AN INVESTIGATION INTO FACTORS INFLUENCING THE SUCCESS OF OVINE ARTIFICIAL INSEMINATION PROGRAMS

THE UNIVERSITY OF SYDNEY

Eloise Ann Spanner

A thesis submitted to fulfil the requirements for the Degree of Doctor of Philosophy,

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Statement of Originality

This is to certify that to the best of my knowledge, the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes. I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

Eloise Spanner

BSc/BAdvSt (Honours) (Animal and Veterinary Bioscience)

Authorship Attribution Statement

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Eloise Spanner (22nd December 2024)

As a supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

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Summary

This thesis investigates the interaction of male and female factors on pregnancy success following intrauterine laparoscopic Artificial Insemination (AI) of sheep. Data were collated from 30,254 individual ewes and 388 sires to design a multifactorial binomial fertility model incorporating a wide range of fertility factors. These fertility factors included the concentration at which sperm was frozen (as a proxy for insemination dose), the percentage of sperm with abnormal morphology, the percentage of viable, acrosome intact sperm, motility and sperm kinematics, and the intra-abdominal fat and uterine tone of ewes at the time of AI. The model was validated with unseen data to predict pregnancy outcomes and compared with ultrasound results under field conditions. Discriminatory and calibration techniques were used to determine the accuracy and sensitivity of the model, and thresholds were suggested for each *in vitro* predictor, which would result in high fertility. Finally, a separate investigation of the impact of cryopreservation techniques on the post-thaw quality of ram spermatozoa was undertaken. Superior quality was observed when semen was frozen between 400–600×10^6 sperm/mL and thawed in tris-citrate-fructose media, one pellet at a time. The results contained within this thesis have tangible applied outcomes for the industry, reducing variation and improving confidence in sheep reproductive technologies.

The results contained in Chapter 2 analysed data collected over three breeding seasons from 24 commercial AI programs, where 24,700 oestrus synchronised Merino ewes were artificially inseminated with semen from 253 sires. Female factors, including age, uterine tone, intra-abdominal fat, time of AI post-CIDR pull, and male factors, such as semen type (fresh or frozen) and package type (pellets or straws), were collected at AI. The results highlighted that ewes with a uterine tone score of 4 or 5 during laparoscopic AI have a significantly higher pregnancy rate than ewes with a 3 or 2 and 1 score. The findings suggest uterine tone assessment is a practical tool for ensuring appropriate synchronisation management and screening of ewes prior to AI. The following chapter builds on this model, examining whether the influence of uterine tone holds significance when included with *in vitro* semen traits of the sires used, further enhancing the predictive accuracy of pregnancy outcomes.

Additional data were collected in Chapter 3 to complete the data set totalling 30,254 Merino ewes from 30 commercial breeding programs around Australia. Alongside the female factors recorded at AI, a subset of the semen inseminated from 388 sires was thawed and

assessed *in vitro* for volume, subjective motility (%), sperm concentration (million sperm/mL), and morphology immediately post-thaw. At 0, 3 and 6 h post-thaw, motility and kinematics (CASA), viability, acrosome integrity, membrane fluidity, mitochondrial superoxide production, lipid peroxidation, intracellular reactive oxygen species, and DNA fragmentation were assessed using flow cytometry. The results indicated that the concentration of sperm frozen, percent of viable spermatozoa with intact acrosomes (6 h post-thaw), and motility (0 h post-thaw) positively influenced pregnancy outcomes. Ewes with a uterine tone and intra-abdominal fat scores of 4 or 5 also resulted in higher likelihood of pregnancy success. An increase in the percent of abnormal sperm morphology decreased pregnancy probability. These findings underscored the critical interplay between male and female fertility traits, with the validation of the model poised to support its application as a valuable decision-making tool to measure the reproductive potential of commercial sheep flocks.

The above model was validated in Chapter 4 using unseen data from 1,269 ewes and 26 sires collected during AI as per the methods in Chapters 2 and 3. A threshold of 0.56 was created to differentiate pregnant ewes from non-pregnant ewes. The predicted pregnancy result per ewe was compared to actual pregnancy results determined approximately 55 days post-AI. The model demonstrated moderate accuracy (74 %), high precision (96 %) and lower specificity (33 %). Additionally, it measured a balanced recall (sensitivity) (76 %) and high F1-score (0.85), suggesting overall good performance, supported by an AUC of 0.62. There was no statistical difference between predicted and actual pregnancy results despite an error value of 26%. The study established optimal thresholds for freezing concentration, abnormal sperm morphology, viable sperm with intact acrosomes (6 h) and CASA PCA (0 h) to be 680.85×10^6 sperm/mL, 15.5 %, 6.31 %, and 18.34 %, respectively. When run through the model, the cumulative likelihood of pregnancy occurring following laparoscopic AI was 64.3%. These findings validated the fertility model as a practical tool for screening semen and ewes before AI, reducing the risk of using sub-optimal semen and increasing the likelihood of successful AI outcomes in the sheep industry.

While overall semen quality is well understood to influence fertility, the effects of *in vitro* cryopreservation processing techniques on sperm quality are less understood. The final study in this thesis investigated how freezing concentrations, thawing diluents, and the number of pellets thawed at once affected sperm quality. When freezing in pellets, our results indicated that a pre-freeze concentration range of $400–600\times10^6$ sperm/mL and thawing in a tris-citrate-fructose media would maximise post-thaw motility and the percent of viable sperm with intact

acrosomes. Similarly, the percentage of sperm with abnormal morphology would also be reduced. Henceforth, it can be recommended that one pellet be thawed in solution with tris-citrate-fructose media to maximise the post-thaw quality of pellet frozen semen.

Combined, the findings in this thesis emphasise that AI success depends on the synergy between *in vitro* processing techniques, the quality of semen post-thaw used to inseminate, and the condition and synchronisation of the ewe at insemination. Unfortunately, variability in AI success will persist across the Australian sheep industry due to environmental conditions, nutritional status and flock management. While achieving 100% predictability in AI success remains elusive, the development of this fertility selection tool can now be used to distinguish sires and ewes of high fertility potential. Selecting animals based on the above criteria will help reduce variability in AI success, increase confidence in reproductive technologies and ultimately increase the number of lambs born to elite flocks, accelerating genetic and production gain throughout the industry.

Table of Contents

List of Abbreviations

°C	degrees Celsius
AI	artificial insemination
ALH	amplitude of lateral head displacement
AO	acridine orange
ART	artificial reproduction technology
AUC	area under the curve
BCF	beat cross-frequency
BSA	bovine serum albumin
BP	bandpass
CASA	computer-assisted sperm analysis
CCD	charge-coupled device
COMET	single-cell gel electrophoresis
DNA	deoxyribonucleic acid
DRY	thawing without media
dsDNA	double-stranded DNA
EY	egg yolk
FACs	fluorescence-activated cell sorting
FITC-PNA	fluorescein isothiocyanate-conjugated peanut agglutinin
FN	false negative
FP	false positive
FR	fresh
FSH	follicle-stimulating hormone

FTC	Salamon's thawing diluent
FTC+EY	Salamon's chill diluent
FZ	frozen
g	gravity, acceleration due to
h	hour
H_2CFDA	dichlorodihydrofluorescein diacetate acetyl ester
H33342	Hoechst 33342
Hz	hertz
H_2O_2	hydrogen peroxide
ICC	intraclass correlation coefficient
IVF	*in vitro* fertilisation/fertilised
LAI	laparoscopic artificial insemination
LIN	linearity
LSD	least significant difference
LH	luteinising hormone
m (prefix)	milli ($\times 10^{-3}$)
M	molar
MACE	mean absolute calibration error
MHz	megahertz
min	minute
mg	milligram
mL	millilitres
mm	millimetres
mW	milliwatt
M540	merocyanine-540

n (prefix)	nano ($\times 10^{-9}$)
nm	nanometre
NSW	New South Wales
nW	nanowatt
PBS	phosphate-buffered saline
PCA	principal component analysis
PCA3	principal component 3
pH	hydrogen ion concentration, -log 10 of
PI	propidium iodide
PMSG	pregnant mare serum gonadotrophin
PNA	arachis hypogaea agglutinin
PPV	positive predictive value
PR	pregnancy rate
PSA	pisum sativm agglutinin
REML	linear regression model
ROC	reviver operating characteristic
ROS	reactive oxygen species
RR	reproductive rate
SA	South Australia
SCD	sperm chromatin dispersion
SCSA	sperm chromatin structure assay
SEM	standard error of the mean
ssDNA	single-stranded DNA
STR	straightness
TN	true negative

TP	true positive
TUNEL	terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labelling
µ (prefix)	micro ($\times 10^{-6}$)
µL	microlitres
µm	micrometres
VAP	average path velocity
VCL	curvilinear velocity
VCP	vasolin-containing protein
VIC	Victoria
VSL	straight-line velocity
v/v	volume/volume
WA	Western Australia
WOB	wobble

List of Tables

List of Figures

List of Publications

The work described in this thesis has resulted in, or contributed to, the following publications and presentations.

Journal Publications

Wilson, H.R., **Spanner, E.A**., McMahon, J., Rickard, J.P., de Graaf, S.P., 2023. 004 Fertility of ram semen frozen in pellets or straws. *Animal - science proceedings* 14, 433. doi: 10.1016/j.anscip.2023.03.005

Spanner, E.A., de Graaf, S.P., Rickard, J.P., 2024. Factors affecting the success of laparoscopic artificial insemination in sheep. *Animal Reproduction Science*, 107453. doi: 10.1016/j.anireprosci.2024.107453

Spanner, E.A., de Graaf, S.P., Rickard, J.P., 2024. Uterine tone influences fertility of Merino ewes following laparoscopic artificial insemination. *Theriogenology*. doi: 10.1016/j.theriogenology.2024.04.002

Robertson, M.J., Chambers, C., **Spanner, E.A**., de Graaf, S.P., Rickard, J.P., 2024. The Assessment of Sperm DNA Integrity: Implications for Assisted Reproductive Technology Fertility Outcomes across Livestock Species. *Biology* 13, 539. doi: 10.3390/biology13070539

Spanner, E.A., de Graaf, S.P., Rickard, J.P., 2024. A multivariate model for the prediction of pregnancy following laparoscopic artificial insemination of sheep. *Scientific Reports* 14, 27556. doi: 10.1038/s41598-024-79253-x

Spanner, E.A., de Graaf, S.P., Rickard, J.P., 2024. The validation and application of an ovine fertility model using standardised *in vitro* thresholds to predict the likelihood of pregnancy. *Under review at Theriogenology.*

Spanner, E.A., Rickard, J.P., de Graaf, S.P., 2024. The effect of freezing and thawing techniques on the *in vitro* quality of ram semen frozen in pellets. *Under review at Animal Reproduction Science.*

Abstracts

Wilson, H.R., **Spanner, E.A**., McMahon, J., Rickard, J.P., de Graaf, S.P. (2023). Fertility of ram semen frozen in pellets or straws. 11th International Ruminant Reproduction Symposium (IRRS), 28th May – 1st June 2023. Galway, Ireland.

Spanner, E.A., Rickard, J.P., de Graaf, S.P. (2023). DNA fragmentation is correlated with abnormal morphology of frozen-thawed ram spermatozoa. Proceedings of the Annual Conference of the Society of Reproductive Biology (SRB) 26th – 29th November 2023. Brisbane, Australia.

Spanner, E.A., Rickard, J.P., de Graaf, S.P. (2024). Predicting the success of laparoscopic artificial insemination in sheep. Proceedings of the 57th Annual Conference of the Society for the Study of Reproduction (SSR) 15th – 19th July 2024. Dublin, Ireland.

Spanner, E.A., Rickard, J.P., de Graaf, S.P. (2023). The probability of pregnancy occurring following laparoscopic artificial insemination of sheep. Proceedings of the Annual Conference of the Society of Reproductive Biology (SRB) 10th – 13th November 2023. Adelaide, Australia.

