberski, D., 2013. The specific response to capacitating stimuli is a sensitive indicator of chilling injury in hypothermically stored boar spermatozoa. *Andrology*, *1*(3), pp.376-386. https://doi.org/10.1111/j.2047-2927.2013.00045.x

Schulze, M., Henning, H., Rüdiger, K., Wallner, U. and Waberski, D., 2013. Temperature management during semen processing: impact on boar sperm quality under laboratory and field conditions. *Theriogenology*, *80*(9), pp.990-998. https://doi.org/10.1016/j.theriogenology.2013.07.026

Schulze, M., Nitsche-Melkus, E., Hensel, B., Jung, M. and Jakop, U., 2020. Antibiotics and their alternatives in artificial breeding in livestock. *Animal Reproduction Science*, *220*, p.106284. https://doi.org/10.1016/j.anireprosci.2020.106284

Szablicka, D., Wysokińska, A., Pawlak, A. and Roman, K., 2022. Morphometry of boar spermatozoa in semen stored at 17 °C—the influence of the staining technique. *Animals*, *12*(15), p.1888. https://doi.org/10.3390/ani12151888

Teves, M.E. and Roldan, E.R., 2022. Sperm bauplan and function and underlying processes of sperm formation and selection. *Physiological Reviews*, *102*(1), pp.7-60. https://doi.org/10.1152/physrev.00009.2020

Thema, M.A., Mphaphathi, M.L., Ledwaba, M.R. and Nedambale, T.L., 2022. Investigation of the efficacy of different semen extenders and in vitro storage period on Windsnyer boar sperm quality equilibrated at 18 °C. Reproduction in Domestic Animals, 57(12), pp.1644-1648. https://doi.org/10.1111/rda.14256

Tomás, C., Gómez-Fernández, J., Gómez-Izquierdo, E. and de Mercado, E., 2014. Effect of the holding time at 15 °C prior to cryopreservation, the thawing rate and the post-thaw incubation temperature on the boar sperm quality after cryopreservation. Animal Reproduction Science, 144(3-4), pp.115-121. https://doi.org/10.1016/j.anireprosci.2013.12.011

Waberski, D., Riesenbeck, A., Schulze, M., Weitze, K.F. and Johnson, L., 2019. Application of preserved boar semen for artificial insemination: past, present and future challenges. *Theriogenology*, *137*, pp.2-7. https://doi.org/10.1016/j.theriogenology.2019.05.030

Waberski, D. and Luther, A.M., 2024. Boar semen storage at 5 °C for the reduction of antibiotic use in pig insemination: pathways from science into practice. *Animal Reproduction Science*, p.107486. https://doi.org/10.1016/j.anireprosci.2024.107486

Wiebke, M., Hensel, B., Nitsche-Melkus, E., Jung, M. and Schulze, M., 2022. Cooled storage of semen from livestock animals (part I): boar, bull, and stallion. *Animal Reproduction Science*, *246*, p.106822. https://doi.org/10.1016/j.anireprosci.2021.106822

Wysokińska, A., Szablicka, D., Dziekońska, A. and Wójcik, E., 2023. Analysis of changes in the morphological structures of sperm during preservation of liquid boar semen in two different seasons of the year. *Animal Reproduction Science*, *256*, p.107297. https://doi.org/10.1016/j.anireprosci.2023.107297

Yeste, M., 2016. Sperm cryopreservation update: cryodamage, markers, and factors affecting the sperm freezability in pigs. *Theriogenology*, *85*(1), 47-64.

Chapter 6: Effects of pre-freezing holding time on post-thaw motility and morphometry of cryopreserved boar epididymal sperm

Thema, M.A., Mkhize, N.R., Sebopela, M.D., Ledwaba, M.R. and Mphaphathi, M.L., 2025. Effect of Pre-Freezing 18° C Holding Time on Post-Thaw Motility and Morphometry of Cryopreserved Boar Epididymal Sperm. *Animals*, *15*(12), p.1691. https://doi.org/10.3390/ani15121691

Abstract

The study investigated the sperm motility and morphometry of pre-freeze and post-thaw boar epididymal semen cooled at increasing holding times at 18 °C. A total of 50 testes of heterogeneous boars were collected (5 testes/day) from the local abattoir and transported to the laboratory at 5 °C within 30 min after slaughter. Semen was retrieved from the caudal part of the epididymis using the slicing float-up method, diluted with Beltsville Thawing Solution extender, pooled in a 50 mL centrifuge tube/5 testes/day, and cooled at 18 °C. Following each holding time (0, 3, 6, 9, 12, 24, and 48 h), the cooled semen sample was re-suspended with Fraction A extender and stored at 5 °C for an additional 45 min. A cooled resuspended semen sample was then diluted with Fraction B extender, loaded into 0.25 mL straws, and frozen using liquid nitrogen vapour. Thawing was accomplished by immersing the semen straws in warm (37 °C) water for 1 min, and the samples were evaluated for sperm motility and morphometry traits using the computer-assisted sperm analyser system. The data were analysed using variance analysis. Descriptive statistics were used to assess sperm morphometry, establishing the minimum and maximum values. Boar epididymal sperm survived for up to 48 h when held at 18 °C. Furthermore, the highest post-thawed sperm motility rates were observed in semen frozen after 3 h of holding time, with a sperm total motility of 85.9%, a progressive motility of 60.3%, and a rapid motility of 33.2%, as compared to other holding times ($P<0.05$). The acceptable ranges for pre-freeze and post-thawed sperm morphology were head length (8.4–9.1 µm), width (4.4-4.8 µm), area (29.9-38.2 µm^2), perimeter (20.1-23.7 µm), midpiece width (1.1-2.8 µm), and sperm shape, which were consistent regardless of the holding time. A holding time of 3 h enhances the cryo-resistance of sperm cooled at 18 °C. Therefore, these findings suggest that boar epididymal sperm can be effectively conserved and can maintain fertilisation capability when cooled for 3 h at 18 °C before freezing.

Keywords: boars, epididymis; cryopreservation; sperm motility; morphometry pre-freeze; post-thaw.

6.1 Introduction

The cryopreservation of epididymal semen might be the only method to protect valuable male genetic material in the event of unanticipated death (Heise et al., 2011). The cryopreservation of boar epididymal semen may assist in conserving the genetic diversity of the threatened or endangered,

making it easier for genetic material to be transported and exchanged over long distances (Bolaji et al., 2021). The cryopreservation technique is frequently employed in reproductive biology labs to make the process of artificial insemination and in vitro fertilisation easier and serves as a backup when infectious diseases arise in farms (Bertol, 2016). However, the use of post-thawed sperm is limited in pigs due to the high susceptibility of boar sperm to cold shock, caused by a high content of polyunsaturated fatty acids in its plasma membrane (Kim et al., 2011). As a result, the cryopreservation of boar sperm may cause sperm damage, oxidative stress, thermal stress, protein denaturation, shrinkage, and irreversible membrane collapse (Khan et al., 2021). Therefore, due to the intolerance of boar sperm to low temperatures, studies have reported improved results when extended ejaculated semen is cooled to 15-20 °C for a minimum of 1-3 h before freezing (Casas & Althouse, 2013).

Diluted boar semen is commonly transported to processing laboratories in a liquid form at an optimum temperature of 15-20 °C (Yeste et al., 2014). To enhance proper transportation and handling of semen, evaluation of the variation in the sperm quality and cryotolerance of other species for up to 24 h of holding time, and the cryotolerance has been recorded (Wasilewska et al., 2016). A holding time has little effect on fresh sperm traits up to 24 h, but a significant effect on post-thaw cryotolerance by reducing metabolic activity as susceptibility to oxidative stress Batista et al., 2012; Santana et al., 2013; Yeste et al., 2014). Torres et al. (2019) recorded holding time as having improved the cryopreservation of ejaculated boar semen and allowed prolonged interaction between the sperm and the seminal plasma components.

The major disadvantage of using post-thaw boar semen is the functional alterations to the sperm, such as their morphometry and motility traits, which may be compromised during freezing. It has been proven that sperm with normal motility, but abnormal morphologies, are incapable of fertilisation (Czubaszek et al., 2019). Sperm morphometry has been associated with factors that can determine fertility (Vernocchi et al., 2014). The study by Talluri et al. (2023) reported smaller boar sperm heads following the cryopreservation of the ejaculated semen. Due to technical discrepancies, subjective sperm morphology assessment in pigs remains troublesome (Finelli et al., 2021). Therefore, to lessen the subjective way of analysing sperm morphometry, the computer-assisted sperm analyser (CASA) system approach was developed and established (van der Horst et al., 2018). The accurate, precise, repeatable, and reliable results have been reported by a few studies when using the CASA system, which had been a challenge for the subjective analyser (Yániz et al., 2015; van der Horst, 2021).

Information about the effects of holding time on post-thaw boar epididymal sperm survival has received less attention. To our knowledge, no published study has documented boar epididymal sperm morphometry and motility induced by holding time at pre-freeze and post-thaw. Therefore, investigating the epididymal sperm motility and morphometry of pre-freeze and post-thaw semen will assist in determining the fertilisation and cryopreservation capability of epididymal sperm in pigs. This

study hypothesises that the holding of boar epididymal sperm at 18 °C for different pre-freeze durations (0, 3, 6, 9, 12, 24 and 48 h) significantly affects cryotolerance and post-thaw sperm motility and morphometric characteristics. This study aims to investigate the variability in the sperm motility and morphometry of pre-freeze and post-thaw semen cooled to 18 °C for prolonged holding times.

6.2. Materials and methods

6.2.1. Statement of animal rights

The experimental procedures were approved and performed in accordance with the ethical standards of the Agricultural Research Council-Animal Production Ethics Committee [APIEC 23/05], University of KwaZulu-Natal Research Ethics Committee [AREC/00007100/2024] and Section 20 of the Animal Diseases Act no 35 of 1984 [12/11/1/18 (6269BM)].

6.3.2. Boar epididymal sperm retrieval

A total of 50 testes of heterogeneous boars were collected (5 testes/day) from the local abattoir and transported to the laboratory at 5 °C within 30 min after slaughter. The testes were further stored at 5 °C for an additional 2 h before being processed. Following storage, the caudal epididymides and the proximal ducts were removed from the testes and cleaned with Sabax® saline NaCl 0.9% solution (Adcock Ingram, South Africa). To avoid blood contamination, superficial blood vessels were punctured so that most of the blood could be wiped off. Then, semen samples were retrieved from the cauda epididymides using the slicing float-up method. The slicing float-up method was performed by slicing the cauda epididymides with a blade to release the semen into the Petri dish (Whitehead Scientific, South Africa) and this was diluted with 5 mL of Beltsville Thawing Solution (BTS; IMV Technologies, France), a semen extender.

6.2.3. Boar epididymal sperm dilution

The retrieved semen samples of good quality (>80% total sperm motility) from 5 cauda epididymis/day were pooled and transferred into 50 mL centrifuge tubes (Whitehead Scientific, South Africa). The semen samples were diluted at a ratio of 1:4 (*v*/*v*) with a BTS extender. The diluted semen samples were cooled to 18 °C and cryopreserved for different holding times (0, 3, 6, 9, 12, 24, and 48 h).

6.2.4. Preparation of boar epididymal sperm freezing extender

The semen holding and freezing extender was prepared one day before use. The boar semen extender used for holding was the BTS extender, which was prepared by dissolving a sachet of BTS extender in a litre of Sabax® (Adcock Ingram, South Africa) distilled water and stored at 18 °C. The chemical composition of the BTS extender is presented in Table 6.1. The semen freezing extender was separated

into fractions A and B and stored at 5 °C. Both Fraction A and Fraction B were prepared according to Thema et al. (2024). The Fraction A extender consisted of BTS + 15% egg yolk, and the Fraction B extender consisted of BTS + 15% egg yolk + 3% Glycerol: *v*/*v*.

Table 6.1: Chemical composition of Beltsville Thawing Solution extender.

Composition	Units g/L
Glucose	37.0
Sodium citrate	6.0
Ethylene diaminuteetraacetic acid	1.25
Sodium bicarbonate	1.25
Gentamycin	-
Penicillin	1.10
Streptomycin	1.10
Potassium chloride	0.75

6.2.5 Boar epididymal sperm cryopreservation and thawing process

Diluted semen with a BTS extender was cooled to 18 °C and subjected to freezing for different holding times (0, 3, 6, 9, 12, 24, and 48 h). At the end of each holding time, the semen was then re-suspended with Fraction A extender and cooled to 5 °C for an additional 45 min. Furthermore, the cooled semen was then diluted with a Fraction B extender (Thema et al., 2024) and loaded into 0.25 mL straws (Embryo Plus, South Africa). The semen straws were exposed to 3 cm above liquid nitrogen for 20 min during cryopreservation (Thema et al., 2023). The frozen semen straws were immediately transferred into the liquid nitrogen tank (-196 °C) for storage. Thawing was accomplished by removing the frozen straws from the liquid nitrogen tank, exposing them to air for 5 s, and immersing the semen straws in warm (37 °C) water for 1 min.

6.2.6. Evaluations of boar epididymal sperm motility traits

Sperm motility traits were analysed following each holding time (0, 3, 6, 9, 12, 24, and 48 h) at pre-freeze and post-thaw using the CASA system (Sperm Class Analyzer® 6.6.80, Microptic S.L, Spain) with different slides. The volume of the 5 µL semen sample at pre-freeze or post-thaw following each holding time was transferred to a warmed (37 °C) microscope glass slide (Labchem Pty Ltd, South Africa) and covered with a warm coverslip (Labchem Pty Ltd, South Africa). Two fields of pre-freeze and post-thaw semen following each holding time with an average of 250 sperm (34.3×10^6/mL/replication) were captured with the aid of a microscope (Nikon®, Toyko, Japan) at a 10X magnification using the CASA system.

6.2.7. Semen staining preparation for boar epididymal sperm morphometry

Semen samples were stained following each holding time (0, 3, 6, 9, 12, 24, and 48 h) at pre-freeze and post-thaw. Prior to staining, the semen sample at pre-freeze or post-thaw stage following each holding time was diluted at a ratio of 1:10 with the BTS extender. The diluted sample was further fixed with 4% Formaldehyde (Sigma Aldrich, United States of America) for 10 min. Then, 10 µL of the fixed sample at pre-freeze or post-thaw following each holding time was smeared on the microscope glass slide and allowed to air-dry overnight at room temperature. During staining, the dried microscope glass slide sample was gently dipped into the SpermBlue® (Ducit, South Africa) tray for 1 minute and into distilled water for 5 seconds (to remove debris and ensure a clean background). A paper towel was used to wipe the back of the microscope glass slide and allowed to air-dry at room temperature. Following drying, the slide was mounted using the Dibutylphthalate Polystyrene Xylene mounting medium (Inqaba Biotechnical Industries (Pty) Ltd, South Africa) and covered with the coverslip.