Webinar

Meat & Livestock Australia (MLA) Sheep Reproduction Strategic Partnership (SRSP) Webinar Series "Let's talk rams" – Part 2: Prediction of ovine AI success. https://www.youtube.com/watch?v=M4UCtyE0VMI

Review of the Literature

The literature review described herein has been published as: Spanner, E.A., de Graaf, S.P., Rickard, J.P., 2024. Factors affecting the success of laparoscopic artificial insemination in sheep. *Animal Reproduction Science*, 107453. doi: 10.1016/j.anireprosci.2024.107453

1.1. GENERAL INTRODUCTION

Artificial insemination is an advanced reproductive technology used to increase the number of lambs born from elite sires to accelerate genetic gain in a flock. It is a method by which semen, collected fresh or frozen from sires with superior genetics, is deposited artificially within the ewes' reproductive tract. It often facilitates a greater number of ewes being inseminated per ejaculate, rapidly improving the efficiency of breeding programs. The mode and success rate of AI is highly dependent on the breed of ewe, method and site of semen deposition, type of semen used, and the number of spermatozoa delivered (Evans 1988). Despite recording acceptable motility and viability (Sánchez-Partida 1999; O' Meara *et al.* 2008), for unknown reasons, frozen-thawed ram spermatozoa are unable to navigate the ewe's cervix in sufficient numbers to achieve pregnancies (10-40%; (Salamon and Lightfoot 1967). As a result, frozen-thawed ram semen must be directly deposited into the ewe's uterine horns via laparoscopy (Killeen *et al.* 1982), bypassing the cervix to achieve acceptable pregnancy rates. When used in conjunction with other reproductive technologies such as semen storage, multiple ovulation embryo transfer and sexed semen, it makes it an extremely versatile and valuable tool for efficient animal breeding.

Within the sheep industry, the success rate of laparoscopic intrauterine AI with frozen semen has been estimated to be approximately 70% (Geenty *et al.* 2014). Unfortunately, this result has been shown to vary significantly between seasons, programs and sires used

(Eppleston and Maxwell 1995; Hill *et al.* 1998; Del Olmo *et al.* 2013; Gibbons *et al.* 2019), with percentages recorded as low as 12% (Hill *et al.* 1998). Furthermore, variability in pregnancy rates of almost 20% have been seen between Merino and cross bred ewes as well as across different locations in Australia over a 5 year period (Geenty *et al.* 2014). This variability has likely contributed to the low uptake of reproductive technologies at an industry level. It has been estimated that less than 1% of the Australian sheep flock is inseminated via AI (Rickard and de Graaf, 2016 Industry survey, unpublished observations), ultimately contributing to a reduced rate of genetic progress compared to other industries (Kleemann and Walker 2005). To improve producer confidence and trust in AI, the industry requires a better understanding of what contributes towards a successful program. This could lead to a reduction in the variability between successful programs, increasing adoption within industry and improving the rate of genetic gain.

There are several factors thought to contribute to the fertility of the ewe under natural joining conditions; related to the health of the sire and quality of the semen (Maxwell *et al.* 1984), the age (Narayan *et al.* 2021), health and nutritional status of the female and management of oestrus (Walker *et al.* 1989) as well as the prevailing environmental conditions near the time of joining (Geenty *et al.* 2014). While some of these factors have been examined as main effects during artificial insemination in a research context, the relative contribution of each of these factors and how they interact within the one artificial breeding program, specifically following laparoscopic AI, within an industry setting is still to be fully elucidated.

Factors related to the sire and quality of semen has been hypothesised to play a major role in the success of any artificial reproductive technology. Salamon's Artificial Insemination of Sheep and Goats (1987) and the Australian Embryo Transfer Society in 1998 established a set of guidelines for the commercial testing of ram semen, recommending that a sample would not meet the industry defined standard if post-thaw sperm motility was lower than 40% and

morphological abnormalities were recorded above 15% (Evans and Maxwell 1987). Today, with the advance of objective semen assessment techniques, there is a wide variety of semen parameters known to collectively define a 'fertile' spermatozoon and how this might contribute to fertility when considering a natural, cervical or laparoscopic AI program. Across species, breeds and even males, in addition to the motility, and morphology of spermatozoa, the velocity, viability, concentration, DNA integrity and level of oxidative stress have all been linked to fertility. Despite these initial predictions, results have been contradictory, with previous studies using different measures of pregnancy success to assess impact on fertility. For example, studies have used hypothetical or pre-determined fertility levels, rather than direct comparisons to pregnancy following insemination. This suggests that further research is required before specific semen parameters can be linked to improving AI success rates and standards potentially updated. Furthermore, understanding how semen factors interact in a multifactorial study alongside female factors recorded during AI is also required.

As mentioned above, the success of an AI program is also dependent on the status of the ewe. Fertility results have been attributed to her body condition, live weight, breed, age (Kleemann and Walker 2005; Hinch and Brien 2014) method of oestrus synchronisation (Langford 1982) and response to exogenous hormones as well as time of AI (Harris *et al.* 1999). Historically, extensive research has shown the importance of the health and condition of the ewe leading up to an AI program (Kleemann *et al.* 2006) on overall success. It is thought that with an increase in the overall size and weight of the Australian ewe (Sawyer *et al.* 2019) as well as changes in wool and meat production trends (Hill *et al.* 1998; Kleemann *et al.* 2006) over the past 10-15 years, it would be worthwhile to revisit these factors in a nation-wide study considering the influence of the modern-day ewe on fertility following AI.

Finally, environmental conditions have long been attributed to physiological and biochemical factors affecting sheep productivity and reproduction (McManus *et al.* 2020). A

complex investigation into the effect of environmental parameters, such as temperature, humidity, and rainfall, on both ewe and ram fertility, leading up to, during and following AI is yet to be investigated. As Australia continues to face a warming, changing climate and lengthening of the seasons (Bi and Parton 2008; Sawyer *et al.* 2019) it remains critical that these parameters are considered alongside male and female factors to develop a cohesive, comprehensive model to predict the success of an AI program.

The current review will summarise the historical and contemporary practises of laparoscopic AI in sheep and will outline contributing male, female and environmental factors considered essential for success. Methods to assess the reproductive potential of the ewe will also be examined, as well as *in vitro* methods of semen assessment. Should trends in the fertility of sheep following AI be established, this could identify recommendations to update semen standards in Australia and help improve the success rate of AI for industry.

1.2. ARTIFICIAL INSEMINATION OF SHEEP

1.2.1. Historical Overview

Investigations into the artificial insemination of sheep first began in the early twentieth century by Ivanov in 1907 and 1912 (Ivanoff 1922). By the 1930s, protocols were well established for vaginal or 'over the rail' and cervical insemination with fresh and diluted semen (Milovanov 1938).

Unfortunately, the widespread use and uptake of AI with frozen-thawed semen was impeded by the poor fertility associated with cervical AI. Pregnancy rates dropped as low as 10-30% seemingly due to the reduced capacity of frozen-thawed sperm to navigate through the cervix (Mattner *et al.* 1969; Lightfoot and Salamon 1970b; Lightfoot and Salamon 1970a). Progress was further inhibited by the inability to penetrate the ewes' cervix with an inseminating pipette. The ovine cervical can usually only be penetrated up to a maximum of 1-1.5cm, with the remainder made up of several misaligned cartilaginous folds (Kershaw *et al.*

2005). Several have tried to overcome the physical barrier of the ewes' cervix by; forced penetration or traction (pulling the entrance of the cervix into the vagina by forceps), otherwise known as 'The Guelph method' (Anderson and Bouma 1973) or surgical incision of cervical folds (Pau *et al.* 2020). Similarly, the use of hormones such as relaxin, oxytocin, prostaglandins (Lightfoot and Salamon 1970a), the use of metabolic stimulants (Maxwell et al. 1995b), increasing the dose of spermatozoa (Lightfoot and Salamon 1970b) and doubling the number of inseminations per ewe (Salamon *et al.* 1977). While some of the above methods did improve lambing rates, logistically they were not feasible or an efficient use of labour and sperm considering large scale production. Several of these methods were also a concern for animal welfare. The focus then turned to developing techniques that enabled insemination to bypass this physical barrier, enabling successful AI with frozen-thawed ram semen.

The first report of intrauterine laparoscopic artificial insemination in sheep was by Killeen and Caffery in the early 1980s (Killen and Caffery 1982). It involves completely circumventing the cervix and depositing semen directly into the uterine horns of the ewe while sedated. Conception rates following laparoscopic AI with frozen semen were comparable to those of natural joining or insemination with fresh semen (Maxwell and Butler 1984; Maxwell 1986a; Maxwell and Hewitt 1986). Today, the protocol has largely remained unchanged and is now widely accepted as the method of artificial insemination with frozen-thawed ram semen in Australia.

1.2.2. Current Industry Practice

There are several steps involved in a successful artificial insemination program; including the collection and preparation of semen, the correct synchronisation of oestrus and nutritional preparation of the ewe, semen deposition, and the successful detection of pregnancy via transcutaneous ultrasound.

There are two methods of semen collection used throughout industry; 1), via artificial vagina in the presence of a teaser ewe (Evans and Maxwell 1987) and 2) via electro-ejaculation using electrical stimulation (Gourley and Riese 1990). Following collection, an initial assessment is performed to determine the quality of the ejaculate by analysing the colour, volume and consistency, as well as wave motion, concentration of sperm, motility and morphology (further explained in section 1.3) (Evans and Maxwell 1987). A "normal" ejaculate collected for artificial breeding programs usually contains 3500-6000×10^6 spermatozoa/mL, appear milky-white, free of contaminants, such as blood or urine (Evans and Maxwell 1987), and record a minimum wave motion score of 3 if used for fresh insemination or 4 for freezing. Suitable ejaculates processed for cryopreservation are diluted with an extender supplemented with 5% glycerol and cooled to 4ºC. Ram semen can be frozen in either pellets (0.2mL in size) containing enough spermatozoa to inseminate three ewes (>75×10^6 motile spermatozoa) or in 0.25mL PVC straws containing enough spermatozoa to inseminate a single ewe (>25×10^6 motile spermatozoa) (Evans and Maxwell 1987). Semen frozen in these forms can be stored indefinitely in liquid nitrogen at a temperature of -196ºC.

Ewes are often artificially inseminated at a synchronised fixed time of oestrus. This requires progesterone or compounds with progesterone-like activity (progestogens) to be administered for 12-14 days prior to AI. At removal, a single injection of equine chorionic gonadotropin (eCG; Minitube) is administrated subcutaneously into the ewe to induce ovulation. Androgenised teasers can also be introduced to ewes when eCG is given to help stimulate oestrus (Signoret *et al.* 1982; Ungerfeld 2012). Most ewes will enter oestrus 24-36 h later, peaking at 48h, (Walker *et al.* 1986). Laparoscopic intrauterine AI of ewes with semen is then normally performed between 48-54h post-CIDR removal (depending on whether fresh or frozen-thawed semen is used), with a minimum of 25 million motile spermatozoa deposited per ewe (Evans and Maxwell 1987).

1.3. SEMEN FACTORS THAT MAY INFLEUNCE FERTILITY FOLLOWING AI

To achieve a successful artificial breeding program, several factors must be considered. Critical to the success of AI, is the preparation of the ewe, the quality of semen used and environmental conditions before, during and after the program. Regarding semen quality, traits including sperm motility/velocity, normal morphology, membrane stability, acrosome and DNA integrity, balanced mitochondrial activity and ROS production are associated with normal spermatozoa (Figure 1.1) and subsequently fertility (Table 1.1). As such, determining potential male, female and environmental factors prior to, during and after AI help develop a cohesive understanding of factors contributing to the success of AI.