6.2.8. Analysis of boar epididymal sperm morphometry

Boar epididymal sperm morphometric traits were evaluated using the CASA system. At least 100 sperm were measured following each holding time for 10 replications (days of collection) (100×10 = 1000) of pre-freeze and post-thaw semen. Overall, 7000 pre-freeze (0, 3, 6, 9, 12, 12, 24, and 48 h) sperm and 7000 post-thaw (0, 3, 6, 9, 12, 24, and 48 h) sperm were analysed for morphometry traits. The microscope glass slide was observed (the focus knob was used to focus on the sperm) and measured for the sperm head area (A; μm^2), head perimeter (P; µm), head length (L; µm), head width (W; µm), head ellipticity (length/width), head elongation (L−W/L+W), head regularity ($\pi \times (L.W/4.A)$, head roughness [$4 \pi \times (A/P^2)$], midpiece width, and shape indices (sperm with a normal head, acrosome, and midpiece, or an abnormal head shape-amorphous, pyriform, or tapered).

6.2.9. Statistical Analysis

The sperm morphometric data were divided into two groups: The first group was the measurements of primary sperm head traits (length, L; area, A; width, W; perimeter, P; ellipticity, L/W; rugosity, 4 πA/P2; elongation, L−W/L+W; and regularity, πL. W/4. A). The second group was the determination of the sperm head indices (head type, normal, shape, acrosome defect, and midpiece defect). The first sperm morphometric data group: In the first sperm morphometric data group, descriptive statistics were used to establish the minimum and maximum values (threshold) using 10–90 percentiles for every morphometric trait in this group.

The data for the first sperm morphometric data group and the sperm motility traits were analysed with repeated-measure analysis of variance using general linear model. The *Shapiro-Wilk*'s test was performed on the standardized residuals to test for deviations from normality. In cases where significant

deviation from normality was observed and due to skewness, outliers were removed until this was normal or symmetrically distributed. Student's t-LSDs (least significant differences) were calculated at a 5% significance level to compare means of the significant source effect of holding time on the first sperm morphometric data group and the motility traits at pre-freeze and post-thaw. The ANOVA analysis was performed using SAS version 9.4 statistical software.

The second sperm morphometric data group was analysed using a Chi-square test to determine the effect of holding time on the sperm percentage variations regarding the sperm with a normal head, acrosome, and midpiece, or an abnormal head shape (amorphous, pyriform, or tapered) evaluated at pre-freeze and post-thaw. The scores were subjected to a 1:1 frequency table, and a Chi-square (χ^2) test was performed to test for equal proportions. Contingency R×C frequency tables were performed for associations between holding times at pre-freeze and post-thaw. The tests were performed for association (patterns) and, where significant differences were found, graphs were constructed to demonstrate the differences in these patterns. The Chi-square analysis was performed using SAS version 9.4 statistical software.

6.3. Results

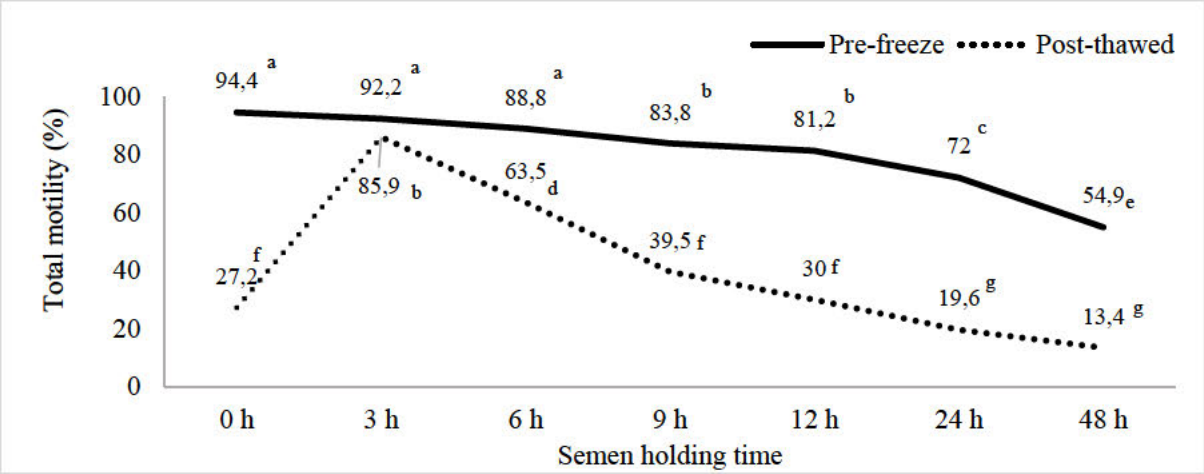

Figure 6.1: The effect of boar epididymal semen holding time on pre-freeze and post-thaw sperm total motility traits using the CASA system (mean±SD).

[a-g]Different letters indicate the significant difference between the pre-freeze and post-thaw groups at specific holding times ($P<0.05$).

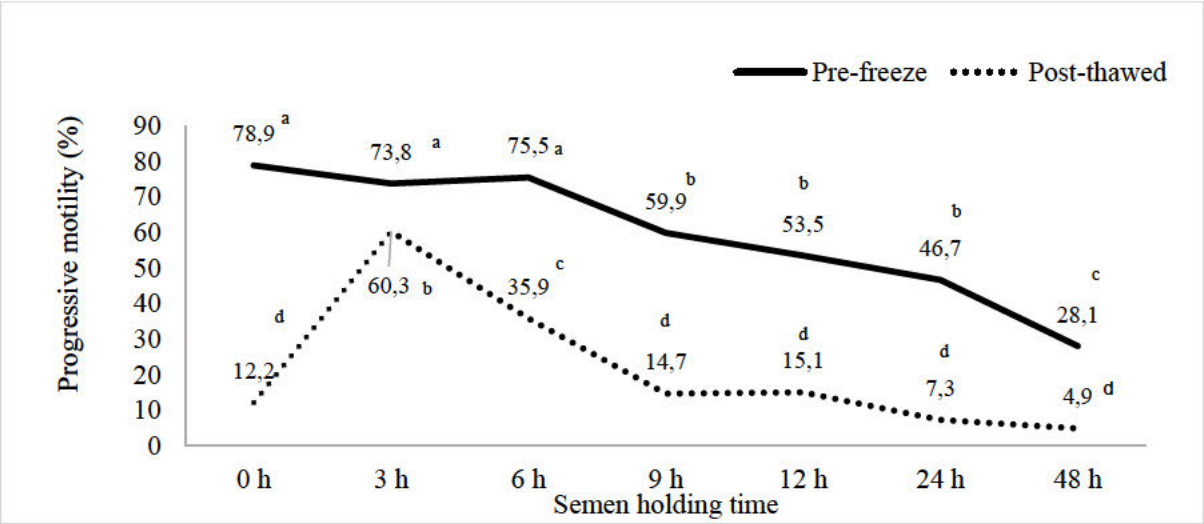

Figure 6.2: The effect of boar epididymal semen holding times on pre-freeze and post-thaw sperm progressive motility traits using the CASA system (mean±SD).

[a-d]Different letters indicate the significant difference between the pre-freeze and post-thaw groups at specific holding times (P<0.05).

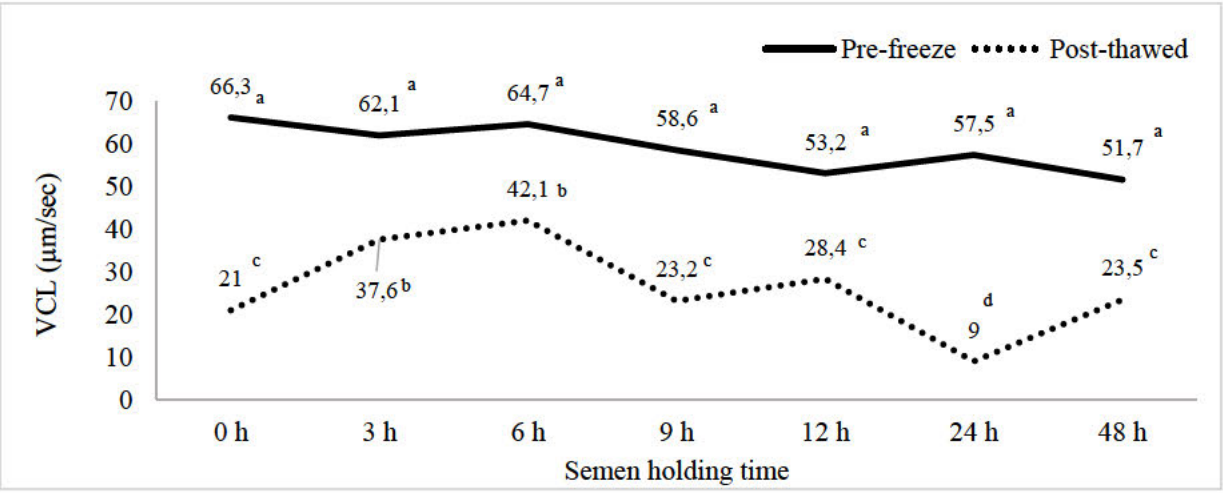

Figure 6.3: The effect of boar epididymal semen holding time on pre-freeze and post-thaw sperm curvilinear velocity traits using the CASA system (mean±SD).

[a-d]Different letters indicate the significant difference between the pre-freeze and post-thaw groups at specific holding times (P<0.05). VCL: Curvilinear velocity.

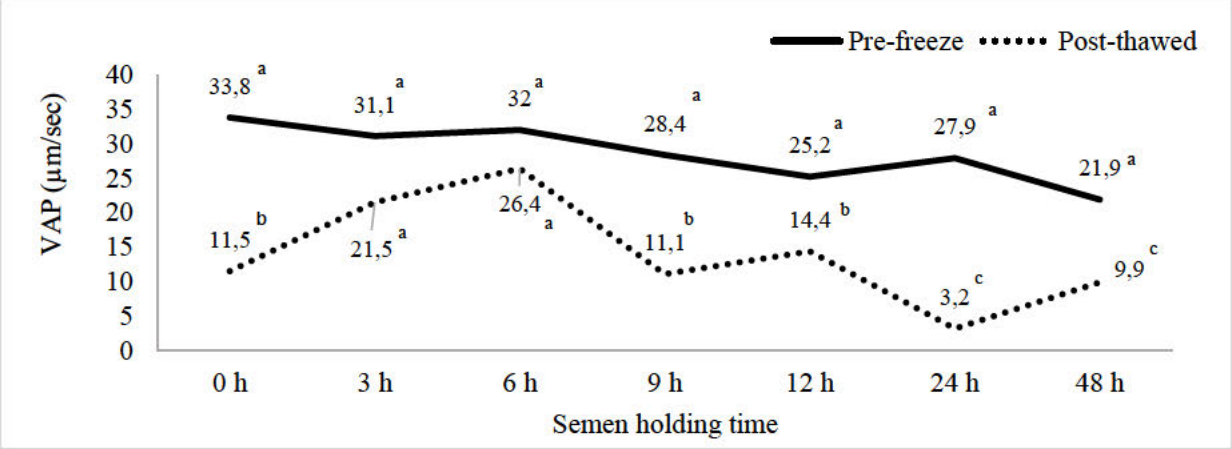

Figure 6.4: The effect of boar epididymal semen holding time on pre-freeze and post-thaw sperm average pathway velocity traits using the CASA system (mean±SD).

[a-c]Different letters indicate the significant difference between the pre-freeze and post-thaw groups at specific holding times (P<0.05). VAP: Average pathway velocity.

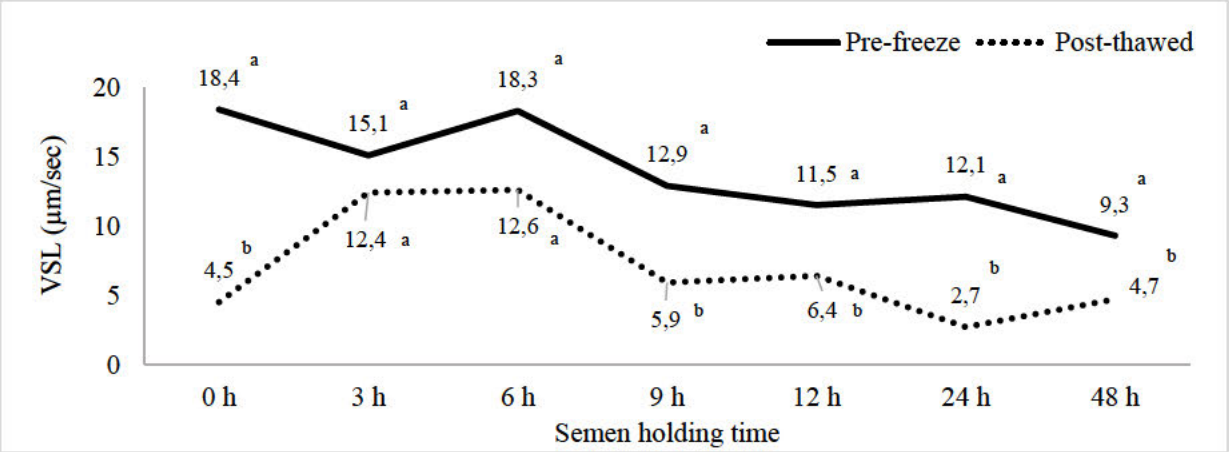

Figure 6.5: The effect of boar epididymal semen holding time on pre-freeze and post-thaw sperm straight line velocity traits using the CASA system (mean±SD).

[a-b]Different letters indicate the significant difference between the pre-freeze and post-thaw groups at specific holding times (P<0.05). VSL: straight line velocity.

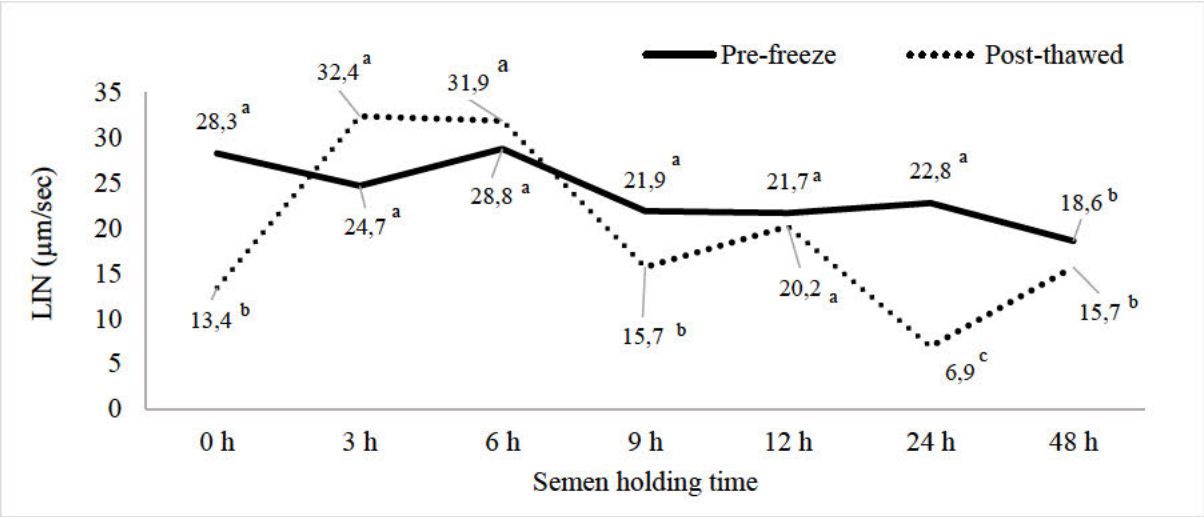

Figure 6.6: The effect of boar epididymal semen holding time on pre-freeze and post-thaw sperm linearity traits using the CASA system (mean±SD).