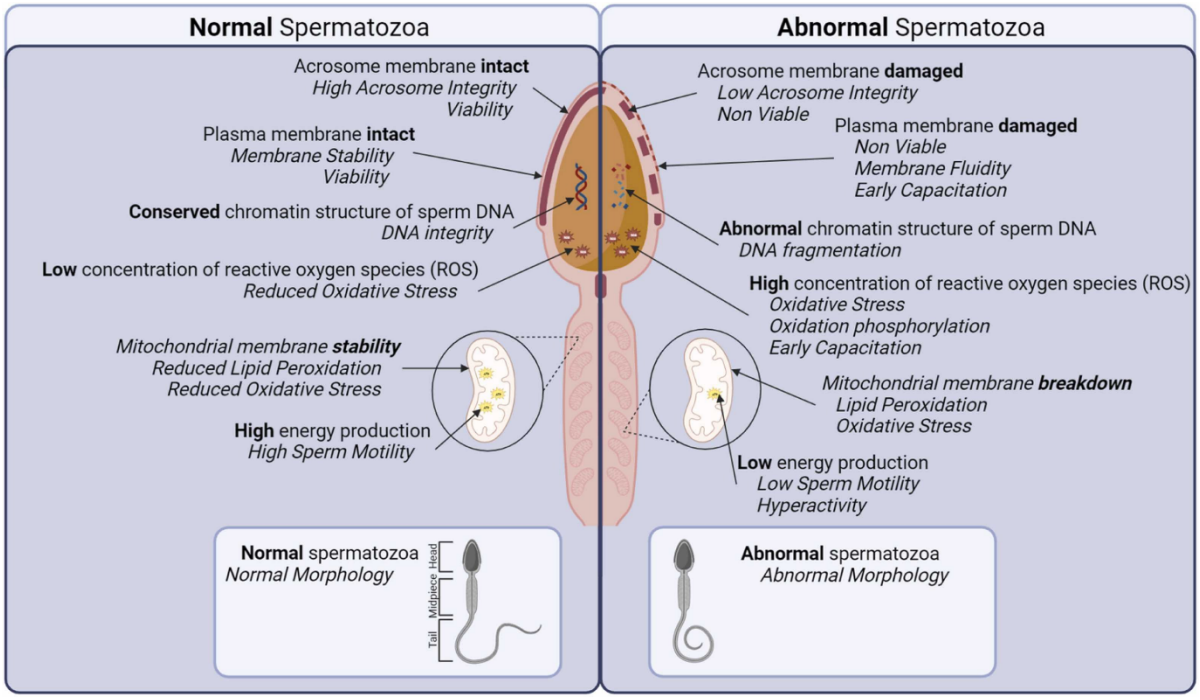

Figure 1.1: Overview of abnormal and normal spermatozoa traits.

Table 1.1: Semen parameters linked to fertility following AI in livestock.

Predictors	Species	Correlation to fertility	Citation
Sperm number per insemination dose	Bull	Correlation between the concentration of spermatozoa $2.5\pm0.2\times10^6$ after "swim-up" of the high fertility group, P=0.042, R R=0.649.	(Gillan *et al.* 2008)
	Bull	A concentration post-swim-up of 4×10^9 improved non-return rates, P=0.03.	(Januskauskas *et al.* 2000)
	Ram	$<100\times10^6$ sperm/ cervical laparoscopic AI dose decreased fertility, P=0.003.	(Macías *et al.* 2020)
	Bull	Semen diluted to $3\text{-}4\times10^6$ sperm per dose resulted in a lower calving rate post-two days of storage than semen diluted to 5×10^6 sperm per dose, P<0.01.	(Murphy *et al.* 2018)
	Ram	Lambing rates via LAI were similar for semen frozen at 200 (57.5%) or 400×10^6 sperm/mL (54.4%), but lower for 800×10^6 sperm/mL (45.5%), P<0.05.	(Alvarez *et al.* 2012)
	Ram	Lambing after LAI increased from 30.6 to 43.3% when inseminated with 90×10^6 to 180×10^6 sperm/mL, respectively, P<0.05.	(Visser and Salamon 1974b)
Morphology	Deer	The percentage of normal sperm correlated to the proportion of pregnant females, P<0.0001.	(Malo *et al.* 2005)
	Ram	The proportion of abnormal spermatozoa negatively reduced the fertility of rams. Previously identified fertility groups from LAI programs showed abnormality percentages as; fertile = 3.78%, subfertile = 22.29% and infertile = 31.78%, P<0.0001.	(Almadaly *et al.* 2019)

Predictors	Species	Correlation to fertility	Citation
	Stallion	The percentage of normal sperm correlated to the percent pregnant cycle (P<0.05, R=0.42) and the percent pregnancy first cycle (P<0.05, R=0.38).	(Love 2011)
	Bull	Correlation to pre-established fertility groups post-thaw (P=0.01, R=-0.762) and after swim-up (P=0.028, R=-0.687).	(Gillan *et al.* 2008)
	Stallion	There is a positive relationship between normal morphology and pregnancy rate post-insemination (P<0.01).	(Morrell *et al.* 2008)
	Bull	There is a positive relationship between normal morphology and pregnancy rate post-insemination (P<0.01).	(Sellem *et al.* 2015)
	Bull	Nonreturn rates were predicted by percentages of normal acrosomes and abnormal heads, P=0.017, and R=0.80.	(Ericsson *et al.* 1993)
	Ram	The proportion of abnormal spermatozoa in the subfertile group (<50% fertility) was 22.29±1.67% compared to the high group (>80% fertility) with 3.78±0.20%, following LAI (P<0.05).	(Almadaly *et al.* 2016)
Motility and kinetic parameters	Stallion	Percent total sperm motility was associated and correlated with pregnancy rates (P=<0.05, R=0.37), pregnancies per cycle (P<0.05, R=0.59) and first cycle (P<0.05, R=0.64).	(Love 2011)
	Ram	Average-path velocity (VAP), the curvilinear velocity (VCL) and the head beat-cross frequency (BCF), all positively influenced the fertility after LAI of preselected highly fertile rams (>50%), P=0.044, 0.027, 0.006, respectively.	(Del Olmo *et al.* 2013)
	Deer	Motility correlated to the proportion of pregnant females inseminate, P<0.0001.	(Malo *et al.* 2005)
	Bull	Post-thaw total motility is a significant factor in non-return rates, P=0.0014.	(Januskauskas *et al.* 2000)

Predictors	Species	Correlation to fertility	Citation
	Bull	Post-thaw subjective motility (P=0.033, R=0.672) and straight-line velocity (P=0.048, R=0.636) correlated to 56 non-return rates.	(Gillan *et al.* 2008)
	Donkey	Sperm motility variables associated (P<0.05) with an increase in the percent pregnant per cycle; motility, progressive motility, curvilinear velocity, straightness, and beat cross frequency (R=0.37, 0.53, 0.44, 0.44).	(Dorado *et al.* 2013)
	Human	The proportion of spermatozoa swimming < 20μm/second (%) reduced IVF results, P<0.005.	(Holt *et al.* 1985)
	Bull	The percentage of motile sperm is associated with variation exhibited in the 59day non-return rates, R=0.58.	(Farrell *et al.* 1998)
Viability	Bull	There is a positive relationship between viability and non-return rates, P=0.048, R=0.635.	(Gillan *et al.* 2008)
	Bull	Post-thaw viability was positively correlated to non-return rates of cows (P=0.0006, R=0.64), decreasing after swim-up (P=0.03, R=0.45).	(Januskauskas *et al.* 2000)
	Bull	There is a correlation between the percentage of non-return rates and the number of viable sperm per AI dosage (P=0.016, R=0.051) and non-return rates and viability (P=0.027, R=0.046). An increase of 1 million viable spermatozoa per dosage increased non-return rates by 0.2% (P=0.016) and increasing viable spermatozoa by 1% increased non-return rates by 0.05% (P=0.027).	(Alm *et al.* 2001)
	Ram	There is a significant (P<0.05) relationship between sperm viability and lambing percentage post cervical AI.	(Santolaria *et al.* 2015)
	Bull	The proportion of live spermatozoa was significantly (P<0.001) lower in below-average compared with average/ above-average fertility bulls. The proportion of dead spermatozoa was significantly (P<0.001) higher in below-average compared with average/ above-average fertility bulls.	(Kumaresan *et al.* 2017)

Predictors	Species	Correlation to fertility	Citation
	Bull	The proportion of live spermatozoa was significantly and positively correlated (R=0.53) to non-return and the proportion of dead spermatozoa (R=-0.53) negatively correlated to non-return rates.	(Kumaresan *et al.* 2017)
	Bull	The percentage of viability of thawed semen correlated with 56d non-return rates at bull and ejaculate R=0.412 and 0.506, respectively.	(Christensen *et al.* 2005)
	Stallion	The high fertility group exhibited higher percentages of live cells (57%) than the low fertility group (30%, $P<0.05$).	(Wilhelm *et al.* 1996)
Acrosome Integrity	Ram	There is a large difference between ram with live spermatozoa with intact acrosomes ($P<0.005$) and reacted acrosome ($P<0.001$) when ewes were cervically inseminated.	(O' Meara *et al.* 2008)
	Bull	Intact acrosomes were positively correlated to 56d non-return ($P<0.05$, R=0.322).	(Sellem *et al.* 2015)
	Bull	Live acrosome-reacted spermatozoa (R=0.50) were positively correlated to non-return rates.	(Kumaresan *et al.* 2017)
	Bull	Semen samples from above-average fertility (>103% nonreturn rate) bulls had significantly ($P<0.05$) higher live acrosome intact spermatozoa and significantly ($P<0.05$) lower dead acrosome intact spermatozoa compared with average/bell-average fertility bulls (<102% nonreturn rate).	(Kumaresan *et al.* 2017)
	Bull	The percentage of acrosome reacted spermatozoa correlated to fertility (R=0.74).	(Januskauskas *et al.* 2000)
DNA Integrity	Stallion	Negative correlation between DNA fragmentation index and pregnancy rate post-AI ($P<0.05$, R=-0.63).	(Morrell *et al.* 2008)
	Stallion	SCSA identified fertility groups, including Percentage seasonal pregnancy rates ($P<0.05$) and Percentage pregnant per cycle ($P<0.01$).	(Love and Kenney 1998)

Predictors	Species	Correlation to fertility	Citation
	Bull	Sperm DNA compaction reduced the percentage of 56d non-return rates (P<0.05).	(Sellem *et al.* 2015)
	Boar	DNA fragmentation reduced farrowing rate (P<0.01, R=-0.55) and number of piglets born (P<0.01, R=-0.54).	(Didion *et al.* 2009)
	Bull	Nuclear shape R=0.86, P< 0.05) separated the high and lower fertility groups (non-return rates).	(Ostermeier *et al.* 2001)
	Bull	The percent of DNA fragmentation was significantly (P<0.05) lower in above-average bulls compared with average and below-average fertility bulls. The proportion of DNA fragmentation negatively correlated R=0.61) to non-return rates.	(Kumaresan *et al.* 2017)
	Ram	SD DNA fragmentation (P=0.001) and MEAN DNA fragmentation (P=0.039) showed a significant negative association with 25-day non-return rate after vaginal insemination.	(Nordstoga *et al.* 2013)
	Bull	DNA fragmentation and male fertility (successful or failed insemination) correlated P<0.001, R=-0.43, -0.50 respectively.	(Karoui *et al.* 2012)
	Bull	DNA fragmentation significantly correlated with fertility results both at batch (P<0.05, R=−0.39), and bull (P<0.01, R=−0.57) levels.	(Januskauska *et al.* 2001)
	Human	Sperm with DNA fragmentation >12% did not result in pregnancy (P<0.05).	(Duran *et al.* 2002)
	Bull	There was a correlation between the sperm TUNEL index and conception rate (P<0.05, R=-0.33).	(Takeda *et al.* 2015)
Oxidative status	Bull	There is a positive relationship between non-return rates and the percentage of sperm oxidative (P<0.05).	(Sellem *et al.* 2015)
	Bull	The proportion of live and H_2O_2− spermatozoa was significantly (P<0.05) lower in below-average compared with above-average bulls (>102% nonreturn rate) and correlated to fertility groups R=0.80.	(Kumaresan *et al.* 2017)