[a-c]Different letters indicate the significant difference between the pre-freeze and post-thaw groups at specific holding times (P<0.05). LIN: linearity.

Figures 6.1-6.6 represent the effect of boar epididymal semen holding time on pre-freeze and post-thawed sperm motility and velocity traits. Semen frozen immediately after retrieval (0 h), had a high pre-freeze total (94.4%) and progressive (78.9%) motility but post-thaw total and progressive motility was poor at 27.2% and 12.2% respectively (P<0.05). However, 3 h holding time did not affect pre-freeze total and progressive motility but it enhances post-thaw total motility. Contrary to expectations, this study finds a significant difference between the pre-freeze and post-thaw sperm total (92.2%; 85.9%) motility recorded at the 3 h holding time. The highest post-thaw sperm progressive motility (60.3%) was recorded in the semen frozen after a 3 h holding time compared to the other holding times (P<0.05). Thereafter, there was a steady decline in total motility and progressive motility with increasing holding time. More than 70% of pre-freeze sperm progressive motility was recorded between the 0 and 6 h holding times and then this decreased during the 9-48 h prolonged holding times. The present study recorded boar epididymal semen as having survived for 48 h of holding time, with >50% sperm total motility recorded at pre-freeze. Furthermore, semen cooled for 3-48 h prior to freezing recorded significant post-thaw sperm VCL, VSL, and VAP movement. Furthermore, there was no significant difference between the post-thaw sperm velocity values recorded when the semen was cooled for 3-6 h prior to freezing.

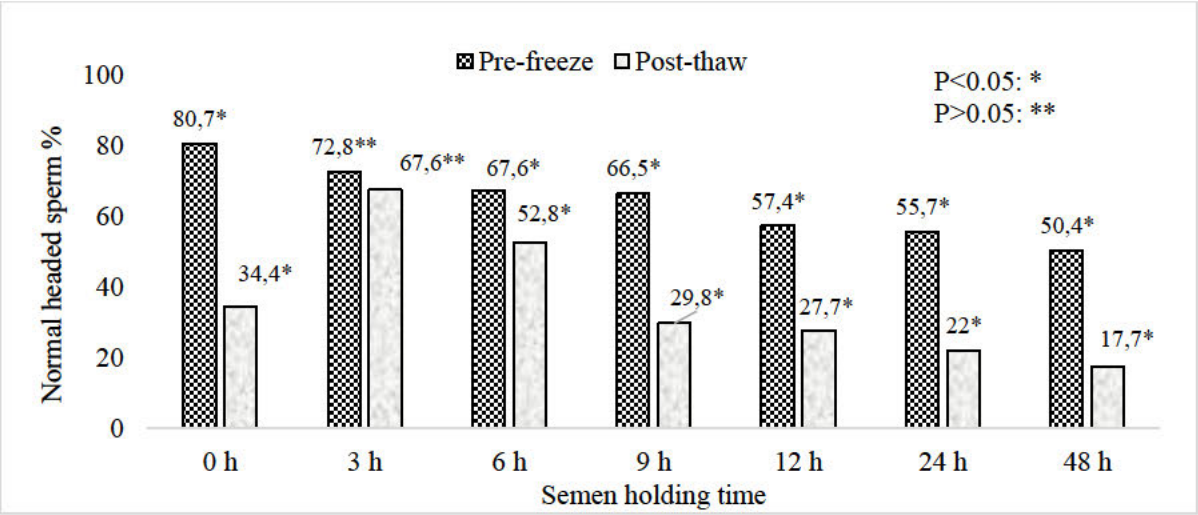

Figure 6.7: The effect of boar epididymal semen holding time on the percentage of pre-freeze and post-thaw sperm with a normal head.

The significant difference (P<0.05) is represented by a star and non significant difference (P>0.05) is represented by two stars within the bars.

Figure 6.7 represents the effect of holding time on the percentage of pre-freeze and post-thaw boar epididymal sperm with normal heads. The present study recorded < 40% of post-thaw sperm with normal heads in the semen frozen immediately after retrieval (0 h). Furthermore, less than 30% of post-thaw sperm with a normal head were recorded in the semen samples frozen for 9–48 h compared to the 3 h holding time (P<0.05). Thereafter, there was a steady decline in post-thaw normal head sperm with increasing holding time. The 3 h holding time, post-thaw semen sample had >60% with normal head sperm (P<0.05). This was significantly higher than other holding time (6, 9, 12, 24 and 48 h), including sperm frozen immediately after retrieval (0 h).

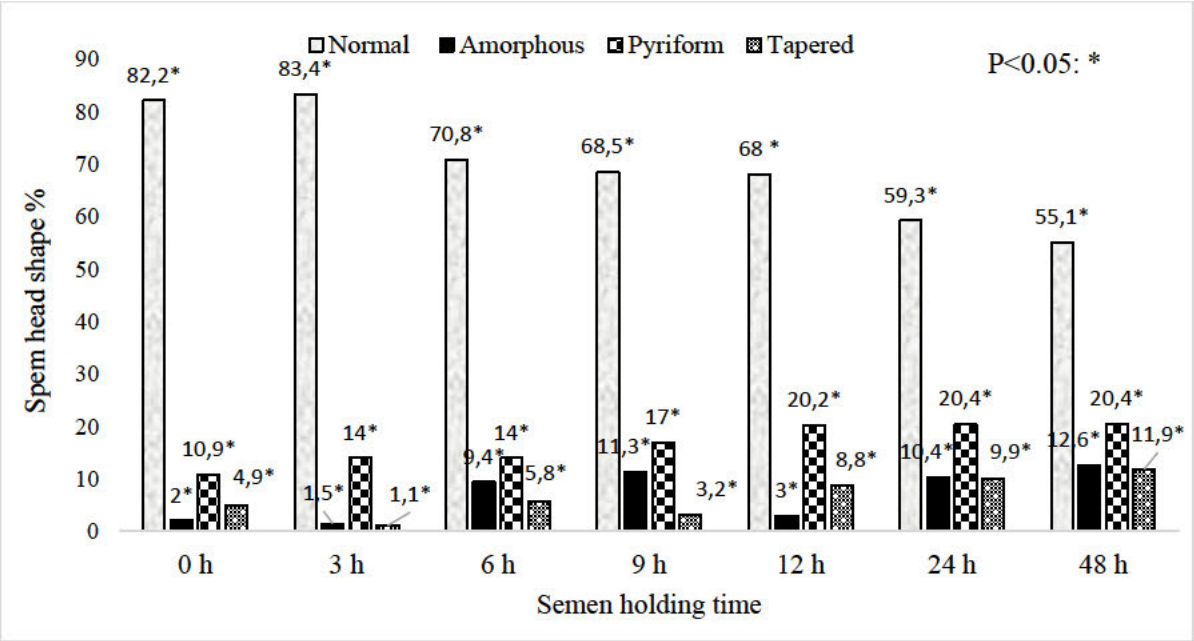

Figure 6.8: The effect of holding time of boar epididymal semen on the percentage of pre-freeze sperm with a normal head shape.

The significant difference (P<0.05) is represented by a star within the bars

Figure 6.8 represents the effect of holding time of boar epididymal semen on the percentage of pre-freeze sperm with a normal head shape. Pre-freeze sperm with a normal head shape decreased with the increasing time of holding the semen samples (P<0.05). Greater than 20% of pre-freeze sperm with a pyriform shape were recorded between 12 and 24 h of holding time. Whereas only <20% of pre-freeze sperm with an amorphous or tapered shape were recorded for the 48 h holding time.

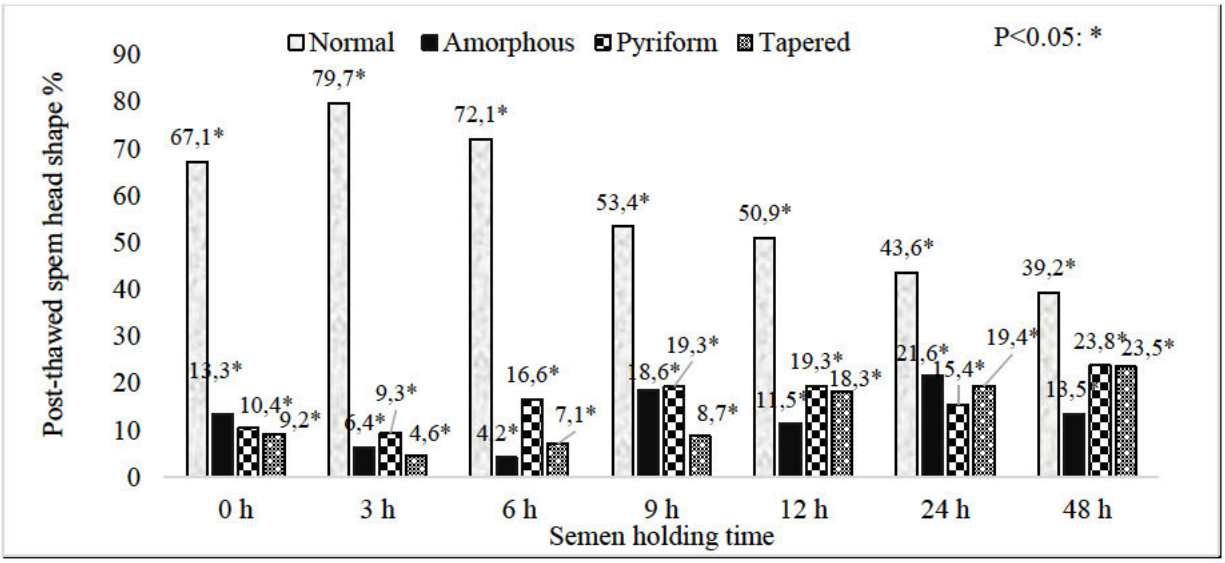

Figure 6.9: The effect of holding time of boar epididymal semen on the percentage of post-thaw sperm with a normal head shape.

The significant difference (P<0.05) is represented by a star within the bars.

Figure 6.9 represents the effect of holding time of boar epididymal semen on the percentage of post-thawed sperm with a normal head shape. The percentage of post-thawed sperm with a normal head shape decreased with increased semen sample holding time (P<0.05). Greater than 70% of the post-thaw sperm with normal head shapes were recorded by semen sample held for 3 and 6 h holding times prior to freezing (P<0.05). Furthermore, at 24 h holding time, post-thaw sperm with amorphous shape were >21.6%. Meanwhile, an increase in post-thaw sperm with tapered (23.5%) and pyriform (23.5%) shapes was recorded in the semen samples frozen for the 48-h holding time.

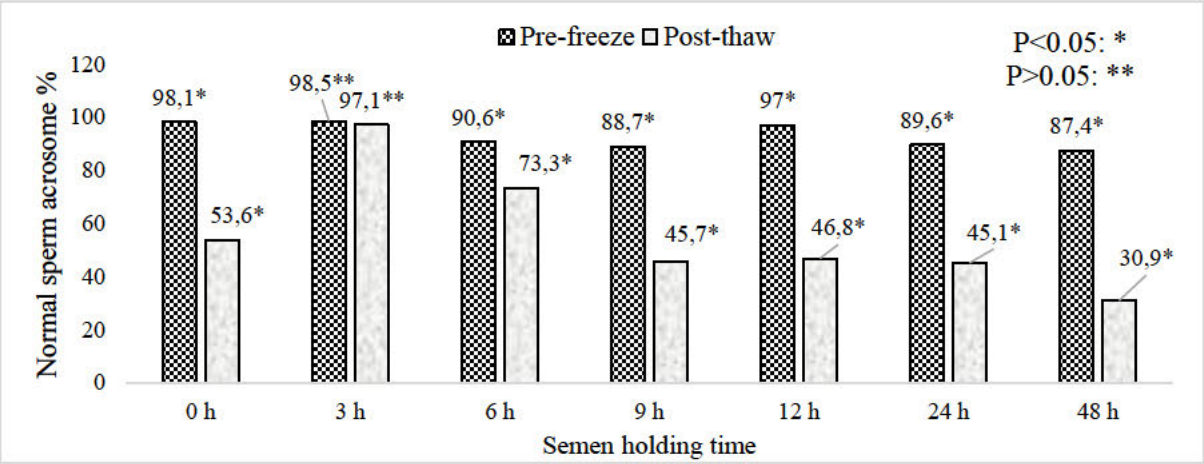

Figure 6.10: The effect of boar epididymal semen holding time on the percentage of pre-freeze and post-thaw sperm with a normal acrosome.

The significant difference (P<0.05) is represented by a star and the non significant difference (P>0.05) is represented by two stars within the bars.

The effect of boar epididymal semen holding time on the percentage of pre-freeze and post-thaw sperm with a normal acrosome is represented in Figure 10. Greater than 80% of the pre-freeze sperm with a normal acrosome were recorded in the present results regardless of holding time. The percentage of post-thaw sperm with a normal acrosome decreased with the increasing holding time of the semen samples (P<0.05). Semen samples frozen for the 9–48 h holding times recorded <50% of post-thaw sperm with a normal acrosome. Furthermore, semen frozen immediately after retrieval recorded <60% of post-thaw sperm with a normal acrosome. Meanwhile, semen exposed to the 3–6 h holding times recorded >70% of post-thawed sperm with a normal acrosome.

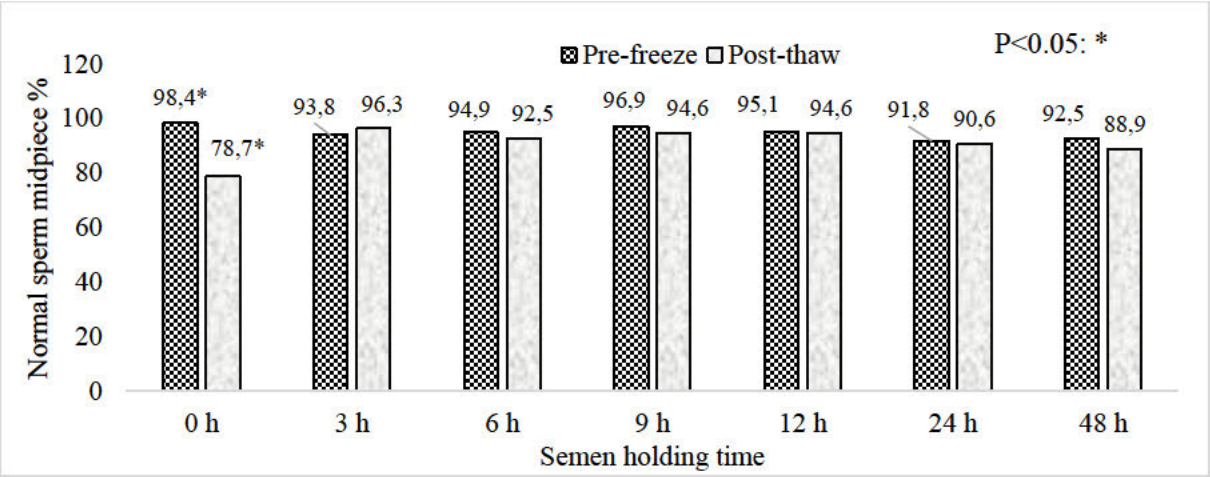

Figure 6.11: The effect of boar epididymal semen holding time on the percentage of pre-freeze and post-thaw normal sperm midpieces.

The significant difference (P<0.05) is represented by a star within the bars.