Predictors	Species	Correlation to fertility	Citation
	Stallion	There is a positive relationship between oxidative stress and successful pregnancies (1490.2% vs. 705.5 pg/ml/24 h).	(Gibb *et al.* 2014)
Mitochondrial Membrane Status	Human	Previously divided high, moderate, and low mitochondrial potential groups measured the *in vitro* fertilisation rates to be higher in the high potential group than in the low potential group (P<0.05).	(Kasai *et al.* 2002)
	Bull	Above-average fertility bulls had significantly (P<0.05) higher MitoSOX+ and JC− sperm population compared with average and below-average bulls.	(Kumaresan *et al.* 2017)
	Boar	Boars with the longest mitochondrial sheath length had significantly higher conception rate (P=0.05) and increased number of piglets born per litter than boars with shorter mitochondrial sheaths (P=0.04).	(Kerns *et al.* 2020)
Plasma Capacitation and Membrane Fluidity	Ram	There is a distinctive difference between ram populations for membranes capacitated (P=0.04), non-capacitated (P<0.001) and dead (P<0.001).	(O' Meara *et al.* 2008)
	Bull	An unstable membrane decreased cow non-return rates (60 days after AI) (P<0.05).	(Hallap *et al.* 2006)
	Ram	Membrane integrity showed the highest correlation with fertility post LAI (R=0.415, P<0.005).	(Pérez-Pé *et al.* 2002)
Proteomics	Bull	Differences in spot intensity between accessory sex gland fluid of pre-documented high fertility and low fertility bulls was measured to be difference across the groups of IVF assays (P<0.05).	(Moura *et al.* 2007)
	Ram	Using Label-free LC-MS/MS, 997 proteins were identified, 840 were shared, 57 and 93 were specific to greater fertility (>89.4% pregnancy rate) and lower fertility (<89.4% pregnancy rate), respectively.	(Hitit *et al.* 2021)
	Bull	The seminal plasma protein PDC-109 was abundant lower conception rate groups when compared to the high conception rates (P<0.05).	(Somashekar *et al.* 2015)

Predictors	Species	Correlation to fertility	Citation
	Boar	The sperm-related transcripts, *FOS, NFATC3, EAF2, BAMBI, PTPRU, PTPN2, ND6* and *ACADM,* are potential markers for predicting the cryopreservation of boar semen.	(Fraser *et al.* 2020)
	Ram	Identified sperm motility marker ProAKAP4 to decrease ram sperm cryopreservation.	(Riesco *et al.* 2020)
	Bull	Gene SIPA1L2 was associated with pregnancy rate in bulls.	(Abril-Parreño *et al.* 2023)
	Bull	The abundance of ZNF706, CRISP2, TNP2, and TNP1 genes was significantly (P<0.05) lower in low-fertile bulls than high-fertile bulls and was correlated with conception rate (P<0.05).	(Prakash *et al.* 2021)
	Stallion	Caseins were found to be more abundant in stallion sperm during high-fertility periods, while epididymal sperm binding protein 1 and clusterin were linked to periods of low-fertility.	(Griffin *et al.* 2022)
	Boar	The level of osteopontin 70 and glutathione peroxidase 5 affected farrowing rate and litter size.	(Michos *et al.* 2021)
	Boar	High sperm levels of CRISP2 were positively correlated with the litter size (R=0.412, P=0.026), live-born piglets (R=0.421, P=0.023) and piglets per litter (R=0.381, P=0.042).	(Gao *et al.* 2021)
	Bull	Proteins C-type natriuretic peptide, TIMP-2, BSP5 and sulfhydryl oxidase were strongly related with high fertility bulls.	(Viana *et al.* 2018)

1.3.1. In Vitro Storage of Semen

The storage or preservation of semen is known to cause substantial damage to spermatozoa, particularly during cryopreservation (Watson 2000a). The dramatic changes in temperature involved in the cooling (5ºC), freezing (-196ºC) and thawing (37ºC) of semen force spermatozoa to transverse across their low critical temperature zone (of -15 to -60 degrees) in quick succession (Söderquist *et al.* 1997), inflicting sub-lethal damage and cold shock to the sperm cell. Compared to other livestock species, ram spermatozoa are more susceptible to cold shock owing to their lower cholesterol: phospholipid ratio (Darin-Bennett and White 1977). This results in a more volatile, permeable membrane, causing a loss of cellular components, increased production of ROS (Kumaresan *et al.* 2017) and an overall decrease in functionality (Sellem *et al.* 2015). Spermatozoa subjected to cryopreservation exhibit elevated metabolic rates, increased membrane fluidity and permeability post-thaw (Watson 2000a). The majority of cells also undergo cryo-capacitation (Salmon *et al.* 2017), displaying membrane reorganisation through the loss of cholesterol and polyunsaturated fatty acids. The increased oxidative stress during freezing and thawing also contributes to greater DNA fragmentation (Peris *et al.* 2004). With a greater metabolic impact, it is inevitable that the percentage of normal morphology of frozen-thawed ram spermatozoa is also reduced, contributing to an increase in the abundance of abnormal heads and midpieces, detached heads and cytoplasmic droplets (O'Connell *et al.* 2002).

The above research clearly demonstrates an effect of cryopreservation on *in vitro* sperm function and largely its impact on fertility following cervical AI. With the exception of research in Norway (Olesen 1993), the biological mechanisms behind the reduced fertility of frozen-thawed ram spermatozoa following cervical AI remains unknown. Hypotheses include specific mucus properties varying between breeds, natural versus synchronised oestrus and even sheep production methods (for a review please see (Fair *et al.* 2019)). Yet none have been linked to

the poor fertility seen in Merino ewes with frozen semen following cervical AI. In relation to laparoscopic AI, frozen-thawed sperm generally results in less pregnancies compared to fresh sperm. For example, (Lightfoot and Salamon 1970b) originally showed pregnancy rates of 38.8% with frozen and 55.8% with fresh semen following uterine insemination. Later confirmed by studies (Langford *et al.* 1979; Jabbour and Evans 1991), who all reported an impact on fertility. Remarkably, (Colas 1975), managed to record similar pregnancy rates with fresh and frozen-thawed ram sperm treatments, where the motility of spermatozoa immediately after, as well as 1 and 3 h after thawing was equal to or above 45, 40 and 30% respectively. To the best of our knowledge, these results have not been repeated with frozen-thawed sperm by other authors.

1.3.2. Sperm Concentration During Storage

The effect of spermatozoa storage concentration on fertility has been explored in a range of species including bull (Nadir *et al.* 1993), dog (Peña and Linde-Forsberg 2000) and ram (Maxwell *et al.* 1980). (D'Alessandro *et al.* 2001) assessed the influence of pre-freezing concentrations of 100×10^6 to $800x\times10^6$ sperm/mL on pregnancy when inseminated into the uterus of ewes via laparoscope. Freezing spermatozoa at 500×10^6 sperm/mL resulted in the greatest levels of sperm viability (71.2%) and acrosome integrity (71.5%). When frozen at 800×10^6 sperm/mL, all sperm parameters were negatively influenced. (Alvarez *et al.* 2012) also demonstrated that freezing concentrations of 200 and 400×10^6 sperm/mL ($20\text{-}40\times10^6$ sperm/0.1mL per insemination dose) would achieve the maximum fertilisation rate, also correlating to greater motility, plasma membrane integrity and permeability when performed on ewes. Although not examined following insemination, a study has reported ram spermatozoa were able to retain normal functioning *in vitro* at greater pre-freeze dilution rates than what are commonly used in industry. In other words, dilution prior to freezing promoted sperm viability and motility post-thaw (Leahy *et al.* 2010). Given there is no current standard

for the concentration at which ram spermatozoa is frozen, pregnancy rates could be confounded by the quality of spermatozoa used for insemination impacted by the concentration at which it was frozen.

1.3.3. Motility of spermatozoa inseminated

Sperm motility is one of the most important parameters used for the evaluation of quality (Van de Hoek *et al.* 2022). It is generally defined as the propagation of transverse waves along the flagellum in proximal-distal direction (Paoli *et al.* 2011). While much of the establishment and maintenance of sperm motility remains outside the scope of this review, the following section will summarise methods used to assess motility and compare literature which has linked motility of different sperm types to fertility following artificial insemination.

Traditionally, motility is assessed subjectively under a phase-contrast microscope with a warm stage, clean slide, and coverslip at approximately 20-50 million sperm/mL. It should be noted that motility assessments of this method are subjective and only assess a limited number of sperm (Decuadro-Hansen 2004; Christensen *et al.* 2005). When investigated in stallions, motility assessment varied up to 30-60% per assessment. As a result, the accuracy of correlations between subjective motility and pregnancy data can be limited (Morrell *et al.* 2008). In an attempt to reduce variability in assessments, computer-assisted sperm analysis (CASA) is an objective semen assessment measure that can accurately assess motility and other kinematics (Mortimer and Mortimer 2013; Palacín *et al.* 2013) using optical microscopy and 2D videomicrography. These parameters include; percentage of motile spermatozoa (TM), progressive (PM), slow and static (Mortimer and Mortimer 2013), average path velocity (VAP), curvilinear velocity (VCL) and straight-line velocity (VSL), the amplitude of lateral head displacement (ALH) and head beat cross frequency (BCF), straightness (STR) and linearity (LIN) (Mortimer 1997).

Given the importance of sperm motility to sperm function, extensive research in several species have focused on the relationship between motility as determined by CASA and fertility. Studies summarised in Table 1.1 include, bulls (Januskauskas *et al.* 2000; Gillan *et al.* 2008; Kumar *et al.* 2016), stallions (Love 2011), deer (Malo *et al.* 2005), rats (Gaddum-Rosse 1981), human (Holt *et al.* 1989), salmon (Gage *et al.* 2004) and rams (Del Olmo *et al.* 2013), These results demonstrate that as total sperm motility and average path velocity increase, the likelihood of pregnancy also increases (Januskauskas *et al.* 2000; Malo *et al.* 2005; Love 2011; Kumar *et al.* 2016). Previous studies have been able to measure a positive correlation with bull sperm motility, recording a Pearson correlation of R=0.34, R=0.37 and R=0.60, respectively (Farrell *et al.* 1998; Januskauskas *et al.* 2000; Dorado *et al.* 2013). Despite remaining significant (P<0.05) positive and linear, the R-value is low and has very little power in explaining such variation between motility and pregnancy rate. Compared to studies in the ram, a correlation between the VAP, VCL and BCF of frozen spermatozoa showed a significant (P<0.05) positive correlation with fertility (R=0.670, R=0.745 and R=0.852, respectively). This differs from studies by (García-Álvarez *et al.* 2010), who did not see any relationship between motility or kinematic parameters in ram following laparoscopic AI. Given sperm motility is strongly governed by the ability of sperm to metabolise substrates and generate ATP, as seen in Figure 1.1 (Freitas *et al.* 2017), it at makes biological sense for velocity to be related to fertility. The contradictory results in the ram studies above are unsurprising given different protocols and diluents are used for assessment. Furthermore, comparison is difficult when studies use different methods of determining fertility such as non-return rate, pregnancy rate or lambing rate. It would benefit future studies to be consistent in using pregnancy data as a measure of fertility.