Figure 6.11 represents the effect of boar epididymal semen holding time on the percentage of pre-freeze and post-thaw normal sperm midpiece. Semen frozen immediately after retrieval recorded the lowest percentage of post-thaw normal sperm midpieces. Overall, >80% of post-thaw and pre-freeze sperm with normal midpieces were recorded throughout 48 h of holding time.

Table 6.2 represents the effect of boar epididymal semen holding time on pre-freeze sperm morphometric traits. The present study recorded acceptable pre-freeze sperm head length (8.7–9.1 µm), width (4.5–4.8 µm), area (33.3–36.6 µm^2), perimeter (21.4–23.7 µm), and midpiece width (1.1–2.8 µm), which fell within the normal range. No significant difference was recorded between the elongation and regularity throughout the semen holding time.

Table 6.2: The effect of boar epididymal semen holding time on pre-freeze sperm morphometric head traits (mean ± SD).

	Holding Time						
	0 h	**3 h**	**6 h**	**9 h**	**12 h**	**24 h**	**48 h**
Pre-freeze sperm head trait							
Length (µm)	9.1 ± 0.8[a]	9.0 ± 0.5[a]	8.9 ± 0.8[b]	8.8 ± 0.6[b]	8.9 ± 0.5[b]	8.9 ± 0.7[b]	8.7 ± 0.6[c]
Width (µm)	4.5 ± 0.5[b]	4.7 ± 0.3[a]	4.7 ± 0.4[a]	4.7 ± 0.3[a]	4.8 ± 0.3[a]	4.6 ± 0.4[b]	4.7 ± 0.3[a]
Area (μm^2)	36.6 ± 5.7[a]	34.8 ± 3.2[b]	34.1 ± 4.0[b]	33.3 ± 3.3[c]	34.6 ± 2.9[b]	33.0 ± 3.9[c]	33.0 ± 3.6[c]
Perimeter (µm)	21.4 ± 2.6[b]	23.7 ± 1.2[a]	23.3 ± 1.8[a]	23.0 ± 1.3[a]	23.5 ± 1.1[a]	23.1 ± 1.7[a]	23.1 ± 1.3[a]
Pre-freeze sperm midpiece trait							
Width (µm)	1.1 ± 0.3[c]	2.8 ± 1.1[a]	2.7 ± 1.2[a]	2.2 ± 1.1[b]	2.6 ± 1.0[a]	2.4 ± 1.2[b]	2.6 ± 1.3[a]
Pre-freeze sperm shape indices							
Ellipticity	2.0 ± 0.4	1.9 ± 0.1	1.9 ± 0.1	1.9 ± 0.1	1.9 ± 0.1	1.9 ± 0.1	1.9 ± 0.1
Elongation	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
Regularity	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0
Roughness	1.0 ± 0.3	0.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.0

[a-c]Values with different superscripts differ significantly in the same row ($P<0.05$).

The effect of boar epididymal semen holding time on post-thaw sperm morphometric head traits is presented in Table 6.3. The present study recorded the highest post-thaw sperm head length (9.1 µm) and area (38.2 µm) in the semen samples frozen for more than 3 h of holding time. Whereas semen frozen immediately after retrieval recorded the lowest post-thaw sperm head length (8.4 µm), area (4.4 μm^2), and width (29.9 µm). Overall, semen frozen for 0–48 h of holding time recorded acceptable post-thaw sperm head length (8.4–9.1 µm), width (4.4–4.6 µm), area (29.9–38.2 μm^2), perimeter (20.1–23.1 µm), and midpiece width (1.0–1.3 µm), which fell within the normal range ($P<0.05$). No significant difference was recorded in post-thaw sperm midpiece elongation and regularity.

Table 6.3: The effect of holding time on the post-thawed boar epididymal sperm morphometric head trait (mean ± SD).

	Holding Time						
	0 h	**3 h**	**6 h**	**9 h**	**12 h**	**24 h**	**48 h**
Post-thawed sperm head trait							
Length (µm)	8.4 ± 0.7[c]	9.1 ± 0.9[a]	8.7 ± 0.7[b]	8.7 ± 0.8[b]	8.7 ± 0.4[b]	8.7 ± 0.6[b]	8.8 ± 0.9[b]
Width (µm)	4.4 ± 0.4[b]	4.5 ± 0.4[a]	4.6 ± 0.4[a]	4.6 ± 0.5[a]	4.6 ± 0.3[a]	4.5 ± 0.4[a]	4.5 ± 0.7[a]
Area (μm^2)	29.9 ± 3.3[c]	38.2 ± 6.9[a]	32.3 ± 3.9[b]	32.4 ± 4.2[b]	32.3 ± 2.7[b]	31.7 ± 3.8[b]	32.9 ± 6.2[b]
Perimeter (µm)	21.9 ± 1.8[a]	20.1 ± 1.9[b]	22.8 ± 1.8[a]	22.9 ± 1.9[a]	22.9 ± 1.0[a]	22.7 ± 1.6[a]	23.1 ± 2.6[a]
Post-thawed sperm midpiece trait							
Width (µm)	1.3 ± 0.5[a]	1.0 ± 0.3[a]	1.0 ± 0.4[a]	1.0 ± 0.3[a]	1.0 ± 0.2[a]	1.1 ± 0.4[a]	1.1 ± 0.5[a]
Post-thawed sperm shape indices							
Ellipticity	2.0 ± 0.2	1.9 ± 0.2	1.9 ± 0.2	1.9 ± 0.8	1.9 ± 0.2	1.9 ± 0.3	2.0 ± 0.6
Elongation	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1
Regularity	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.0	0.9 ± 0.1	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0
Roughness	1.2 ± 0.1	0.8 ± 0.1	0.8 ± 0.0	0.8 ± 0.1	0.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.1

[a-c]Values with different superscripts differ significantly in the same row ($P<0.05$).

6.4. Discussion

This study set out with the aim of investigating the variability in the sperm motility and morphometry for pre-freeze and post-thaw semen cooled to 18 °C following varying holding times. The present study recorded boar epididymal pre-freeze and post-thaw sperm as having survived up to 48 h of holding time. However, semen frozen immediately after retrieval (0 h) and for prolonged holding times (9-48 h) recorded less cryo-resistance with the lowest post-thawed sperm total and progressive motility percentages. A possible explanation for these results might be that semen retrieved from the epididymis contains epididymal fluid instead of seminal plasma, Rodriguez-Martinez et al. (2022) suggesting that holding time plays a significant role in minimising the sensitivity of the sperm to cold shock through seminal plasma proteins (Batista et al., 2012).

One unanticipated finding was that >80% sperm total motility was recorded in post-thaw sperm frozen for the 3 h holding time. This study produced results that corroborate the findings of a range of previous works in this field. This study also accords with the observations by Okazaki et al. (2012), which recorded 80% of post-thaw sperm retaining total motility in boar epididymal semen compared to ejaculated semen. A possible explanation for these results might be that holding time protects the boar sperm against cold shock through the maintenance of the lipid architecture of the plasma membrane

(Casas & Althouse, 2013). The observed increase in post-thawed sperm total and progressive motility recorded for the 3 h holding time could be attributed to the epididymal sperm in the testis mixing with the epididymal fluids and remaining quiescent until they are ejaculated in semen (Batista et al., 2012). It is suggested that the protein profile in the epididymal fluid could be responsible for the increase in boar epididymal sperm cryo-resistance (Tomás et al., 2014). In addition, the cauda part of the epididymis consists of immobilin and antioxidants, which play a role in increasing epididymal fluid viscosity (James et al., 2020).

In accordance with the present results, previous studies demonstrate high cryotolerance in the sperm of the boar epididymis compared to the ejaculated semen. This is supported by studies on cryopreservation of epididymal semen in humans (Monteiro et al., 2011) and equines (Talluri et al., 2023), where it had recorded higher sperm motility rates were recorded following thawing. Moreover, these results are supported by our previous studies on the cryopreservation of Windsnyer (Thema et al., 2023) and large white boar semen (Ledwaba et al., 2022), in which the ejaculated sperm was also demonstrated to have lower cryotolerance. Although these results differ from Torres et al. (2019) and Tomás et al. (2014), who also reported a 24 h holding time to be ideal for the freezing of ejaculated boar semen. Although a 24 h holding time has been reported to be the best in studies on the cryopreservation of ejaculated semen, their results recorded the lowest post-thaw sperm motility rates irrespective of the holding time. These discrepancies between the cryotolerance of ejaculated and epididymal sperm may be related to the electrolytes in the seminal plasma of the ejaculated semen, which may have a negative effect on the sperm and lead to a decrease in their quality during cryopreservation in comparison with epididymal semen (Talluri et al., 2023). Seminal plasma may increase the risk of premature capacitation and membrane destabilisation, which can compromise sperm cryo-resistance (Hermo et al., 1994).

The highest percentage of post-thaw sperm with normal heads and acrosomes was recorded in the semen frozen after 3 and 6 h of holding time. This study highlights that cryopreservation can have a greater impact on the sperm head shape and acrosome integrity, especially in the semen frozen immediately after retrieval and following increased holding time (9-48 h). The changes in the sperm head shape were associated with a prolonged holding time, cold shock, oxidative stress, and premature capacitation, which may be responsible for the low fertility rate. This finding corroborates the ideas of Prinosilová et al. (2012), who suggested that the sperm plasma membrane is very sensitive and may break in the acrosomal area during cryopreservation.

6.5. Conclusions

The present study recorded boar epididymal post-thaw sperm as having survived up to 48 h of holding time pre-freeze. However, the pre-freeze holding time of the boar epididymal semen at 18 °C for 3-6 h resulted in significantly higher sperm total motility and morphometry. Additionally, the 3 and 6 h

holding times were able to improve the cryotolerance of the boar epididymal sperm. The freezing of semen immediately after retrieval and for a prolonged holding time beyond 6 h negatively affected sperm quality, due to the absence of the seminal plasma in the epididymal semen. This suggests a protective role of seminal plasma in minimising the oxidative stress of epididymal sperm frozen immediately after retrieval and for a prolonged holding time (9-48 h). It was further observed that the boar epididymal sperm exhibits better cryotolerance compared to results for ejaculated sperm obtained in other studies. These results show that the study was successful as it was able to overcome the challenge of cryopreserving boar sperm predicting high fertilisation rate. Therefore, further research should be carried out to investigate the fertilising ability of post-thaw epididymal boar sperm.

6.6 References

Bertol, M.A., 2016. Cryopreservation of epididymal sperm. *Cryopreservation in eukaryotes. (Eds F Marco-Jiménez, H Akdemir)*, IntechOpen. London. pp.121-135. https://dx.doi.org/10.5772/65010

Batista, M., Santana, M., Alamo, D., González, F., Niño, T., Cabrera, F. and Gracia, A., 2012. Effects of incubation temperature and semen pooling on the viability of fresh, chilled and freeze-thawed canine semen samples. *Reproduction in Domestic Animals*, *47*(6), pp.1049-1055. https://doi.org/10.1111/j.1439-0531.2012.02014.x

Bolaji, U.F.O., Ajasa, A.A., Ahmed, R.O., Bello, S.F. and Ositanwosu, O.E., 2021. Cattle conservation in the 21st Century: A mini review. *Open Journal of Animal Sciences*, *11*(02), p.304. https://doi.org/10.4236/ojas.2021.112023

Casas, I. and Althouse, G.C., 2013. The protective effect of a 17 °C holding time on boar sperm plasma membrane fluidity after exposure to 5 °C. *Cryobiology*, *66*(1), pp.69-75. https://doi.org/10.1016/j.cryobiol.2012.11.006

Czubaszek, M., Andraszek, K., Banaszewska, D. and Walczak-Jędrzejowska, R., 2019. The effect of the staining technique on morphological and morphometric parameters of boar sperm. *PloS one*, *14*(3), p.e0214243. https://doi.org/10.1371/journal.pone.0214243

Finelli, R., Leisegang, K., Tumallapalli, S., Henkel, R. and Agarwal, A., 2021. The validity and reliability of computer-aided semen analyzers in performing semen analysis: a systematic review. *Translational Andrology Urology*, *10*(7), 3069-3079. https://doi.org/10.21037/tau-21-276

Heise, A., Thompson, P.N. and Gerber, D., 2011. Influence of seminal plasma on fresh and post-thaw parameters of stallion epididymal spermatozoa. *Animal Reproduction Science*, *123*(3-4), pp.192-201. https://doi.org/10.1016/j.anireprosci.2010.11.017

James, E.R., Carrell, D.T., Aston, K.I., Jenkins, T.G., Yeste, M. and Salas-Huetos, A., 2020. The role of the epididymis and the contribution of epididymosomes to mammalian reproduction. *International journal of molecular sciences*, *21*(15), p.5377. https://doi.org/10.3390/ijms21155377

Khan, I.M., Cao, Z., Liu, H., Khan, A., Rahman, S.U., Khan, M.Z., Sathanawongs, A. and Zhang, Y., 2021. Impact of cryopreservation on spermatozoa freeze-thawed traits and relevance omics to assess sperm cryo-tolerance in farm animals. *Frontiers Veterinary Science*. *8*, 609180. https://doi.org/10.3389/fvets.2021.609180

Kim, S., Lee, Y.J. and Kim, Y.J., 2011. Changes in sperm membrane and ROS following cryopreservation of liquid boar semen stored at 15 °C. *Animal Reproduction Science*, *124*(1-2), pp.118-124. https://doi.org/10.1016/j.anireprosci.2011.01.014

Monteiro, G.A., Papa, F.O., Zahn, F.S., Dellaqua Jr, J.A., Melo, C.M., Maziero, R.R.D., Avanzi, B.R., Alvarenga, M.A. and Guasti, P.N., 2011. Cryopreservation and fertility of ejaculated and epididymal stallion sperm. *Animal Reproduction Science*, *127*(3-4), pp.197-201. https://doi.org/10.1111/j.1365-2605.2007.00794.x

Okazaki, T., Akiyoshi, T., Kan, M., Mori, M., Teshima, H. and Shimada, M., 2012. Artificial insemination with seminal plasma improves the reproductive performance of frozen-thawed boar epididymal spermatozoa. *Journal of Andrology*, 33(5), pp.990-998. https://doi.org/10.2164/jandrol.111.015115

Prinosilová, P., Sedlackova, M., Kopecká, V. and Hlavicová, J., 2012. Boar sperm head membrane damage during cryopreservation evaluated by electron microscopy. *Research in Pig Breeding*, *6*(2), pp.58-61.

Rodriguez-Martinez, H., Roca, J., Alvarez-Rodriguez, M. and Martinez-Serrano, C.A., 2022. How does the boar epididymis regulate the emission of fertile spermatozoa?. *Animal Reproduction Science*, pp.106829. https://doi.org/10.1016/j.anireprosci.2021.106829

Santana, M., Batista, M., Alamo, D., González, F., Niño, T., Cabrera, F. and Gracia, A., 2013. Influence of cool storage before freezing on the quality of frozen–thawed semen samples in dogs. *Reproduction in Domestic Animals*, *48*(1), pp.165-170. https://doi.org/10.1111/j.1439-0531.2012.02124.x

Talluri, T.R., Jhamb, D., Paul, N., Mehta, S.C., Singh, J., Dedar, R.K., Legha, R.A. and Pal, Y., 2023. Cryopreservation and freezability of epididymal and ejaculated stallion spermatozoa. *The Indian Journal of Animal Sciences*, *93*(7), pp.691-696. https://doi.org/10.56093/ijans.v93i7.132542

Thema, M.A., Mphaphathi, M.L., Ledwaba, M.R. and Nedambale, T.L., 2023. Sperm cryopreservation in Windsnyer boars; principles, technique, and updated outcomes. *Animal Reproduction*, *20*(3), p.e20220100. https://doi.org/10.1590/1984-3143-AR2022-0100

Thema, M.A., Sithole, A., Mphaphathi, M.L., Ledwaba, M.R., Sebopela, M.D. and Mkhize, N.R., 2024**.** Improving cryosurvivability of boar epididymal sperm quality using various chicken egg yolk concentration for freezing. *Cryobiology*, *117*, p.105079. http://dx.doi.org/10.1016/j.cryobiol.2024.105079

Tomás, C., Gómez-Fernández, J., Gómez-Izquierdo, E. and de Mercado, E., 2014. Effect of the holding time at 15°C prior to cryopreservation, the thawing rate and the post-thaw incubation temperature on the boar sperm quality after cryopreservation. *Animal Reproduction Science*, *144*(3-4), pp.115-121. https://doi.org/10.1016/j.anireprosci.2013.12.011

Torres, M.A., Monteiro, M.S., Passarelli, M.S., Papa, F.O., Dell'Aqua Jr, J.A., Alvarenga, M.A., Martins, S.M. and de Andrade, A.F., 2019. The ideal holding time for boar semen is 24 h at 17°C prior to short-cryopreservation protocols. *Cryobiology*, *86*, pp.58-64. https://doi.org/10.1016/j.cryobiol.2018.12.004

van der Horst, G., 2021. Status of sperm functionality assessment in wildlife species: from fish to primates. *Animals*. *11*, 1491. https://doi.org/10.3390/ani11061491

van der Horst, G., Maree, L. and du Plessis, S.S., 2018. Current perspectives of CASA applications in diverse mammalian spermatozoa. *Reproduction, Fertility and Development*, *30*(6), pp.875-888. https://doi.org/10.1071/RD17468

Vernocchi, V., Morselli, M.G., Consiglio, A.L., Faustini, M. and Luvoni, G.C., 2014. DNA fragmentation and sperm head morphometry in cat epididymal spermatozoa. Theriogenology, 82(7), pp.982-987. https://doi.org/10.1016/j.theriogenology.2014.07.014

Wasilewska, K., Zasiadczyk, Ł., Fraser, L., Mogielnicka-Brzozowska, M. and Kordan, W., 2016. The benefits of cooling boar semen in long-term extenders prior to cryopreservation on sperm quality characteristics. *Reproduction in Domestic Animals*, *51*(5), pp.781-788. https://doi.org/10.1111/rda.12751

Yániz, J.L., Soler, C. and Santolaria, P., 2015. Computer assisted sperm morphometry in mammals: A review. *Animal Reproduction Science*, *156*, pp.1-12. https://doi.org/10.1016/j.anireprosci.2015.03.002

Yeste, M., Estrada, E., Rivera del Alamo, M.M., Bonet, S., Rigau, T. and Rodriguez-Gil, J.E., 2014. The increase in phosphorylation levels of serine residues of protein HSP70 during holding time at 17°C is concomitant with a higher cryotolerance of boar spermatozoa. *PloS one*, *9*(3), p.e90887. https://doi.org/10.1371/journal.pone.0090887

Chapter 7: Assessing *in vitro* fertilisation ability of the cryopreserved boar epididymal sperm on oocytes matured in two media

M.A. Thema, N.R Mkhize, M.D Sebopela, M.R. Ledwaba and M.L. Mphaphathi., (*Under review*). Assessing *in vitro* fertilisation ability of pre-freeze and post-thawed boar epididymal sperm on oocytes matured in two media.

Abstract

This study aimed to investigate the fertilisation potential of post-thawed boar sperm retrieved from the epididymis sperm utilising pig oocytes that have undergone maturation in a North Carolina State University 23 (NCSU-23) and modified NCSU-23 (mNCSU-23) media. Sperm was retrieved from the caudal part of the epididymis with the aid of the slicing float-up method and diluted with Beltsville Thawing Solution extender. Only sperm samples exhibiting >90% sperm total motility were subjected to holding prior cryopreservation process and in vitro fertilisation. Oocytes were retrieved from the ovaries and sperm from the 6-cauda epididymis collected from the local abattoir. A total of 160 oocytes were equally allocated for media (NCSU-23 and mNCSU-23) and matured for 44 h, on the day of fertilisation that is when the testes were collected for epididymal sperm retrieval. The retrieved epididymal sperm sample were pooled, analysed for sperm quality, split for *in vitro* fertilisation and cryopreservation following 3 h of holding. The same batch of sperm sample frozen were re-analysed at post-thaw following three months of storage and *in vitro* fertilised with the oocytes matured with two media. Sperm motility and morphometric traits were evaluated utilising computer-assisted sperm analyser prior to IVF. Data was analysed with the aid of analysis of variance. The statistical analysis was performed using SAS version 9.4 statistical software. There was no significant difference in the prevalence of zygotes with 2 pronucleus (normal fertilisation/ early embryo cleavage) in pre-freeze (26; 30%) and post-thawed (29.6; 22.4%) sperm in both oocytes matured with mNCSU-23 or NCSU-23 media. Additionally, oocytes matured in the mNCSU-23 media exhibited higher overall IVF rates of 91.2% with pre-freeze sperm and 81.6% with post-thawed sperm. The overall IVF rate increased due to the higher number of >2 pronucleus (abnormal fertilisation) recorded in the study as a result of polyspermy. Whereas oocytes matured in NCSU-23 media and fertilised with post-thawed sperm recorded lower IVF rates of 60.8%. The findings reveal that the normal fertilisation rate was achieved with the pre-freeze and post-thawed boar epididymal sperm using oocytes matured with mNCSU-23 and NCSU-23 media. Both mNCSU-23 and NCSU-23 media can be used for IVF and embryo production. However, polyspermy is still a challenge in pig IVF. Therefore, future studies in pigs are recommended to address this challenge.

Keywords: Cryopreservation, embryo production, porcine, reproduction, semen

7.1 Introduction

The *in vitro* fertilisation (IVF) success using cryopreserved boar sperm appears to be low due to the osmotic shock and oxidative stress that occur during semen freezing (Zeke et al., 2012). Cryopreservation of semen is a crucial strategy for preserving valuable male genetic material, particularly when collection through ejaculation is no longer possible. In such cases, epididymal sperm recovery provides an important alternative (Bertol et al., 2013). Post-mortem sperm recovery is vital for germplasm conservation efforts and biodiversity preservation (Strand et al., 2016). Although previous studies in various animal species have described the fertilisation success of epididymal sperm (Gill et al., 2016; Raad et al., 2018), limited information exists regarding boar epididymal sperm to fertilise mature oocytes *in vitro*. In pigs, the use of post-thawed epididymal sperm for fertilisation is restricted due to high susceptibility of boar sperm to cold shock, which impairs their functional competence (Benson et al., 2012; Daigneault et al., 2014). Maintaining boar sperm functional status after thawing is critical for successful IVF and subsequent to embryo production (Martecikova et al., 2010).

Despite sufficient motility, the ability of post-thawed epididymal boar sperm to fertilise during IVF is infamously poor. Oocyte maturation is a key determinant of developmental competence and IVF success (Varghese et al., 2011; Lee et al., 2015). Because oocytes remain arrested in meiotic prophase I until puberty, precise control during *in vitro* maturation is essential to resume meiosis and achieve fertilisation competence (Gilchrist, 2010). Several attempts have been made to improve the IVF protocol in the past few decades. Despite extensive research on improving porcine IVF, the rates of embryonic development to the blastocyst stage have been extremely low (Ledwaba et al., 2022). Nonetheless, many aspects of the IVF protocol remain unknown because there have been insufficient extensive studies evaluating the efficacy of media, particularly intended for IVF in porcine, using post-thawed epididymal sperm.

It is known that several IVF protocol elements, including the media's composition, impact the outcomes of fertilisation in pig embryo production systems (Romar et al., 2016). The North Carolina State University 23 (NCSU-23) has been widely used in the porcine *in vitro* embryo production as a maturation and culturing media (Pyoos et al., 2018); however, improper in vitro oocyte maturation media may leads to inadequate cytoplasmic development, which hinders further embryo development (He et al., 2024).The most common oocyte maturation media used in pigs is Tissue culture medium 199, NCSU-37 and 23 (Gil et al., 2010), with NCSU-23 showing superior results in promoting oocytes nuclear and cytoplasmic maturation that develop into embryos (Pyoos et al., 2018). Despite the superior results reported with NCSU-23 maturation media compares to other media, its current formulation contains glucose as the primary energy source (Chen et al., 2021), porcine follicular fluid and 2% antibiotic to mediate osmolality and prevent contamination and sorbitol (Pawlak et al., 2018; Malik et

al., 2023). The composition of sorbitol in media, may induce oxidative stress and disrupt the oocytes intracellular redox balance while leads to inadequate cytoplasmic development noted by Zhang et al. (2021). Although, the composition of glucose may provide energy during cytoplasmic and embryo development, its concentration in the NCSU-23 is may be insufficient for promoting developmental competency Wen et al., 2020. Collado-Fernandez et al. (2012) emphasised that oocyte maturation requires a large quantity of energy to support its dynamic cellular processes. Wen et al. (2020) noted that glucose releases metabolites from the pentose phosphate pathway and glycolysis to promote pig oocyte maturation. This defines the importance of nutritional composition in the oocyte maturation media, which may affect metabolic pathways during embryonic developmental competency (Gandhi et al., 2025).

To address these limitations, modifications (increased amount of glucose concentration and removal of sorbitol) to the current standard NCSU-23 media it is therefore propose in the present study to support improvement of IVF protocols. Although the existing studies have primarily focused on oocyte maturation and embryo development in current standard NCSU-23, there is limited evidence on how the used to modified maturation media may impact IVF success using cryopreserved epididymal sperm in porcine sperm. This improvement of IVF protocols is necessary to produce high-quality oocytes and improve IVF success consistently (Lin et al., 2014). This study aimed to investigate the fertilisation potential of post-thawed boar epididymal sperm utilising pig oocytes that have undergone maturation in a North Carolina State University 23 (NCSU-23) and modified NCSU-23 (mNCSU-23) media. Further hypothesise that, pre-freezed and post-thawed boar epididymal sperm significantly influences *in vitro* fertilisation success when oocytes are matured in NCSU-23 and mNCSU-23 media.

7.2 Materials and methods

7.2.1 The study area and design

The study was conducted at the Germplasm Conservation and Reproductive Biotechnologies section in the Agricultural Research Council, Irene, in Gauteng Province. The area is located at 25º 53′ 59.6″ South latitude and 28º 12′ 51.6″ East longitude in Pretoria, South Africa. The 6 testes of 7-8 months old boars of heterogeneous/unknown breed were all collected in one day from the local abattoir. Oocytes were retrieved from the ovaries and sperm from the 6-cauda epididymis collected from the local abattoir. A total of 10 ovaries of 7-8 months heterogeneous/ unknown breeds were collected from a local abattoir. A total of 160 oocytes were equally allocated for media (NCSU-23 and mNCSU-23) and matured for 44 h, on the day of fertilisation that is when the testes were collected for epididymal sperm retrieval. The retrieved epididymal sperm sample were pooled, analysed for sperm motility, split for *in vitro* fertilisation and cryopreservation following 3 h of holding. The same batch of sperm sample frozen were re-analysed at post-thaw following three months of storage and *in vitro* fertilised with the oocytes

matured with two media [distributing the oocytes in 8 replication (20x8=160) per media]. Following maturation, 100% cumulus cells expansion was observed in both two maturation media. Oocytes matured in mNCSU-23 media recorded 81%, maturation rate and 46.2% with NCSU-23 media based on the polar body extrusion.

7.2.2 Statement of animal rights

The experimental procedures were approved and performed in accordance with the ethical standards of the Agricultural Research Council-Animal Production Ethics Committee [APIEC 23/05], University of KwaZulu-Natal Research Ethics Committee [AREC/00007100/2024] and Section 20 of the Animal Diseases Act no 35 of 1984 [12/11/1/18 (6269BM)].

7.23 Preparation of the boar epididymal sperm freezing extender

The sperm freezing extender was prepared a day before use in 50 mL tubes (Nest®, Biotechnology, China). The freezing extender was separated into two fractions (fraction A and B). Fraction A of the freezing extender consisted of 75% (75 mL) Beltsville Thawing Solution (BTS) supplemented with (15 mL; v/v) chicken egg yolk. Fraction B consisted of (15 mL; v/v) chicken egg yolk, 82% (82 mL) BTS, and 3% (3 mL) glycerol.

7.2.4 Boar epididymal sperm retrieval and processing

The six testes of heterogeneous boars were collected from the local abattoir and transported to the laboratory at 5 °C within 30 minutes after slaughter on the day of fertilisation. The testes were further stored at 5 °C for 2 h before processing. Following storage, the caudal epididymides and the proximal ducts were removed from the testes and cleaned with saline solution (NaCl 0.9%). To avoid blood contamination, superficial blood vessels were punctured so that most of the blood could be wiped off. The slicing float-up procedure involves using a scalpel blade to slice the cauda part of the epididymis to release the sperm in a Petri dish, suspend it with 5 mL of a warm BTS extender (37 °C), and gently agitate the mixture for approximately 10 minutes (Turri et al., 2012; Falomo et al., 2016).

7.2.5 Boar epididymal sperm dilution and processing

Sperm retrieved from six cauda epididymis were evaluated for sperm motility immediately after retrieval and >90% total motility was recorded. Sperm samples retrieved from six cauda epididymides were pooled, transferred into 50 mL centrifuge tubes (Whitehead Scientific, South Africa) analysed for sperm motility, split for *in vitro* fertilisation and cryopreservation following 3 h of holding

7.2.6 Boar epididymal sperm cryopreservation and thawing process

Diluted sperm sample (102.9×10^6 sperm/mL) in a 50 mL centrifuge tube was pre-cooled (pre-freeze) at 18 °C for 3 h, re-suspended with Fraction A of extender at a ratio of 1:2 resulting in 51.45×10^6 sperm /mL, and further cooled at 5 °C for 45 minutes. Cooled sperm was then diluted with a Fraction B extender (1:2) resulting into 25.73×10^6 sperm/mL and loaded into 0.25 mL straws containing 6.43×10^6 sperm/0.25 mL. Semen straws were exposed 3 cm above the liquid nitrogen for 20 minutes (Thema et al., 2023). The frozen semen straws were immediately transferred into the liquid nitrogen tank (-196 °C) and stored for 3 months. Thawing was accomplished by removing the frozen semen straw from the liquid nitrogen tank, exposing it to the air for 5 seconds, and immersing the semen straw in warm (37 °C) water for 1 minute.