1.3.4. Number of spermatozoa per insemination dose

The total number of spermatozoa within a given insemination dose is equally as important. It is calculated using the sperm concentration (sperm/mL) and the corresponding volume (mL) for insemination. When protocols were first established for the AI of ewes with ram semen, extensive research focused on the most suitable sperm dose for the type of semen used as well as site of semen deposition. Laparoscopic AI requires a smaller sperm dose compared to cervical AI, given it is deposited closer to the site of fertilisation in the uterine horns (Maxwell *et al.* 1984). Furthermore, inseminate doses should also be correct for the motility of samples, altering the sperm dose.

A study by (Visser and Salamon 1974b) has compared 90 and 180×10^6 motile spermatozoa per insemination dose and measured an increase in lambing from 25.3% to 32.0%, respectively following cervical AI. Pregnancy further increased to 56.1% when a second insemination of 180×10^6 occurred (a total of 360×10^6 sperm per ewe). Moving closer to the site of fertilisation via laparoscopic AI, (Maxwell 1986a) saw insemination of $0.5\text{-}50\times10^6$ motile spermatozoa result in lambing rates of 27-62%, with 20×10^6 motile spermatozoa yielding the highest result of 76.8% (Maxwell 1986b; Maxwell 1986a). Increasing doses above 40×10^6 motile spermatozoa per ewe did not improve lambing rates (Valee *et al.* 1987). Alternatively, previous studies have measured no difference in conception rate when motile spermatozoa doses of frozen-thawed sperm was reduced from 52.2×10^6 to 13.0×10^6 per uterine horn or compared between $80\text{-}320\times10^6$ sperm, respectively (Findlater *et al.* 1991; Eppleston *et al.* 1994). Additionally, (D'Alessandro *et al.* 2001) also failed to see a significant effect of semen dose (20, 40, 60, 80, 160×10^6 sperm/ewe) on fertility, however lambing rates did tend to decline with an insemination dose of 160×10^6. This study did not consider the variable motility and viability results of each treatment group that may have confounded the results (D'Alessandro *et al.* 2001). As such, the most successful result in laparoscopic AI of sheep is

still achieved using an insemination dose of 20 million motile spermatozoa per ewe (Maxwell 1986a).

1.3.5. Morphology of spermatozoa inseminated

The predictive value of sperm morphology was first proposed by Krueger et al. (1988), who found an inverse relationship between oocyte fertilisation and human sperm morphology. It is governed by the idea that spermatozoa must be morphologically normal to reach and fertilise an egg in a natural or artificial breeding program (García-Vázquez *et al.* 2016). The biophysical features of sperm morphology also play a vital role in the selection criterion of the female tract, mucus and cilia (physical barriers) enabling the successful transit of spermatozoa (Maddison *et al.* 2016). Morphological abnormalities can be broken down into three main groups (Figure 1.1); head deformities (pear-shaped, detached heads), midpiece (bent midpiece, proximal droplet), and tail damage (coiled tail, distal reflect), and the techniques used to measure these abnormalities can vary.

Sperm morphology can be assessed using a variety of techniques. One method involves using a smear staining technique, where 100-200 fixed sperm are stained with eosin nigrosine. Any unstained sperm remain morphologically acceptable (Malo *et al.* 2005; Almadaly *et al.* 2021). Alternatively, morphology can be measured by counting 200 sperm on a wet mount under the phase-contrast microscope (Gillan *et al.* 2008). Independent of the methodology chosen, variability and much debate remain across the industry regarding morphology classification as predictors of fertility.

The correlation of morphology and fertility has been examined in a number of species including; deer (Malo *et al.* 2005), bull (McGowan *et al.* 2002; Gillan *et al.* 2008) and stallions (Morrell *et al.* 2008; Love 2011), see Table 1.1. A vast amount of morphology work has been completed in cattle, (Johnson 1997) work explored the variety of ways in which spermatozoa morphology can be classified ranging from major defects (proximal cytoplasmic droplets,

pyriform heads, coiled tails, middle piece defects, etc.) to minor defects (distal cytoplasmic droplets, detached heads, bent tails, abnormal heads etc.) (Johnson 1997). Reports have also shown a significant negative correlation of bull sperm morphology to an adjusted estimate of fertility as 0.762 (Gillan *et al.* 2008). A study with rams (Almadaly *et al.* 2016), saw high fertile ram groups (>80% field fertility) had the lowest percentage of abnormal cells following electro-ejaculation. These results were repeated by (Almadaly *et al.* 2019) who further saw morphology abnormalities correlated to males which recorded pregnancy rates as low as 19.17% lambing rates. Considering the process of AI, it raises questions as to whether factors including motility and morphology are as important if spermatozoa are deposited within the uterine horns, bypassing female selection mechanisms. Today, a frozen-thawed sample will not pass commercial semen assessment standards, if it records abnormalities greater than 15% (Evans and Maxwell 1987) however, it would be interesting to further allocate abnormalities into subsections. Determining whether there are any relationships between specific abnormalities and fertility could help determine the minimum requirements for sperm morphology for successful AI.

1.3.6. Viability of spermatozoa inseminated

Viability refers to the condition of the plasma membrane surrounding spermatozoa. Responsible for maintaining homeostasis within the cell, spermatozoa must be viable to successfully transport male gametes to the site of fertilisation, bind to and penetrate the zona pellucida, and fuse with the oocyte during fertilisation (Januskauskas *et al.* 2000). The outer membrane of a sperm cell is made up of a permeable phospholipid bilayer. A spermatozoa becomes abnormal when its membrane becomes damaged (Figure 1.1), resulting in the ability to take up fluorochromes (Resli *et al.* 1983; Kumar *et al.* 2016). This can, therefore, be used to objectively measure the viability of cells by calculating the amount of fluorescence emitted,

(Gillan *et al.* 2005). These assays can be done subjectively using a fluorescent microscope or objectively using flow cytometry.

Flow cytometry has been used to assess the link between sperm viability and fertility in several species (Table 1.1). There has been only one study that has been able to quantify a viability threshold to increase 56d non-return rates when inseminating dairy bulls, including a viability score of 75% for fresh semen combined with a post-thaw threshold of 40% (Christensen *et al.* 2005). At these thresholds, non-return rates would increase by 0.32 percentage units, but 18.7% of the semen batches would be rejected (Christensen *et al.* 2005). Bull sperm viability was also positively correlated to an adjusted estimate of bull fertility by (Gillan *et al.* 2008), recording an R-value of 0.635. Despite using a small sample size, (Wilhelm *et al.* 1996) when assessing stallion sperm viability using propidium iodide (PI) saw the percentage of live cells matched to stallions in the high fertility group. Similarly, (O' Meara *et al.* 2008) highlighted similar trends in ram spermatozoa following cervical AI. Future investigations into laparoscopic AI to understand better how the viability of ram spermatozoa influences AI success.

1.3.7. Capacitation and membrane fluidity of spermatozoa inseminated

The capacitation of spermatozoa refers to the chemical process that allows the sperm to attain the final step of maturation, penetrate and fertilise the oocyte (Yanagimachi *et al.* 1994). As spermatozoa enter the oviduct, they swim out of seminal plasma, shedding off decapacitation factors (Talevi and Gualtieri 2010), initiating changes on the sperm membrane. Beginning cascading events (Gadella 2008) involving the increased transportation of calcium across the cell membrane (Lishko and Mannowetz 2018) and seeing an increase in ROS levels (Figure 1.1) (de Lamirande and Lamothe 2009). This activates signal transduction to induce hyperactivity of spermatozoa.

Capacitation can be measured by assessing the amount of calcium-mediated changes using fluorescent antibiotic chlortetracycline (CTC) (Gillan *et al.* 2005). Designed by (Thundathil *et al.* 2000), cells are counted under a phase-contrast microscope represented by CTC scanning patterns outlined by (Fraser *et al.* 1995) When the CTC is neutral, it will impeach the cell membrane, non-reactive to intracellular free calcium (Thundathil *et al.* 2000). When negatively charged, the CTC will bind with calcium, producing fluorescent patterns including non capacitated (F-), capacitated (B-), and acrosome-reacted (AR-) patterned spermatozoa (Thundathil *et al.* 2000). As summarised in Table 1.1, greater unstable membranes as measured by CTC reduced non-return rates in cows (Hallap *et al.* 2006). Unfortunately, this assessment is limited by the variability and subjective nature of the CTC results.

Alternatively, (O' Meara *et al.* 2008) measured the membrane fluidity by the change in phospholipid scrambling. Using a hydrophobic stain, merocyanine 540 (M540) alongside YO-PRO-1 (Langner and Hui 1993), an increase in phospholipid scrambling can suggest that capacitation has occurred to quantify plasma membrane stability. Previously, results have showed capacitated spermatozoa could be correlated to the number of dead spermatozoa (Table 1.1), indicating a reduction in fertility potential (O' Meara *et al.* 2008). Despite this, the results have been unsuccessfully in determining a threshold of capacitated sperm that will reduce fertility.

When capacitation occurs, the production of phosphorylation of tyrosine is induced. Spermatozoa require tyrosine phosphorylation of the plasma membrane to bind to the zona pellucida and aid the acrosome reaction (Naz and Rajesh 2004). The tyrosine phosphorylation of proteins has been associated with sperm capacitation in several species, but it remains unclear for rams in relation to fertility. A study on rams (Pérez-Pé *et al.* 2002) proved that cold shock induces protein tyrosine phosphorylation alongside decreasing plasma membrane integrity. The addition of seminal plasma proteins before cold shock not only improved sperm

survival but also promoted a decrease in protein tyrosine phosphorylation (Pérez-Pé *et al.* 2002). This suggests a potential for tyrosine phosphorylation as measure the capacitation of spermatozoa, impact of cryopreservation and *in vivo* fertility. Having the ability to screen vulnerable sires for cryo-capacitation post-thaw, could help reduce the risk of failed AI problems.

1.3.8. Acrosome integrity of spermatozoa inseminated

Acrosomal enzymes are released following capacitation as part of the acrosome reaction, enabling penetration of the zona pellucida and oocyte fertilisation (Abou-Haila and Tulsiani 2000; Januskauskas *et al.* 2000). The acrosome integrity of spermatozoa is measured using plant lectin-labelled fluorescent probes, Pisum sativum agglutinin (PSA) and Arachis hypogaea agglutinin (PNA) (Gillan *et al.* 2005). Both are capable of binding to a damaged and intact acrosome (Figure 1.1), respectively and was first used by (Maxwell and Johnson 1997). To mimic the accumulation of lysophospholipid (inducing an acrosome reaction) in boars, when measured by a flow cytometer they observed an increase in FITC-PSA. Alternatively, to assess the number of intact acrosomes using flow cytometry, (Januskauskas *et al.* 2000) followed protocols using PNA-FITC. Any fluorescence detected will indicate a damaged acrosome, as PNA targets the inner leaflet of the outer acrosomal membrane and cannot bind to an intact membrane. The study by (Januskauskas *et al.* 2000) saw the above-average fertility bulls to have greater acrosome intact bull spermatozoa when correlated to cow non-return rates (Table 1.1), accounting for much of the variability in bull fertility. A fluorescent triple stain combining phycoerythrin-conjugated peanut agglutinin (PE- PNA)/SYBR-14/PI used by (Sellem *et al.* 2015) showed the percent of acrosome intact sperm correlated with fertility when assessed on bovine (Table 1.1). Of specific interest to rams, O'Meara et al. (2008) found no relationship between acrosome integrity and *in vivo* fertility, despite significant variation between rams with intact and reacted acrosomes when cervically inseminated.