7.2.7 Evaluations of boar epididymal sperm motility traits

Pre-freeze (sperm pre-cooled at 18 °C for 3 h before freezing) and post-thawed sperm samples were analysed prior to the IVF process using the CASA system. The volume of 5 µL sperm sample was transferred to a warmed (37 °C) microscopic glass slide and covered with a warm microscopic coverslip. Two fields were analysed per sample (pre-freeze and post-thaw) of sperm with an average of 34.3×10^6 sperm/mL for pre-freeze and 12.86×10^6 sperm/mL of post-thawed sperm per treatment/replication were captured with the aid of a microscope (Nikon®, Japan) at 10X magnification using the computer-assisted sperm analyser (CASA; Microptic, Sperm Class Analyzer® 6.6.80) system.

7.2.8 Staining preparation for boar epididymal sperm morphometry analysis

Pre-freeze and post-thawed sperm samples were stained for sperm morphometry prior to the IVF process. Prior to staining, the sperm samples were diluted at a ratio of 1:10 (1 mL sperm: 10 mL BTS) with the BTS extender. The diluted sample was further fixed with 4% Formaldehyde for 10 minutes. Then, 10 µL of the fixed sample was smeared on the microscope glass slide and allowed to air dry overnight at room temperature. During staining, the dried microscope glass slide sample was gently dipped in the SpermBlue® tray for 1 minute and in distilled water for 5 seconds (to remove debris and ensure a clean background). The paper towel was used to wipe the back of the microscope glass slide and allowed to air dry at room temperature. Following drying, the slide was mounted using Dibutylphthalate Polystyrene Xylene mounting medium (Associated Chemical Enterprises, South Africa) and covered with the coverslip.

7.2.9 Analysis of boar epididymal sperm morphometry

Boar epididymal sperm morphometric traits were analysed using the CASA system. At least 200 sperm were measured at pre-freeze and post-thaw before IVF for morphometry traits. The microscope glass slide was observed (the focus knob was used to focus sperm) and measured for the sperm head area (A; μm^2), head perimeter (P; µm), head length (L; µm), head width (W; µm), head ellipticity (length/width), head elongation (L-W/L+W), head regularity (πx (L.W/4.A), head roughness [4 π x (A/P^2)] and midpiece width.

7.2.10 Preparation of the *in vitro* maturation and fertilisation media

The washing, maturation and IVF media were prepared the day before the ovaries' collection. The washing media used in the current study were modified Dulbecco's Phosphate Buffered Saline (mDPBS) and modified Medium 199 (mM199). The mM199 were prepared by supplementing 450 mL M199 + 10% fetal bovine serum + 5 mL antibiotic. The mDPBS was prepared by supplementing 500 mL DPBS + 10% bovine serum albumen (BSA) + 5 mL antibiotic + 200 µL phenol red. For maturation, NCSU-23 and mNCSU-23 media were prepared according to Table 7.1. Porcine follicular fluid prepared for maturation media were obtained from ovarian follicles. Stocks A and B were prepared according to Table 7.2 and 7.3 for both maturation media. For fertilisation, the media used was modified Tris buffered media (mTBM), which was prepared with 113.1 mM sodium chloride, 3 mM potassium chloride, 7.5 mM calcium chloride dihydrate, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate, 1 mM caffeine, and 0.1% BSA.

Table 7.1: Composition of the NCSU-23 and mNCSU-23 media prepared in a 50 mL centrifuge tube.

Chemical composition	NCSU-23 media	mNCSU-23
Stock solution A	36 mL	36 mL
Stock solution B	9 mL	9 mL
PFF	5 mL	5 mL
Glucose	0.0045 g	0.0085 g
Sorbitol	0.0984 g	-
B-ME stock solution	50 µL	50 µL
L-Cysteine stock solution	500 µL	500 µL
Antibiotic (Antimycotic 2%)	1 mL	1 mL
FSH	100 µL	100 µL
LH	10 Ml	10 µL

PFF: porcine follicular fluid; FSH: follicular stimulating hormone; LH: luteinising hormone, mNCSU: modified North Carolina State University

Table 7.2: Preparation of stock solution A for NCSU-23 and mNCSU-23 media

Components	g/100 mL
NaCl	0.7942
KCl	0.0446
$CaCl_2.2H_2O$	0.0312
KH_2PO_4	0.0202
$MgSO_4.7H_2O$	0.0366
Glutamine	0.0182

NaCl: sodium chloride; KCl: potassium chloride; $CaCl_2.2H_2O$: calcium chloride dihydrate; KH_2PO_4: monopotassium phosphate; $MgSO_4.7H_2O$: magnesium sulfate heptahydrate

Table 7.3: Preparation of stock solution B for NCSU-23 and mNCSU-23 media

Components	g/100 mL
N_aHCO_3	1.0530
Phenol red	40 µL

$NaHCO_3$: sodium hydrogen bicarbonate

7.2.11 Ovaries' collection and oocytes' retrieval

Six dishes (Falcon 1008) of washing media (mDPBS and mM199) were prepared before the ovaries collection and were placed in an incubator (38.5 °C). Each of the overall ovaries (10) were collected from a local abattoir in a thermos flask containing warm 0.9% NaCl saline solution at 38 °Con the two different days. On arrival at the laboratory, the ovaries were washed with 0.9% saline water at 38 °C to eliminate excess blood and sprayed with 70% alcohol to prevent contamination. The ovaries were then placed in a dish containing warm saline water and maintained in a water bath at 38 °C. The oocytes were retrieved by slicing in a petri plate and transferred into 50 mL centrifuge tubes containing 5 mL mDPBS. The supernatant was then gently extracted from the pellet using a Pasteur pipette.

7.2.12 Pig oocytes searching and maturation process

Six dishes (Falcon 1008) of warm washing media (mDPBS and mM199) were placed on the warm plate at 38.5 °C. The retrieved oocytes washed three times in mDPBS and mM199 graded using a stereomicroscope (Olympus BX71, Taipei, Taiwan). Only Grade A (with three layers of cumulus cells) and Grade B (with at least two layers of cumulus cells) were subjected to in vitro maturation with NCSU-23 and mNCSU-23 media in an incubator (Thermo® CO_2, ThermoFisher Scientific™, United State of America) for 44 h at 38.5 °C.

7.2.13 Pig cumulus cells expansion and thawing of boar epididymal semen

After 44 h of incubation, matured oocytes in NCSU-23 and mNCSU-23 media were identified by expanded cumulus cells and polar body extraction and were used for IVF. Thawing of semen was accomplished by removing the frozen semen straws from the liquid nitrogen tank, exposing them to the air for 5 seconds, immersing the semen straws in warm (37 °C) water for 1 minute, and transferring them into the 15 mL centrifuge tube (Thema et al., 2023). Post-thawed sperm were evaluated for sperm motility and morphometry traits before the IVF process.

7.2.14 Sperm capacitation and the *in vitro* fertilisation process

Matured oocytes in NCSU-23 and mNCSU-23 media were *in vitro* fertilised with either liquid stored or cryopreserved sperm. For pre-freeze samples, sperm was diluted with BTS following retrieval and exposed to holding at 18 °C for 3 h prior to IVF. The same batch of the sperm sample was frozen and used for IVF after 3 months of storage. The sperm capacitation was induced on either liquid stored or cryopreserved sperm 0.25 mL sperm (1.61×10^6 sperm/mL) placed in a 15 mL centrifuge tube and added with 6 mL of pre-warmed (37 °C) mTBM media. The samples were centrifuged at 37 °C for 2 minutes at a speed of 2000 rpm. Following centrifugation, the pellet was suspended in mTBM media at the same level as the pellet to wash the sperm and was centrifuged for the second time. After centrifugation, the supernatant was removed second time, and the sperm pellet was diluted (1:5) with mTBM media. For IVF a total concentration of 0.32×10^6 sperm/mL sperm was transferred to each droplet of 25 matured oocytes.

Five wash drops (100 µL) of mTBM were prepared in a separate 1008 falcon® petri dish for NCSU-23 and mNCSU-23 treatments and coated with 3 mL of mineral oil before IVF. Another 2 drops of 50 µL of the same media were generated in a separate dish and used as IVF drops. First, matured oocytes from each media treatments were washed five times in 3 mL of mineral oil-covered pre-warmed mTBM media droplets prepared in a 1008 falcon® petri dish. Following washing, the oocytes matured in NCSU-23 and mNCSU-23 media were dispersed equally into two separate petri plates containing two 50 µL drops of the mTBM media covered with 3 mL of mineral oil. Each 50 µL drop of mTBM in separate dishes on matured oocytes of maturation media treatments received 50 µL of capacitated diluted sperm and incubated at 38.5 °C for 18 h.

7.2.15 Assessment of the pronucleus following *in vitro* fertilisation

At the end of 18 h of IVF, the pronucleus were checked. To denude them, the fertilised oocytes were vortexed in a preheated (38.5 °C) mM199 solution for three minutes. After that, the oocytes were placed in a petri dish with mM199. The 25 mg (0.025 g) of Hoechst 33342 (Sigma B226, St. Louis, MO, USA) was used to stain the oocytes following the procedure stated by Ledwaba et al. (2022). The presumptive

zygotes were evaluated under the inverted microscope for a total fertilisation rate at 0 pronucleus (PN), 1PN, 2PN, and >2PN.

7.2.16 Statistical analysis

The two-way factorial analysis of variance was used to test the main effects of the type of sperm (pre-freeze and post-thaw) and oocyte maturation media (NCSU-23 and mNCSU-23), as well as their interaction effect on the IVF rate using general linear model. To identify if the effect of sperm cryopreservation was dependent on the oocyte's maturation media, the interaction effect was carefully examined. Sperm motility and morphometry was analysed using paired student t-test. A paired t-test was used to analyse the sperm motility and morphometric traits from the same pooled sample at pre-freeze and post-thaw, enabling a direct evaluation of the impact of cryopreservation.

The *Shapiro-Wilk*'s test was performed on the standardised residuals to test for deviations from normality. Fisher LSDs (Least significant differences) were calculated at a 5% significance level to compare means of significant sources for pairwise comparison. In the sperm morphometric data group, descriptive statistics were used to establish the minimum and maximum values (threshold) using 10-90 percentiles for every morphometric trait in this group. All data analyses were performed using SAS version 9.4 statistical software.

7.3 Results

Sperm motility traits of boar epididymal sperm at pre-freeze and post-thaw are presented in Table 7.4. Total and progressive motility was higher in a pre-freeze sample to cryopreserved epididymal boar sperm ($P<0.05$). However, non-progressive motility and static sperm percentage increased following thawing, different ($P<0.05$) from pre-freeze. Curvilinear velocity (VCL; 67.1. vs 37.6 µm/sec; $P<0.05$) recorded at pre-freeze differ from after thawing treatment. The other parameters such as average pathway velocity (31.1-21.5 µm/sec), straight-line velocity (15.1-12.4 µm/sec), straightness (56-48.7%), linearity (32.4-24.7%), wobble (57.6-50.1), and amplitude of lateral head (1.8-1.4 µm/sec) recorded no difference.

Table 7.4: The pre-freeze and post-thawed sperm motility trait of boar epididymides (mean ± SD).

Sperm motility trait	Pre-freeze (Thema et al., 2025)	Post-thawed	P-Value
Total motility (%)	92.2±3.4[a]	85.9±6.4[b]	<0.05
Progressive motility (%)	73.8±8.6[a]	60.3±10[b]	<0.05
Non progressive motility (%)	18.4±7.5[b]	25.6±19.2[a]	<0.05
Static (%)	7.8±3.4[b]	14.1±6.4[a]	<0.05
VCL (µm/sec)	62.1±16.9[a]	37.6±9.2[b]	<0.05
VAP (µm/sec)	31.1±8.3[a]	21.5±5.1[a]	>0.05
VSL (µm/sec)	15.1±3.5[a]	12.4±3.8[a]	>0.05
STR (%)	48.7±6.7[a]	56±7.8[a]	>0.05
LIN (%)	24.7±3.8[a]	32.4±6.1[a]	>0.05
WOB (%)	50.1±2.2[a]	57.6±11.6[a]	>0.05
ALH (µm/sec)	1.8±0.4[a]	1.4±0.2[a]	>0.05

[a-b] Values with different superscripts differ significantly in the same row ($P<0.05$). TM: total motility, PM: progressive motility, NPM: non progressive motility, VCL: curvilinear velocity, VSL: straight line velocity, VAP: average pathway velocity, LIN: linearity, STR: straightness, WOB: wobble, ALH: amplitude of lateral head, Sec: seconds.

Sperm morphometry traits of boar epididymides at pre-freeze and post-thaw are shown in Table 7.5. Sperm head dimensions, including head length (9.0–9.1 µm), width (4.5–4.7 µm), area (34.8–38.2 μm^2), perimeter (20.1–23.7 µm) and midpiece width (1.0–1.1 µm) fell within the threshold. No significant difference was recorded between post-thawed and pre-freeze sperm midpiece, elongation, and regularity.

Table 7.5: The sperm morphometry trait of boar epididymides at pre-freeze and post-thaw (mean ± SD).

Sperm morphometric trait	Pre-freeze	Post-thawed	P value
	Sperm head trait		
Length (µm)	9.0±0.5[a]	9.1±0.9[a]	>0.05
Width (µm)	4.7±0.3[a]	4.5±0.4[a]	>0.05
Area (μm^2)	34.8±3.2[b]	38.2±6.9[a]	<0.05
Perimeter (µm)	23.7±1.2[a]	20.1±1.9[b]	<0.05
	Sperm midpiece trait		
Width (µm)	1.1±0.3[a]	1.0±0.3[a]	>0.05
	Sperm shape indices		
Ellipticity	1.9±0.2[b]	1.0±0.3[a]	<0.05
Elongation	0.3±0.0[a]	1.0±0.3[a]	>0.05
Regularity	0.9±0.0[a]	1.0±0.3[a]	>0.05
Roughness	0.8±0.0[b]	1.0±0.3[a]	<0.05

[a-b]Values with different superscripts differ significantly in the same row (P<0.05).

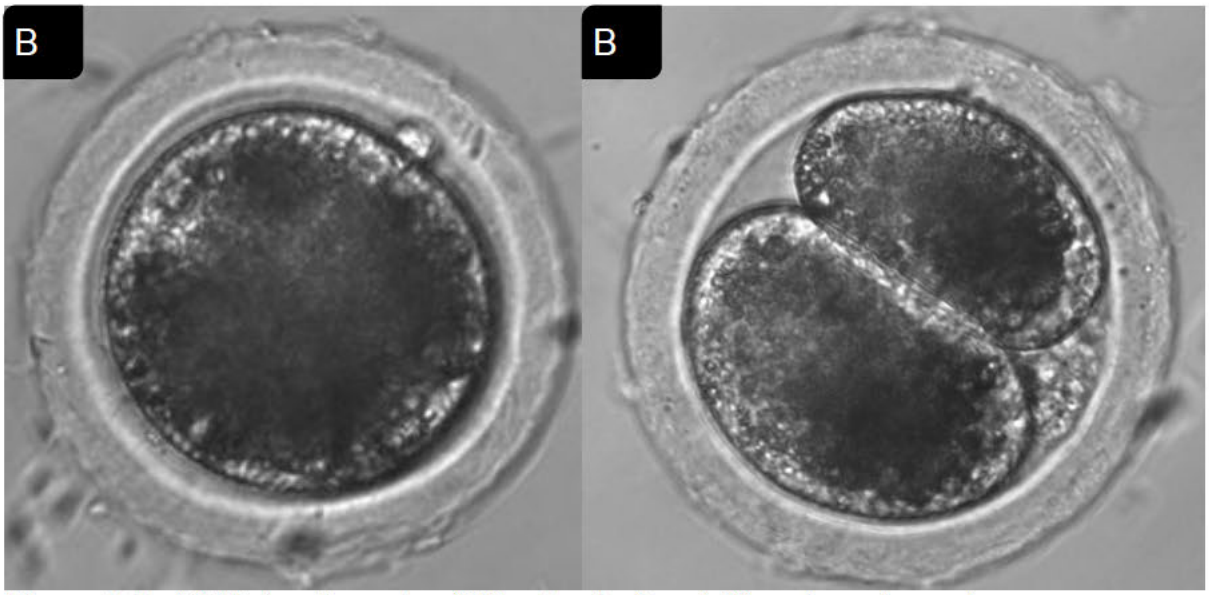

Figure 7.1: A) Matured oocytes (PB extrusion) and B) early embryo cleavage

The fertilisation ability of pre-freeze and post-thawed boar epididymal sperm utilising oocytes that have undergone maturation at NCSU-23, and mNCSU-23 media, is represented in Table 7.6. Overall, the interaction between the sperm type and oocytes maturation media showed significant effect on the total IVF rate. Normal fertilisation (2PN; 22-30%)/ early embryo cleavage (Figure 7.1) recorded in the study did not differ (P>0.05) between the oocytes matured in two media, irrespective of the type of sperm (pre-freeze and post-thawed) used to fertilise. The overall IVF rate was higher in the oocytes matured

with mNCSU-23 media fertilised with pre-freeze and post-thawed sperm, followed by the NCSU-23 oocytes fertilised with pre-freeze sperm (P>0.05). The total fertilisation percentage increased by the highest number of polyspermy recorded.

Table 7.6: Fertilisation ability of pre-freeze and post-thawed boar epididymal sperm utilising gilts oocytes matured in NCSU-23 and mNCSU-23 media (mean ± SD).

Modified type	Treatments	IVF Oocytes (n)	0 PN (%)	1 PN (%)	2 PN (%)	>2 PN (%)	Total IVF Rate (%)
Modified NCSU-23	**Pre-freeze**	160	8.8±6.4^{c}	8.8±7.7^{a}	26.0±15.2^{a}	56.4±19.2^{a}	91.2±5.9^{a}
	Post-thawed	160	18.4±8.3^{b}	6.0±3.1^{a}	29.6±17.7^{a}	46.0±19.3^{a}	81.6±8.3^{a}
NCSU-23	**Pre-freeze**	160	19.6±7.6^{b}	6.0±3.7^{a}	30.0±20.5^{a}	44.4±17.6^{a}	80.4±15.1^{a}
	Post-thawed	160	39.2±15.1^{a}	11.6±3.7^{a}	22.4±15.2^{a}	26.8±17.3^{b}	60.8±15.1^{b}
P-value			<0.05	>0.05	<0.05	<0.05	>0.05

$^{a-c}$Values with different superscripts differ significantly in the same column (P<0.05). mNCSU-23: modified North Carolina State University 23, IVF: *in vitro* fertilisation, PN: pronucleus, 1PN: abnormal fertilisation, 2PN: normal fertilisation, >2PN: polyspermy, Total IVF rate: 1PN+2PN+>2PN

7.4 Discussion

This study evaluated the fertilising competence of post-thaw and pre-freeze sperm using oocytes matured in NCSU-23 and mNCSU-23. A higher proportion of oocytes reached maturation when cultured in mNCSU-23 compared to standard NCSU-23 media. Similarly, Xie et al. (2016) reported increased oocyte maturation rates in mice under comparable glucose-enriched conditions. These results align with the findings of Wen et al. (2020), who demonstrated that elevated glucose metabolism promotes meiosis resumption and progression to the metaphase II stage. Similarly, Collado-Fernandez et al. (2012) emphasised that oocyte maturation requires a large quantity of energy to support its dynamic cellular processes. Wen et al. (2020) noted that glucose releases metabolites from the pentose phosphate pathway and glycolysis to promote pig oocyte maturation. Previous studies have shown that the concentration of energy substrates in maturation media influences meiotic maturation (Wen et al., 2020), a finding supported by the current study. The removal of sorbitol in the mNCSU-23 medium may have eliminated the oxidative stress, as excessive sorbitol is known to impair the intracellular redox balance (Zhang et al., 2021).

In this study, oocytes matured using NCSU-23 and mNCSU-23 maturation media recorded significant 2PN (normal fertilisation/early embryo cleavage) using pre-freeze and post-thawed sperm. Surprisingly, 2-8 cell divisions (early cleavage rate) were observed at the IVF in both maturation media treatments despite the absence of specific *in vitro* culture conditions such as a modular chamber. The observed increase in early embryo cleavage could be attributed to the embryo quality and that it's likely

to develop into the blastocyst stage rather than late-cleaving counterparts (He et al., 2024). The decrease in the cytoplasmic to nuclear volume ratio is crucial in activating specific genes. While the pattern of cleavage divisions differs between species, mid-blastula transition occurs when the cleavage rate decreases (Ajduk & Zernicka, 2016). The transition from fertilisation to cleavage is caused by the activation of mitosis-promoting factor, which regulates the biphasic cell cycle of early blastomeres (Marlow et al., 2010).

These studies suggest that embryos that cleave early will likely complete the first mitotic division early post-insemination, resulting in a higher pregnancy rate (Chen et al., 2019). The most interesting finding was the high post-thaw total motility (85.9%) of boar epididymal sperm recorded prior to the IVF process. In accordance with the present results, previous studies have emphasised the conservation of epididymal sperm as an important method for preserving boar genetic resources (Turri, 2012).

The current study differs from the report by Hallberg et al. (2024) which recorded a proportion of oocytes being penetrated and developed into cleavage to be 39%, using the ejaculated semen of Hampshire boars. On the other hand, a study by Serrano-Albal et al. (2024), also recorded a greater average of 38.5–53.5% oocytes with normal penetration/developed to cleavage. A high abnormal fertilisation rate (>80%) was observed in oocytes matured with mNCSU-23, particularly when using pre-freeze and post-thawed sperm, followed by oocytes matured with NCSU-23, fertilised with pre-freeze sperm, primarily due to increased incidences of polyspermy (>2PN formation).

The pig provides an example of a high incidence of polyspermy penetration during traditional IVF (Coy & Avilés, 2010). The present findings seem consistent with other studies that found more polyspermy than normal penetration in pigs (Gil et al., 2010). Polyspermy penetration compromises early embryonic development, ultimately reducing the success of IVF in pigs. A possible explanation for polyspermy in pigs might be the presence of caffeine in the IVF media, which may induce premature acrosome reaction in sperm, leading to multiple penetration of the sperm into the oocyte's zona pellucida (Stival et al., 2016). One potential strategy to reduce polyspermy involves replacing caffeine in the IVF medium with alternative compounds that promote zona pellucida binding without inducing premature acrosome reactions (Coy & Avilés, 2010). In the IVF process, a large concentration of sperm is often used which increases the likelihood of polyspermy penetration (Kitaji et al., 2015). To reduce the concentration of sperm in the IVF process, the application of intracytoplasmic sperm injection (ICSI; Briski & Salamone, 2022) instead of the traditional IVF technique should be practised. Although ICSI is a widespread option to avoid polyspermy (Grupen, 2014), ICSI is a key step in the fertilisation process that may assist in sperm selection (Stival et al., 2016).

7.5 Conclusion

This study was able to improve the current global obstacle in cryopreserving boar sperm. This will strengthen genetic resource banking programmes in South Africa, and beyond. It was recorded that both pre-freeze and post-thaw epididymal sperm retained the ability to fertilise oocytes matured in NCSU-23 and mNCSU-23 media, leading to early embryonic cleavage. However, polyspermy remained a significant limitation in pigs' IVF outcomes. Future research should focus on reducing polyspermy during fertilisation. This could be done through ICSI or modulation of oocyte maturation conditions. Exploring the epigenetic and molecular integrity of embryos derived from post-thaw epididymal sperm is a worthy future research direction.

7.6 References

Ajduk, A. and Zernicka-Goetz, M., 2016. Polarity and cell division orientation in the cleavage embryo: from worm to human. *MHR: Basic Science of Reproductive Medicine*, *22*(10), pp.691-703. https://doi.org/10.1093/molehr/gav068

Benson, J.D., Woods, E.J., Walters, E.M. and Critser, J.K., 2012. The cryobiology of spermatozoa. *Theriogenology*, *78*(8), pp.1682-1699.

https://doi.org/10.1016/j.theriogenology.2012.06.007

Bertol, M.A.F., Weiss, R.R., Thomaz-Soccol, V., Kozicki, L.E., Fujita, A.S., Abreu, R.A.D. and Green, K.T., 2013. Viability of bull spermatozoa collected from the epididymis stored at 18-20 C. Brazilian Archives of Biology and Technology, 56(5), pp.777-783. https://doi.org/10.1590/S1516-89132013000500008

Briski, O. and Salamone, D.F., 2022. Past, present and future of ICSI in livestock species. *Animal Reproduction Science*, *246*, p.106925. https://doi.org/10.1016/j.anireprosci.2022.106925

Chen, H., Einstein, L.C., Little, S.C. and Good, M.C., 2019. Spatiotemporal patterning of zygotic genome activation in a model vertebrate embryo. *Developmental Cell*, *49*(6), pp.852-866. https://doi.org/10.1016/j.devcel.2019.05.036

Chen, P.R., Redel, B.K., Kerns, K.C., Spate, L.D. and Prather, R.S., 2021. Challenges and considerations during in vitro production of porcine embryos. *Cells*, *10*(10), p.2770. https://doi.org/10.3390/cells10102770

Collado-Fernandez, E., Picton, H.M. and Dumollard, R., 2012. Metabolism throughout follicle and oocyte development in mammals. *International Journal of Developmental Biology*, *56*(10-12), pp.799-808. https://doi.org/10.1387/ijdb.120140ec

Coy, P. and Avilés, M., 2010. What controls polyspermy in mammals, the oviduct or the oocyte?. *Biological Reviews*, *85*(3), pp.593-605. https://doi.org/10.1111/j.1469-185X.2009.00117.x

Daigneault, B.W., McNamara, K.A., Purdy, P.H., Krisher, R.L., Knox, R.V. and Miller, D.J., 2014. Novel and traditional traits of frozen-thawed porcine sperm related to in vitro fertilization success. *Theriogenology*, *82*(2), pp.266-273. https://doi.org/10.1016/j.theriogenology.2014.04.006

Falomo, M.E., Rossi, M. and Mantovani, R., 2016. Collection, storage and freezability of equine epididymal spermatozoa. *Italian Journal of Animal Science*, *15*(3), pp.386-389. https://doi.org/10.1080/1828051X.2016.1210485

Gil, M.A., Cuello, C., Parrilla, I., Vazquez, J.M., Roca, J. and Martinez, E.A., 2010. Advances in swine in vitro embryo production technologies. *Reproduction in Domestic Animals*, *45*, pp.40-48. https://doi.org/10.1111/j.1439-0531.2010.01623.x

Gill, P.K. and Desai, N., 2016. Testicular sperm can effect early embryo morphokinetics. Fertility and Sterility, 105(2), p.e4. https://doi.org/10.1016/j.fertnstert.2015.12.028

Gilchrist, R.B., 2010. Recent insights into oocyte–follicle cell interactions provide opportunities for the development of new approaches to in vitro maturation. *Reproduction, Fertility and Development*, *23*(1), pp.23-31. https://doi.org/10.1071/RD10225

Grupen, C.G., 2014. The evolution of porcine embryo in vitro production. *Theriogenology*, *81*(1), pp.24-37. https://doi.org/10.1016/j.theriogenology.2013.09.022

He, L.N., Xu, Q., Lin, J., Liu, Y. and Chen, W., 2024. Predictive strategies for oocyte maturation in IVF cycles: from single indicators to integrated models. *Middle East Fertility Society Journal*, *29*(1), p.33. https://doi.org/10.1186/s43043-024-00193-7

Hallberg, I., Morrell, J.M., Malaluang, P., Johannisson, A., Sjunnesson, Y. and Laskowski, D., 2024. Sperm quality and in vitro fertilizing ability of boar spermatozoa stored at 4 °C versus conventional storage for 1 week. *Frontiers in Veterinary Science*, *11*, p.1444550. https://doi.org/10.3389/fvets.2024.1444550

Kitaji, H., Ookutsu, S., Sato, M. and Miyoshi, K., 2015. A new rolling culture-based in vitro fertilization system capable of reducing polyspermy in porcine oocytes. *Animal Science Journal*, *86*(5), pp.494-498. https://doi.org/10.1111/asj.12327

Ledwaba, M.R., Mphaphathi, M.L., Thema, M.A., Pilane, C.M. and Nedambale, T.L., 2022. Investigation of the efficacy of dithiothreitol and glutathione on in vitro fertilization of cryopreserved Large White boar semen. *Animals*, *12*(9), p.1137. https://doi.org/10.3390/ani12091137

Lee, J., Park, J.I., Im Yun, J., Lee, Y., Yong, H., Lee, S.T., Park, C.K., Hyun, S.H., Lee, G.S. and Lee, E., 2015. Rapamycin treatment during in vitro maturation of oocytes improves embryonic development after parthenogenesis and somatic cell nuclear transfer in pigs. *Journal of Veterinary Science*, *16*(3), pp.373-380.

https://doi.org/10.4142/jvs.2015.16.3.373

Lin, Z.L., Li, Y.H., Xu, Y.N., Wang, Q.L., Namgoong, S., Cui, X.S. and Kim, N.H., 2014. Effects of growth differentiation factor 9 and bone morphogenetic protein 15 on the in vitro maturation of porcine oocytes. *Reproduction in Domestic Animals*, *49*(2), pp.219-227. https://doi.org/10.1111/rda.12254

Malik, P., Mukherjee, S. and Mukherjee, T.K., 2023. *Mammalian cell culture types and guidelines of their maintenance.* In Practical Approach to Mammalian Cell and Organ Culture (pp. 233-259). Singapore: Springer Nature Singapore. https://doi.org/10.1007/978-981-19-1731-8_6-2

Marlow, F.L., 2010. '*Maternal control of development in vertebrates: my mother made me do it*!' Editor: Daniel Kessler, Developmental biology, University of Pennysylvania. *Morgan and Claypool Publishers Life Sciences*, pp.157.

Martecikova, S., Hulinska, P., Reckova, Z., Pavlik, A., Jeseta, M. and Machatkova, M., 2010. Effect of acrosome reaction progress in frozen-thawed boar spermatozoa on the efficiency of in vitro oocyte fertilization. *Veterinární medicína*, *55*(9), pp.429-437.

Pawlak, P., Warzych, E., Cieslak, A., Malyszka, N., Maciejewska, E., Madeja, Z.E. and Lechniak, D., 2018. The consequences of porcine IVM medium supplementation with follicular fluid become reflected in embryo quality, yield and gene expression patterns. *Scientific Reports*, *8*(1), p.15306. https://doi.org/10.1038/s41598-018-33550-4

Pyoos, G.M., Maqhashu, A.M., Scholtz, M.M. and Nedambale, T.L., 2018. The comparison of three media on the in vitro maturation rate of pig oocytes. *South African Journal of Animal Science*, *48*(6). https://doi.org/10.4314/sajas.v48i6.4

Raad, G., Nader, C., Azoury, J., Tanios, J., Bader, R., Dahdouh, E., Rizk, K., Zayat, M.C., Azoury, J. and Azoury, J., 2018, July. Is there a difference in embryo morphokinetics between ejaculated and non-ejaculated spermatozoa, 33, pp.205-206.