1.3.9. Oxidation status of sperm inseminated

Reactive Oxygen Species (ROS) are continually produced within the mitochondria during cellular metabolism to create energy following the enzymatic reduction of oxygen (Ciftci *et al.* 2009). Oxidative and antioxidative systems are in place to combat excessive production levels of ROS (Forman and Torres 2001; Tremellen 2008). Semen cryopreservation is one of the leading causes of ROS production (Qamar *et al.* 2020). Previous research has found that if seminal plasma is removed during the freezing process, spermatozoa can overproduce oxidative-free radicals that contribute to the modification of cellular proteins and lipid structure, disenabling cellular metabolism (Newsholme *et al.* 2016). As such, spermatozoa undergoing oxidative stress result in a cascade of events that decrease viability, morphology, motility, and membrane integrity of sperm (Figure and Table 1.1) (Cassani *et al.* 2005; Tremellen 2008), consequently reducing the fertilisation capabilities of spermatozoa.

Using flow cytometry, a combination of Bodipy and PI mixture in a sperm suspension can quantify the abundance of membrane lipid peroxidation as an indirect measure of oxidative stress. Measured by reacting the stain with free radicals, emissions from the cells will shift from red (non-oxidised cells) to green (oxidised). Similarly, by using a MitoSOX red stain in combination with SYTOX, Green (Aitken and Fisher 1994; Aitken *et al.* 2012) measured the intracellular generation of superoxide radicals within the mitochondria in humans by penetrating spermatozoa with a damaged plasma membrane (cell viability). Other stains described by (Martínez-Pastor *et al.* 2010) saw (Del Olmo *et al.* 2015) included a combination of PI and YO-PRO-1 to quantify cell apoptosis. As a direct consequence of ROS, cells with membrane damage will ultimately die, as they cannot maintain homeostasis within the cell. A final stain to measure ROS production includes H_2DCFDA (Martínez-Pastor *et al.* 2010), targeting hydrolysing and oxidising events within cells.

A surprising positive relationship was discovered by (Gibb *et al.* 2014) between stallion fertility and oxidative stress, by which sperm with greater oxidation phosphorylation levels result in greater pregnancies. The authors proposed that fertile spermatozoa produce high levels of oxidation phosphorylation and oxidative stress is a by-product of hyperactive mitochondrial activity (Gibb *et al.* 2014). Lipid peroxidation was positively correlated to motility and average path velocity in the stallions (Gibb *et al.* 2014). In bovine, however, the overproduction of ROS will consequently reduce motility, inhibiting survival and fertilisation (Sellem *et al.* 2015). Although it remains apparent that oxidative stress is a vital metabolic parameter of ram sperm, such research is yet to be conducted to look at the relationship between oxidative stress and fertility of ram sperm. As such, the industry requires the ability to determine the threshold for intracellular ROS production to explain and predict successful AI results.

1.3.10. DNA integrity

Sperm DNA integrity is defined as the abnormal chromatin structure of sperm DNA predisposed to acid-induced denaturation *in situ* (Morrell et al. 2008). It can be assessed by a number of different assays including the; Sperm Chromatin Structure Assay (SCSA) (Evenson *et al.* 1999), Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) (Sharma *et al.* 2013), Single-cell gel electrophoresis (COMET) (Kodama *et al.* 1997), sperm chromatin dispersion (SCD) test or (HALO) (Frydman *et al.* 2008) and finally Chromomycin A3 (CMA3) (Manicardi *et al.* 1995).

The most well-known and commonly used assay is SCSA. It applies a low pH treatment which produces denatured cells. These are then measured by SCSA and acridine orange (AO) (Evenson and Jost 2000). Designed by (Darzynkiewicz 1990), the degree of chromatin integrity is commonly analysed by flow cytometry, capturing the shift from green (stable, double-stranded DNA) to red (denatured, single-stranded DNA (Morrell *et al.* 2008). The SCSA method has been used to examine the correlation of DNA integrity with fertility (both under

natural and artificial insemination conditions) in multiple species, including ram (Del Olmo *et al.* 2015), human (Evenson *et al.* 1999), bull (Januskauska *et al.* 2001; Gillan *et al.* 2008; Sellem *et al.* 2015), stallion (Love and Kenney 1998; Morrell *et al.* 2008), canine (Hesser *et al.* 2017) and boar (Didion *et al.* 2009). To date there is no strong evidence linking DNA integrity to ram sperm fertility, at least in studies using SCSA on samples from pre-determined fertility groups (Table 1.1).

The TUNEL assay has been studied intensely in human DNA, (Duran *et al.* 2002) found DNA fragmentation to be greater in couples experiencing unexplained pregnancy loss (Table 1.1) (Duran *et al.* 2002). It has also been used in some livestock species, namely bulls (Takeda *et al.* 2015), however yet to be explored in rams.

The Halo Sperm assay test is designed, so that sperm with fragmented DNA is unable to produce dispersed DNA loops observed in sperm with non-fragmented DNA, following acid denaturation and removal of nuclear proteins (Fernandez-Capetillo *et al.* 2003). It has been used predominantly in human studies (Table 1.1) to demonstrate both a significant (Bounartzi *et al.* 2016; Al Omrani *et al.* 2018) and insignificant (Anifandis *et al.* 2015; Le *et al.* 2021) correlation between sperm DNA fragmentation and pregnancy outcome, suggesting further investigation is required.

In relation to the Chromomycin A3 (CMA3) test, spermatozoa with normal chromatin integrity (CMA3 negative) display faint fluorescence compared to CMA3 positive sperm with weakened chromatin packing. Yet to be correlated to livestock field fertility, studies found that CMA3-positive sperm links to fertility in human IVF trials (Iranpour *et al.* 2000; Nasr-Esfahani *et al.* 2001; Gu *et al.* 2009; Lazaros *et al.* 2011).

While literature suggests that poor sperm DNA integrity is linked to infertility, the lack of significance in the ram is likely due to variability in the susceptibility of sperm to DNA damage (Evenson *et al.* 1999). Spermatozoa are reported to have tightly packed chromatin

(Figure 1.1), suggesting they are relatively resistant to DNA damage, even after freezing (Peris-Frau *et al.* 2021b; de la Iglesia *et al.* 2023). This presents a limitation to subsequent studies, as to assess DNA fragmentation, a threshold must be met with the use of a positive control (Peris-Frau *et al.* 2021a). It is important that assays used to assess DNA integrity as a predictor of fertility, need to be relatively cost and time effective given the large sample size needed to accurately detect significance. Many of the assays listed above, although sensitive to DNA fragmentation are not viable for large operations.

1.3.11. Proteomics and transcriptomics of spermatozoa

Over the course of the review, it is apparent that the predictive values of some sperm characteristics on fertility are low, which has led to the investigation of fertility biomarkers, through both the use of proteomics (the study of protein expression) and transcriptomics (the study of RNA transcripts produced by the genome which regulate expression, function, and regulation of genes). 'Omic' technologies can qualitatively and quantitatively identify biomolecules within a cell.

The use of transcriptomics is a relatively new diagnostic tool at least in the investigation of livestock sperm fertility. Of note, is the exploration of molecular mechanisms related to spermatogenesis which have led to the discovery of small noncoding RNAs (sncRNA) acquired through interactions with extracellular vesicles in the epididymis (Salilew-Wondim *et al.* 2020). MicroRNA (miRNA) have also emerged to influence the endometrial milieu within the uterus, altering embryo development, implantation, and the maternal recognition of pregnancy in pigs (Kaczmarek *et al.* 2020). In bulls, (Sellem *et al.* 2021), observed the expression of sncRNA (miRNA, rsRNA and tsRNA) to increase along the epididymis, suggesting a biological role for sperm maturation and attainment of fertility. Some studies have even begun linking expression of specific genes to high and low fertility in bulls (Prakash *et al.* 2021; Abril-Parreño *et al.* 2023) and pigs (Álvarez-Rodríguez *et al.* 2020; Fraser *et al.* 2020). Here, authors

identified several differentially expressed RNA present in spermatozoa which could be used as non-invasive molecular markers for predicting fertility. For example, the transcriptional abundance of ZNF707, CRISP2, TNP2 and TNP1 in sperm has been linked to impaired oxidative phosphorylation in low fertility bulls (Prakash *et al.* 2021), while micro RNA's miR-221 and mir-621 has been linked to high-fertility pig sperm (Álvarez-Rodríguez *et al.* 2020). In sheep, transcriptomes have been compared environments (Dalrymple *et al.* 2015) and breeds (Hodge 2021) but none have conclusively linked transcript expression to sperm fertility following AI. Given the success noted in other species, this is a gap in the research that must be addressed. On the other hand, the use of proteomic data to predict fertility has been suggested to be more accurate than transcriptomics since distinct genes are expressed in a cell-dependent manner, and mRNA is not always translated to a protein (Kovac *et al.* 2013).

Proteomics has been applied to study a wide range of gametes (oocyte, sperm, and embryo), and fluids (epididymal, oviductal and seminal plasma) in a range of mammalian species. Many of these studies compare different phenotypic sperm types such as fresh versus frozen, to link expression to function. Specific to ram spermatozoa, (Rickard *et al.* 2015), isolated proteins from spermatozoa associated with freezing resilience and (Pini *et al.* 2018) measured the protective effect of the binder of sperm or BSP proteins during cryopreservation (Table 1.1). Additionally, (Riesco *et al.* 2020) was successful in identifying a novel biomolecule related to sperm motility (ProAKAP4) and saw a significant decrease in ram sperm expression following freezing. Nonetheless, these studies still lack any correlation to field fertility. Summarised in Table 1.1, proteomic biomarkers have been correlated to fertility traits in other livestock species such as; cattle (Viana *et al.* 2018), pigs (Gao *et al.* 2021; Michos *et al.* 2021) and stallions (Griffin *et al.* 2022). More recently, advanced proteomic tools have been applied to spermatozoa to deepen our understanding of differences in the proteome of fertility phenotypes. Intact cell matrix-assisted laser desorption/ionisation time-of-flight

(MALDI-TOF) – mass spectrometry (MS), has been used as a molecular test to diagnose male infertility in chickens (Soler *et al.* 2020). Soler et al. (2016) identify proteins related to energy metabolism, structure, and movement between fertile/subfertile male chickens (Soler *et al.* 2016). Except for bovine oocytes, this technique has yet to be applied to spermatozoa from other species and holds tremendous potential for identifying unknown biomolecules possibly linked to the fertility of ram spermatozoa following AI. This will allow producers to optimise reproductive management whilst maintaining high productivity through good selection and optimal gamete handling protocols.

1.4. FEMALE FACTORS THAT MAY INFLUENCE FERTILTY FOLLOWING AI

1.4.1. Age of the ewe

Fertility and fecundity of ewes vary with age. There is a linear trend in which fertility increases from ewes lambs to yearlings. (Hulet and Price 1975; Gaskins *et al.* 2005). Generally, ewe fertility will gradually increase until the age of 6, then will fall with increasing age (Laster *et al.* 1972; Shorten *et al.* 2013). Previous research, shows that ewe age affects pregnancy rates (Laster *et al.* 1972, Kleeman et al. 2005; Gaskins *et al.* 2005), twinning (Narayan *et al.* 2021), ovulation (Shorten *et al.* 2013) and litter size (Hanrahan 1982; Shorten *et al.* 2013; Narayan *et al.* 2021). (Anel *et al.* 2005) observed ewes to have greater fertility following laparoscopic AI, between 1.5 and 4.5 years of age, calculating lambing rates to decrease by 2.07% per year thereafter. (Narayan *et al.* 2021), also saw 5-year-olds record a significantly greater pregnancy rate than 6-, 4- and 3-year-olds, following laparoscopic AI. Both these papers remain consistent with the data on natural joining programs, however, it remains unclear how the relationship between age and fertility is impacted by other variables. For example, do ewes aged between 2 and 5 years old have a better primed reproductive tract for fertilisation, gestation, and parturition? Or do they respond better to the exogenous hormones used for oestrus synchronisation, resulting in more successful AI pregnancies?