Romar, R., Funahashi, H. and Coy, P., 2016. In vitro fertilization in pigs: new molecules and protocols to consider in the forthcoming years. *Theriogenology*, *85*(1), pp.125-134. https://doi.org/10.1016/j.theriogenology.2015.07.017

Serrano-Albal, M., Aquilina, M.C., Kiazim, L.G., Zak, L.J., Griffin, D.K. and Ellis, P.J., 2024. Effect of two different sperm selection methods on boar sperm parameters and in vitro fertilisation outcomes. *Animals*, *14*(17), p.2544.
https://doi.org/10.3390/ani14172544

Strand, J., Ragborg, M.M., Pedersen, H.S., Kristensen, T.N., Pertoldi, C. and Callesen, H., 2016. Effects of post-mortem storage conditions of bovine epididymides on sperm characteristics: investigating a tool for preservation of sperm from endangered species. Conservation Physiology, 4(1), p.cow069. https://doi.org/10.1093/conphys/cow069

Stival, C., Puga Molina, L.D.C., Paudel, B., Buffone, M.G., Visconti, P.E. and Krapf, D., 2016. Sperm capacitation and acrosome reaction in mammalian sperm. In: Buffone, M. (eds) Sperm

acrosome biogenesis and function during fertilization. Advances in Anatomy, *Embryology and Cell Biology*, *220*, pp.93-106. Springer, Cham. https://doi.org/10.1007/978-3-319-30567-7_5

Thema, M.A., Mphaphathi, M.L., Ledwaba, M.R. and Nedambale, T.L., 2023. Sperm cryopreservation in Windsnyer boars; principles, technique, and updated outcomes. *Animal Reproduction*, *20*, p.e20220100. https://doi.org/10.1590/1984-3143-AR2022-0100

Thema, M.A., Mkhize, N.R., Sebopela, M.D., Ledwaba, M.R. and Mphaphathi, M.L., 2025. Effect of Pre-Freezing 18 °C Holding Time on Post-Thaw Motility and Morphometry of Cryopreserved Boar Epididymal Sperm. *Animals*, *15*(12), p.1691. https://doi.org/10.3390/ani15121691

Turri, F., 2012. Developing the use of epididymal semen for farm animal cryobanks and some field applications. https://tesidottorato.depositolegale.it/handle/20.500.14242/170363 Accessed 26022025

Turri, F.E.D.E.R.I.C.A., Madeddu, M., Gliozzi, T.M., Gandini, G. and Pizzi, F., 2012. Influence of recovery methods and extenders on bull epididymal spermatozoa quality. *Reproduction in Domestic Animals*, *47*(5), pp.712-717. https://doi.org/10.1111/j.1439-0531.2011.01948.x

Varghese, A.C., Ly, K.D., Corbin, C., Mendiola, J. and Agarwal, A., 2011. Oocyte developmental competence and embryo development: impact of lifestyle and environmental risk factors. *Reproductive Biomedicine Online*, *22*(5), pp.410-420. https://doi.org/10.1016/j.rbmo.2010.11.009

Wen, J., Wang, G.L., Yuan, H.J., Zhang, J., Xie, H.L., Gong, S., Han, X. and Tan, J.H., 2020. Effects of glucose metabolism pathways on nuclear and cytoplasmic maturation of pig oocytes. *Scientific Reports*, *10*(1), p.2782. https://doi.org/10.1038/s41598-020-59709-6

Xie, H.L., Wang, Y.B., Jiao, G.Z., Kong, D.L., Li, Q., Li, H., Zheng, L.L. and Tan, J.H., 2016. Effects of glucose metabolism during in vitro maturation on cytoplasmic maturation of mouse oocytes. *Scientific Reports*, *6*(1), p.20764. https://doi.org/10.1038/srep20764

Zeke, J., Konc, J., Kanyo, K., Kriston, R. and Cseh, S., 2012. Birth and clinical pregnancy from fresh and frozen oocytes fertilized with cryopreserved testicular spermatozoa. *Systems Biology in Reproductive Medicine*, *58*(3), pp.165-167. https://doi.org/10.3109/19396368.2012.656797

Zhang, Y., Yan, Z., Liu, H., Li, L., Yuan, C., Qin, L., Cai, L., Liu, J., Hu, Y. and Cui, Y., 2021. Sorbitol accumulation decreases oocyte quality in aged mice by altering the intracellular redox balance. *Aging (Albany NY)*, *13*(23), p.25291. https://doi.org/10.18632/aging.203747

Chapter 8: General conclusions and recommendations for further research

8.1 Introduction

In boar reproductive biotechnology, conservation of genetic material at post-mortem is becoming more important with the idea of maintaining the genetic makeup of valuable species. This study investigated sought to establish scientifically grounded protocols for recovery, preservation, and fertilisation ability of boar epididymal sperm, thereby advancing reproductive biotechnology and genetic resource conservation. The sperm retrieval method, semen extenders, testicular storage temperatures, period, sperm pre-freeze holding times, cryopreservation, oocytes maturation media and *in vitro* fertilisation (IVF) results were addressed through this experiment. Discovery of the retrieval method and semen extender that can result in high sperm yield that are viable and may be preserved without the sperm motility and morphometry being compromised was a critical challenge.

The 15-18 ℃ testicular storage temperature elevated metabolic activity, speed up cellular deterioration and impaired sperm motility, acrosome and morphology. While 15-18 ℃ is an optimum storage temperature for preserving boar semen in a liquid form and supports sperm quality. It was a challenge to freeze boar epididymal sperm, immediately after retrieval because, pre-freeze holding temperature of 18 ℃ improves cryotolerance of the boar epididymal sperm. The overarching purpose was to provide a reproducible framework that can inform the establishment of epididymal sperm cryobanks for genetic conservation, and assisted reproduction, with implications extending beyond pigs to other species. The study also analysed the IVF fertilising ability of pre-freeze and post-thawed epididymal sperm on oocytes subjected to *in vitro* maturation in North Carolina State University 23 (NCSU-23) and modified NCSU-23 (mNCSU-23) media.

8.2 General discussion and conclusion

This study demonstrated that high quality boar epididymal sperm can be retrieved after death and be conserved for use in assisted reproductive technologies. The slicing float-up method using Beltsville Thawing Solution (BTS) extender have proved to be the most effective, slicing float-up yielding more sperm with higher motility, normal morphology and morphometry compared to the flushing method.

This study showed the effectiveness of antibiotics concentration in the BTS extender which played a big role in maintaining sperm pH and sperm viability during sperm recovery. The BTS extender also contains glucose which have proven as suitable sugar supplementation in several semen preservation studies than fructose in Andromed® extender. This type of sugar in the BTS extender play a vital role in interacting with the phospholipid membranes at a low hydration while causes stabilisation of membranes. It is also utilised by sperm as an energy source through glycolysis and mitochondrial oxidative phosphorylation to support sperm motility and morphology at recovery and during preservation. Additionally, even without seminal plasma in the epididymal sperm, the composition of

the ion chelators in BTS extender, such as ethylenediaminetetra acetic acid where able to protect sperm against oxidative damage and lipid peroxidation during recovery.

Studies in boars reported the effect of testicular storage time on the epididymal sperm viability but none of these studies compared epididymides processed immediately after death stored at 5 and 18 °C. Our findings confirmed that viable boar epididymal sperm can be successfully recovered from testes stored at 5 °C for at least 2-24 h and not immediately after death. The predominant effect of testicular storage at 5 °C was a reduction in the metabolic rate of the sperm, which aided to prolong their survival time. The present study highlights the necessity of maintaining lower testicular storage temperatures to protect not just the testes or epididymis from degrading easily, but also functional features linked to sperm motility and morphometry.

In the current study, liquid preserved boar epididymal sperm stored at 18 °C maintained superior motility and normal morphometry for up to 48 h compared to samples stored at 5 °C. The storage temperature between 15 and 20 °C is an optimum temperature for liquid preserving boar sperm. In terms of cryopreservation, pre-freeze holding times (3-6 h) at 18 °C significantly improved post-thaw sperm motility, morphometry and cryotolerance, indicating the importance of sperm holding before freezing. The improved cryotolerance of boar epididymal sperm cooled for 3-6 h at 18 °C before freezing is attributed to the protective effect of epididymal fluid, containing low bicarbonate levels and absence of relative high levels. This explains why epididymal sperm withstand the cryopreservation following 3-6 h holding irrespective of the exposure of the seminal plasma. The 3 h holding time at 15-18 °C has been developed in pigs ejaculated semen cryopreservation protocol with the intention for the sperm to withstand damage caused by the freezing process, cryoprotectant toxicity and improving cryotolerance.

The current study was able to overcome the current global challenge of cryopreserving boar sperm. It was recorded that both pre-freeze and post-thawed epididymal sperm retained the ability to fertilise oocytes matured in NCSU-23 and mNCSU-23 media, leading to early embryonic cleavage. However, polyspermy remained a significant limitation in pigs IVF outcomes. Taken together, these findings demonstrate the feasibility of conserving boar genetic material post-mortem while illuminating the cellular mechanisms governing sperm survival during temperature transitions. The results substantiate the establishment of boar epididymal sperm cryobanks and contribute to global efforts in animal germplasm preservation.

8.3 Challenges and limitations

Low sperm yield during sperm recovery, which is dependent on the type of retrieval technique used. The flushing method may result into low sperm yield in pigs sperm recovery due to the few cuts performed. Although flushing technique could be the acceptable standard for collecting epididymal

sperm from other animals, it may not allow the release of all sperm because it relies on single incision and pushing fluid through epididymal duct leaving some sperm behind.

The nutritional composition in the semen extenders such as Andromed® extender used in the current study which had a negative impact on the acrosome of the sperm. The Andromed® extender is a protein-free and contains a high saturated and unsaturated phospholipid content. While for epididymal sperm with the absence of seminal plasma requires protein and unsaturated fatty acid composition from the semen extenders for the purpose of sperm maintenance and protection against oxidative stress and cold. Therefore, the composition of semen extenders during sperm recovery is one of the key elements that may negatively or positively influence the retrieved epididymal sperm quality.

The damage of sperm acrosome resulting to amorphous shape with the prolonged testicular storage time at 18 °C. This sperm acrosome damage, amorphous abnormalities and decrease in sperm motility can be explained by both the metabolic depletion due to the increasing testicular storage temperature (18 °C). It was shown that lower liquid storage temperature (5 °C) increased boar epididymal sperm membrane permeability and cold shock susceptibility due to the high polyunsaturated fatty acid in the boar sperm plasma membrane. The lower temperature on the boar sperm may lead to the membrane fluidity and permeability being affected. Storing boar sperm at 5 °C with the prolonged storage time may reduce sperm motility, viability and normal morphology. Although, cold storage temperature is commonly known with its ability to inhibit bacterial growth but may be regarded critical for liquid preservation of boar sperm because of its sensitivity to chilling injury.

The absence of the seminal plasma and immaturity of some of the sperm in the epididymal semen sample unlike ejaculated semen. While, boar sperm is known to have high polyunsaturated fatty acid, this limitation makes the boar epididymal sperm more vulnerable to oxidative stress and cryodamage if freeze immediately after recovery or exposed to the prolonged holding time (9-48) before cryopreservation).

The present of egg yolk in the freezing extender of boar semen interfered with analyses of post thaw sperm motility and morphometry with computer assisted-sperm analyser (CASA). Despite the protective effects of egg yolk on boar sperm during cryopreservation, its thickness particles musk the motion of the sperm which may affect the image capturing and the accuracy of the kinematics and morphometric analysis with CASA.

The incidence of polyspermic fertilisation rate during IVF process which may limit the production of viable embryos and increase the embryo loss percentages. High polyspermy during IVF revealed the persistent challenge of achieving normal fertilisation (2PN) in porcine IVF systems causing abnormal chromosome numbers in embryos. Normal penetration fertilisation involves the development of a block on the plasma membrane and zona pellucida hardening immediately after the leading sperm penetration preventing polyspermy (additional sperm from entering the oocyte).

8.4 Recommendation for future research

This study highlights the possibility of epididymal sperm recovery following 2-48 h animal death when epididymides stored at 5 °C, with the scarce information on boars, the long-term epigenetic integrity and healthy development of offspring from epididymis must be addressed in the future research. The recovery of boar epididymal sperm using slicing float-up and BTS extender can have a positive effect on the boar sperm at the stage of recovery and preservation. The supplementation of protective components such as antioxidants in the extenders it is recommended to provide protection against oxidative stress and cold shock while maintains the quality of the sperm during handling and preservation. The 3-24 h prolonged holding of boar epididymal semen sample at 18 °C before freezing favours bacterial proliferation and maintained cryo-survivability of the sperm while resulted in the improved fertilisation rates. It is further recommended that future studies can improve the cryotolerant of boar epididymal sperm with 3-24 h pre-freeze at 18 °C.

Additional research is suggested to investigate a protective role of seminal plasma addition to the epididymal sperm in minimising the oxidative stress of epididymal sperm frozen immediately after retrieval and for prolonged holding time (9-48 h). The prolonged holding time before freezing has been observed to allow a prolonged interaction between boar sperm and seminal plasma components in the studies on other species while limited in pigs. Seminal plasma is a specific substrate that consists of proteins in the ejaculated semen and play a vital role in enhancing, maintaining sperm motility and morphology during preservation.

It is suggested that future studies should research on the alternative cryoprotect to egg yolk with same qualities for cryopreserving boar sperm and be CASA friendly to improve the sperm motility and morphometry analysis.

Polyspermy still remains a significant issue in the pig IVF systems, strategies such as ICSI and artificial insemination should be implemented in the future studies to test the fertilising ability of post-thawed boar epididymal and to prevent polyspermy to happen. The main advantage of fertilisation by intra cytoplasmic sperm injection (ICSI) is the possibility of injecting one sperm into the cytoplasm or zona pellucida. The utilisation of ICSI may limit the transmission of diseases while prevent abnormal fertilisation that lowers fertilisation rate. The use of ICSI technique has been used to overcome polyspermy in other species, however its use in porcine it is still underutilised because of the challenges in optimising the process.

8.5 Overall contribution

This thesis provides a scientific and technological foundation for the preservation of boar epididymal sperm, demonstrating that viable, fertilisation-competent sperm can be retrieved and cryopreserved post-mortem. It demonstrates the promise of quality post-mortem sperm rescue and cryopreservation in

boars, prompt sperm recovery, cooling and proper testicular storage the most important factors affecting the ability to recover quality samples. Results of this study may improve the use of epididymal sperm in local breed gene banking. It further integrates developmental biology, cryobiology, and reproductive biotechnology. This work offers new insights into cellular resilience and the management of thermal stress in gametes. Beyond its immediate application in porcine systems, the study contributes to broader conservation biology by offering adaptable methodologies for safeguarding the genetic integrity of endangered or elite livestock breeds, bridging the gap between post-mortem gamete biology and practical genetic resource banking. The *in vitro* production and cryopreservation of epididymal sperm could facilitate a global exchange of valuable genetic material and accelerate breeding programs.