1.4.2. Nutritional status of the ewe

It is well understood that the nutritional status of a ewe will directly affect their reproductive performance (Meikle *et al.* 2018). Nutritional partitioning is regulated by endocrine signals, which in turn, modulates reproductive functionality and subsequently fertility (de Brun *et al.* 2020). More specifically, the metabolic status of the ewe prior to pregnancy is crucial to provide a suitable environment for conception and early embryo development (Abecia *et al.* 2006). As such, the plane of nutrition leading up to AI as well as body weight, condition and internal fat levels of ewe can all contribute to the success or failure of an AI program.

1.4.2.1. Plane of nutrition

The influence of level of nutrition prior to and during mating of ewes and its effect on ovulation and lambing percentage has been under investigation over many years. Briefly, a rising level of nutrition prior to conception sees an increase in fertility (Bennett *et al.* 1964; Coop and Clark 1969; Edey 1970). Similarly, by improving nutrition during late pregnancy research has shown the plane of nutrition is critical to avoiding pregnancy toxaemia of the ewe, and contribute to important factors including birth weight, survival, and growth (Wallace 1948; Foote *et al.* 1959; Coop and Clark 1969; Cloete 1990). Of note, these studies are performed under natural mating conditions in various breeding times, thus, future research should focus on comparing reproductive performance in artificial breeding programs.

The type of feed given is also important. Well recognised is the use of lupins as a feed supplement in ewes. (Brien *et al.* 1977; Cloete 1990) both demonstrated that although ovulation rates increased, there was no evident change on pregnancy rate. (Brien *et al.* 1977) also compared irrigated pasture and hay supplements which measured no change in ovulation or embryo survival despite lower insemination rates. Research showed supplementation pre-mating has little effect of liveweight and body conditioning however can improve reproductive performance when pasture availability decreases (Gunn *et al.* 1992a; Gunn *et al.* 1992b). As

such, its critical for producers to consider the type of supplementation when seasonal conditions have limited the availability of natural pasture and the impact not only on pregnancy rates but pregnancy survival to full term.

1.4.2.2. Bodyweight of a ewe

Whether considering a natural or artificial breeding program, Body Condition Score (BCS) underpins a ewes' assessment of their nutritional status and health. BCS throughout research, has acted as the gold standard for ewe fertility assessment. Research indicates that a ewe BCS of 2.5-3.5 is considered "fertile" and ready to breed (Cranston *et al.* 2011; van Burgel *et al.* 2011). Being a subjective assessment, however, BCS lacks standardisation and cannot reflect the ewe's internal condition (Summers *et al.* 2012). Alternatively, another way to quantify an ewe's nutritional status is the live weight (measured in kilograms). In nearly all studies of merino sheep, live weight has been a successful indicator of reproductive growth and maturity (Foster *et al.* 1985; Rosales Nieto *et al.* 2013b). Previous research suggests that earlier puberty (Rosales Nieto *et al.* 2013a), ovarian stimulation (Avdi *et al.* 2003), body condition score, longer postpartum in anoestrus (Wright *et al.* 1990) and reduction in the ram effect (Todorov and Nedelkov 2015) have all been positively correlated to live weight (Debus *et al.* 2022). (Edey 1968) found mature ewes experience a 5% increase in ovulation with a 2.5kg increase in liveweight. As well (Cahill and Blockey 1974) observed a change in liveweight and ovulation rate in maiden ewes, differing between breed and season. Whereby ovulations increased from a liveweight of 27kg (Cahill and Blockey 1974).

1.4.2.3. Visual intra-abdominal fat scores of ewes

A novel parameter and possible indicator of ewe fertility is the measure of internal fat within the abdominal cavity of the ewe, otherwise known as intra-abdominal fat. Like uterine tone, intra-abdominal fat is assessed by the technician performing AI. Subjectively assessed from 1 (little to no fat present in the abdominal region) to 5 (extensive amount of fat present in the

abdominal area). Research into the role of intra-abdominal fat on pregnancy success in sheep is limited and, as such, opens a potential pathway for explaining variability within an AI breeding program. Breeding programs using over-conditioned animals, have shown that excessive fat mobilisation can cause fatty infiltration of the liver and fatal ketosis after a feeding reduction period. Large amounts of intra-abdominal fat in ewes can further limit free space in the abdomen for the rumen to expand to receive a high-fibrous diet (Kerr 2018), consequently making them unable to ingest the calories needed to support pregnancy and continued health. Given much focus has revolved around the role of body condition and body weight of ewes, it would be interesting to examine whether body condition score, body weight and intra-abdominal fat scores are correlated and have an overall effect on fertility following AI.

1.4.3. The synchronisation of oestrus in the ewe for AI

Oestrous synchronisation is vital for any successful AI program. Without cycling ewes, even if the most fertile spermatozoa are deposited within the reproductive tract, the ewe will not fall pregnant. As explored in section 2.2, the most common method of oestrus synchronisation is the use of progesterone CIDRs (12-14 days) followed by a single dose of eCG, however other protocols have been shown to influence synchrony thus could contribute to variation in resulting pregnancy rates.

To determine the effectiveness of CIDRs and fluorogestone acetate sponges, (Walker *et al.* 1989) reviewed the ability of each to induce the preovulatory lutenising hormone (LH) surge and ovulation. Effectively reviewed in Table 1.2, CIDRs saw an earlier response and ovulation compared to sponges, 55 and 60 h, respectively. Moreover, (Fukui *et al.* 1999) showed that CIDR's produced less variation around the time of ovulation and a greater tendency for greater conception rates, 69% compared to 58.6% when using other intravaginal devices. More recently (Swelum *et al.* 2015) measured pregnancy, fertility, twinning rate and fecundity to be significantly greater in the CIDR group when compared to sponges as did

(Santos-Neto *et al.* 2015). Conversely, (Ainsworth and Downey 1986; Wheaton *et al.* 1993) reported the percentage of ewes lambing and twinning rate, to be similar between sponges and CIDR-treated ewes. While (Wilson and Maxwell 1989), saw FGA sponges return a greater pregnancy result than CIDRs in Autumn (54.7 v 67.4%), but not spring (59.2 v 56.2%), suggests a seasonal impact on effectiveness of oestrus synchronisation. Then again, (Hill *et al.* 1998) saw no difference between pregnancy rates when inseminated during the months of March, April and May compared to the summer months. The type of progestogen used could also be a reason for the variability. (Hill *et al.* 1998) saw a lower conception rate when using medroxyprogesterone acetate (MAP; 64.6%) compared to flurorogestone acetate (30mg FGA; 74.7%) and CIDR (71.7%). Within Australia, the limited availability of sponges has meant most of the sheep industry relies on CIDRs for oestrous synchronisation. CIDRs are more accessible, quick to insert, and a more efficient form of progesterone (Wheaton *et al.* 1993). Nevertheless, it is important to note that protocols must be adapted depending on the progesterone used and the form of administration.

Subsequent research has continued to determine the impact of a single gonadotrophin injection to induce ovulation (Evans and Maxwell 1987; de Graaf 2010) and subsequent fertility as per Table 1.2. Equine chorionic gonadotropin (eCG) studies in sheep have observed a positive effect of the administration of eCG post the removal of the progesterone pessary on an increase in ovulation rate (Langford 1982; Walker *et al.* 1986; Naqvi and Gulyani 1998), conception and lambing rates (Langford 1982; Swelum *et al.* 2015). (Hill *et al.* 1998) explored the effect of eCG doses and saw a greater pregnancy rate following administration of 250 IU (72.9%) or 300 IU (79.1%) compared to 200 IU (62.4%). A greater eCG dose at CIDR removal does normally lead to a super-ovulatory effect or increase in proliferation, measured as twins and triplets (Quintero-Elisea *et al.* 2011). Traditionally there were also concerns over the length of progesterone exposure and the impact on sperm transport in the uterus (Quinlivan and

Robinson 1969; Hawk 1973; Evans and Armstrong 1984), therefore other methods were considered such as the use of prostaglandin 2-alpha (PGF2α) in conjunction with progesterone pessaries and eCG as well as a single dose of Gonadotrophin Releasing Hormone (GnRH).

Administering GnRH at the expected time of oestrus onset or just before the luteinising hormone (LH) surge such as 36 h after removal of progesterone device (Nancarrow *et al.* 1984; Eppleston *et al.* 1991) did not demonstrate any improvement in the number of pregnant ewes compared to current protocols (Fukui *et al.* 1985), despite seeing improved synchronisation of ewes (Smith *et al.* 1986). It is believed to have a strong feedback mechanism on the follicle and oocyte, tightening the window between LH surge and ovulation within the flock, reducing biological variation between animals. Since GnRH has been used in AI programs which have used compromised or very low doses of spermatozoa (1×10^6 sperm/dose), such as that involved in sex-semen programs (Hollinshead *et al.* 2003; de Graaf *et al.* 2007) or in multiple-ovulation embryo transfer (MOET) programs (Walker *et al.* 1986; Ryan *et al.* 1992), where timing is critical to success. Limited authors have reported success or benefits using GnRH in non-super ovulated ewes using standard fresh or frozen-thawed ram spermatozoa.

PGF2α can be administered, usually twice 7 days apart to induce luteal regression (Boland *et al.* 1978). Following luteolysis of the corpus luteum, the reduction in progesterone levels is thought to initiate the follicular phase of the cycle. Unfortunately, it has not been successful due to poor fertility and embryonic loss (Hackett *et al.* 1981) when compared to standard synchronisation protocols with progestogen, progesterone and eCG (Olivera-Muzante *et al.* 2011). Other studies, however, have shown no impact on fertilisation (Gonzalez-Bulnes *et al.* 2005), whilst other have explored the efficiency of administering PGF2a in combination with the ram effect.

1.4.3.1. The use of teasers to induce and detect oestrus

In addition to the use of exogenous hormones, the use of androgenised wethers (wethers given testosterone supplements) or vasectomised rams (rams who still produce pheromones but are infertile) have been used to help induce the onset of oestrus in ewes for an AI program. The 'ram effect' first described by (Underwood 1944) has been used to hasten puberty, induce oestrus during seasonal anoestrus, modify the LH surge (Lindsay *et al.* 1975) and induce ovulation in sexually quiescent females (Martin *et al.* 1986), as mentioned in Table 1.2. Others have reported it to have a minimal effect on ewes ovulated, with many regressing prematurely and ewes displaying no oestrus behaviours (Signoret *et al.* 1982). There are also several factors thought to contribute to the ewe response including, stress, age, breed, time of anoestrus and sexual experience (reviewed by (Fabre-Nys *et al.* 2015)).

In relation to their use during an AI program, teasers have been introduced at various stages in the cycle at different concentrations (6% of teasers to number of ewes in the mob) to improve oestrus synchrony. (Evans *et al.* 2004) observed that ram exposure during the last three days of sponge treatment advanced timing of oestrus and ovulation but decreased fertility, possibly due to ewes being in late oestrus or having poor oocyte quality at the time of mating. This was confirmed by (Hawken *et al.* 2005) as well as able to improve number of viable embryos collected and number of ewes lambing by introducing rams 36 h post-sponge removal. Vasectomised rams introduced simultaneously with the second dose of PGF2a have advanced the onset of oestrus and increased the proportion of ewes which responded to synchronisation compared to ewes which were not exposed to rams. This is not a substitute for application of PGF2a, as the presence of teasers alone was not strong enough to initiate an oestrous response (Ungerfeld 2011). Nevertheless, when considering a laparoscopic AI program with fresh or frozen ram semen, by exposing ewes to vasectomised rams or androgenised wethers at progesterone pessary removal, following eCG administration, will result in acceptable fertility.

Reported by (Maxwell *et al.* 1984; Maxwell and Barnes 1986), this protocol is regularly used today. It is necessary to use at least 4% vasectomised rams or androgenised teasers at the time of pessary removal. Ewes must then be observed closely over the next 48h, marked, and drafted off. This observation period can be time-consuming and labour-intensive with excessive handling of ewes. Given markings can rub onto other ewes, this can likely contribute to variable success rates and impact on fertility, if relied upon to detect and determine time of insemination.

1.4.3.2. Uterine tone as a marker of oestrus in ewes

Determining oestrus in the ewe externally presents its own challenges. An area lacking research on how the internal conditions of the ewe can suggest synchronisation success and response to exogenous hormones. Practically, uterine tone is subjectively assessed by the technician performing AI, classifying tone from 1 (flaccid and pale) to 5 (turgid and pink). During the oestrus cycle, multiple changes occur within the uterine tissues due to hormone response, placental growth, and preparation for birth (Cosson 2013; Akbulut and Çelik 2021). Altering its tone, shape, and form (Spencer *et al.* 2004; Cosson 2013). (Griffin *et al.* 1992) measured a direct enhancement of uterine tone in mares that may have resulted from a diestrus surge in oestrogens within the bloodstream in preparation for follicular development and fertilisation. Uterine tone in nonbred and pregnant mares peaked at Days 6 and 13 after ovulation. Similarly measured in cattle, comparing the uterine tone of cows mated or non-mated (Bonafos *et al.* 1995) and sheep experiencing dystocia (reviewed by (Jacobson *et al.* 2020)). Limited by the fact that only a few studies have considered uterine tone as a marker of oestrus and uterine health and impact on AI success.

There are logistical and welfare challenges around studying uterine tone or the impact of tone on pregnancy success given it requires observing the internal organs of the animal. The procedure of inseminating ewes is a useful model given a laparoscope is already used and

enables accurate visualisation of the uterine horns and ovaries. Commercially this is not practical which limits its use across the industry. Nevertheless, future research would help understand how the ewe's uterus changes following oestrus synchronisation, time of ovulation and thereby improve the success of AI.

1.4.4. Timing of insemination

The timing of insemination is pivotal to achieve fertilisation (Evans and Maxwell 1987). For AI to be successful, the deposition of semen into the female reproductive tract must occur early enough to facilitate sperm transport to the site of fertilisation but also late enough that preserve the viability and survivability of spermatozoa. Generally, ewes are expected to enter oestrus within 36-48h following pessary removal and will ovulate 60 h post-pessary removal (Maxwell and Butler 1984; Walker *et al.* 1986).

The time of insemination or deposition of semen will depend on several factors including the type of oestrus (natural vs synchronised), the method of oestrus synchronisation, the type of AI and the type of semen used. As the type of oestrus and method of synchronisation will affect ovulation time, and the method of insemination and sperm type will influence sperm survivability and transport, all these factors will consequently impact the success of AI to varying degrees.

Despite the traditional belief that synchronisation protocols saw a decrease in sperm transport through the female tract, there are no significant differences in fertility between ewes inseminated to a natural or synchronised oestrus (Donovan *et al.* 2004b). With the exception in Norway, where inseminating frozen sperm to a natural oestrus can result in greater cervical AI results (Olesen 1993). Nevertheless, when inseminating to a natural oestrus, the use of teasers becomes paramount. Traditionally, ewes marked by a raddled teaser in the morning would be drafted off and inseminated 12h later (Maxwell 1986b). This method is achievable when performing cervical AI given the producer can perform the procedure themselves with

limited equipment. Conversely, there are even a few reports of inseminating frozen semen via laparoscopy to a natural oestrus, with varying levels of success varying from 42-80% pregnancy rate (Takenaka 1985; Azzarini and Valledor 1988). Compared to the sheep industry with Australia, this method is not feasible given the size of flocks to be inseminated and the extensive nature of Australian sheep production. As such, most of the industry utilises fixed-time AI to a synchronised oestrus. This enables the bulk of the flock to be inseminated at the same time, creating a tighter lambing window. Fixed time AI can differ depending on the type of pessary used for synchronisation (CIDR vs sponge) and location of semen deposition. Most research in the 60-80s used sponges, however few studies have repeated this research with CIDRs over recent years.

As summarised in Table 1.2, when using fresh semen, (Maxwell 1986b) cervically inseminated a sperm dose of 0.1mL (or 100×10^6 motile spermatozoa) 55h following sponge removal and recorded a pregnancy rate of 60%. Of note, when inseminating fresh semen laparoscopically, it is important to consider the dose of semen used and the synchronisation process so the process of the AI procedure and use of semen becomes economically feasible.

Alternatively, as spermatozoa become more compromised and undergo periods of liquid storage or are frozen or even sex-sorted, the timing of insemination becomes much more critical, needing to be deposited closer to the time of ovulation. Cervical AI with frozen semen is rarely performed or encouraged owing to the poor fertility associated (Maxwell *et al.* 1980). When inseminating frozen semen via laparoscopy, (Maxwell 1986b) determined that the optimum time for insemination in ewes treated with a progesterone sponge (FGA or MAP sponge) and 400IU of eCG was 60-72h after sponge removal, or slightly after ovulation, seeing pregnancy results of approximately 53-57%. This same study also saw inseminating much earlier 24-35h after sponge removal coincided with greater rates of embryonic loss. Similar results were seen by (Maxwell and Barnes 1986). The work of others (see Table 1.2) has also

observed a similar pregnancy rate when inseminating earlier or before ovulation, 54-60 h (Maxwell 1986b; Findlater *et al.* 1991), 56-60 h (Nehring *et al.* 1989), 57-81h (Walker *et al.* 1989) and 52-60 h (fresh) and 55-60 h (frozen) (Gourley and Riese 1990), recording pregnancy rates between 50 and 67%. (Hunter and Nichol 1993), measured spermatozoa to remain fertile within the cervix for up to 50 h after deposition. Consequently, pregnancy rates following LAI AI with frozen-thawed ram semen at 60 h can be similar to if not greater than pregnancy rates following cervical AI with fresh ram semen (Maxwell and Butler 1984). While these studies were all performed using sponges, the inseminate dose did vary from 20-50×10^6 motile spermatozoa/dose. Previously, more variation in pregnancy rates seems to occur when insemination occurred after 50-56 h post-sponge removal (Evans 1988).

In comparison to sponges, limited studies exist to determine the optimal time of AI post-CIDR removal. Generally, the onset of oestrus is thought to occur earlier when using CIDRs compared to sponge (Clark *et al.* 1984; Walker *et al.* 1989; Swelum *et al.* 2015), and commercially it is recommend ewes be inseminated 48-54h post=CIDR removal. This remains to be confirmed following laparoscopic AI and observe whether other factors such as dose of eCG, uterine tone or intrabdominal fat score can alter this response and affect field fertility.

Table 1.2: Ewe parameters linked to fertility in livestock.

Author	AI Technique	Semen Type	Fertility Measure	Hormones Administered	Timing of AI	Correlation to Fertility Measure
(Swelum *et al.* 2015)	Laparoscopic intrauterine	Fresh	Pregnancy and number of fetuses	Fluorogestone acetate (FGA) sponges and controlled internal drug release (CIDR)	48–50 h after progestogen removal	Pregnancy, fertility, twinning rates, and fecundity were significantly ($P \leq 0.05$) higher in the CIDR group (77.86, 75.57, 34.34, and 1.02, respectively) compared to the FGA group.
(Vilariño *et al.* 2010)	n/a	n/a	Ewes marked by teaser wethers	DICO and a CIDR-G	n/a	No difference between DICO and CIDR-G treated ewes in the onset of oestrus (34.5±2.8 and 30.0±7.7h) and time to ovulation (60.0±9.1 and 54.9±6.4h), $P = 0.09$.
(Walker *et al.* 1989)	n/a	n/a	Observations of the ovaries using the laparoscope	CIDR, 60 mg medroxy-progesterone acetate (MAP) and FGA	n/a	CIDR-progesterone treated ewes commenced ovulation earlier than ewes treated with either MAP or FGA (51, 69, 63 h, $P<0.001$)
(Hill *et al.* 1998)	Laparoscopic intrauterine	Fresh and Frozen	Trans-abdominal ultrasound at 60 days post-AI	CIDR-G, MAP and FGA sponges	48–58h after sponge removal	Pregnancy rates obtained with MAP sponges (64.6%) were significantly lower than FGA sponges (74.7%, $P<0.01$). No difference between FGA sponges and CIDR-G (71.7%, $P>0.05$).

Author	AI Technique	Semen Type	Fertility Measure	Hormones Administered	Timing of AI	Correlation to Fertility Measure
		(pellets and straws)		PMSG dose of 200IU, 250IU, 300IU and >375IU Folligon PMSG and pregnecol PMSG		200 IU resulted in significantly (P<0.05) lower pregnancy rates (62.4%) compared with 250 IU (72.9%), 300 IU (79.1%) and ≥ 375 IU (69.4%). The pregnancy rate for ewes administered Folligon PMSG (71.9%) was significantly higher (P<0.001) compared to ewes treated with pregnecol PMSG (65.8%). Ewes inseminated with fresh semen had a higher pregnancy rate (82.2%, P<0.001) than those inseminated with semen frozen in pellets (69.5%) or straws (71.6%).
(Maxwell and Barnes 1986)	Laparoscopic intrauterine	Frozen	Ewes marked by rams with crayon Pregnancy is determined after slaughter	Intravaginal progestagen-impregnated sponges (P sponge) and CIDR	60 h after sponge removal	Ewes came into oestrus between 24 and 48h after sponge or CIDR removal, with 96.0% of all ewes in oestrus at 48h. Pregnancy and fetus numbers were not different for ewes treated with sponges (60%) or CIDRs (54%, P>0.05).
(Fukui *et al.* 1999)	Laparoscopic intrauterine	Frozen (pellet)	Lambing rates	MAP, FGA, CIDR, P sponge	44–52 h after progesterone removal	There was no difference in lambing rates; 45.0, 41.5, 57.9 and 39.5% between MAP, FGA, CIDR and P sponge (P>0.05)

Author	AI Technique	Semen Type	Fertility Measure	Hormones Administered	Timing of AI	Correlation to Fertility Measure
(Walker *et al.* 2023b)	Laparoscopic intrauterine	Chilled semen	Transabdominal ultrasound 50 post-AI.	CIDR	44 h after pessary removal	The pregnancy rate was highest in autumn (90.2%) when luteolysis occurred during Days 7–9 of the CIDR insertion period compared to Days 1–6 (77.8%, *P*=0.16), 10–12 (68.8%, P<0.05) or Days ≥13 (71.2%, *P*<0.05).
(Ainsworth and Downey 1986)	Natural	Natural	The number of ewes marked by ram. Number of lambs born	FGA, CIDR	n/a	For CIDR and FGA-treated ewes, 92% and 91% of ewes were marked by the ram. Fertility and litter size were similar for both treatments.
(Walker *et al.* 1986)	n/a	n/a	Repeated laparoscopy to view ovaries	FSH, PMSG	n/a	FSH and PMSG treatments significantly affected ovulation rates (8.4 and 7.3, *P*<0.05). After progestogen removal, the median time to ovulation was 60 and 54h, respectively, *P*<0.05.
(Naqvi and Gulyani 1998)	n/a	n/a	Laparoscopic examination of ovaries	PMSG, PMSG + GnRH, PMSG + FSH, PMSG + GnRH + FSH	n/a	Treatment with PMSG and GnRH increased the ovulation rate (9.1 corpora lutea vs 3.0 with PMSG alone *P<0.05*). The use of PMSG and FSH decreased the proportion of ewes with corpora lutea (4/8 vs 7/8) and the number of ovulations (2.4±0.6 vs 9.1±2.6, *P<0.05*).

Author	AI Technique	Semen Type	Fertility Measure	Hormones Administered	Timing of AI	Correlation to Fertility Measure
(Langford 1982)	Cervical insemination	Chilled semen	Progesterone concentration 18 days post-AI Lambing rates	With and without PMSG	54, 57 or 60 h and double insemination at 54 and 60 h post-sponge removal	Conception rates with single insemination at 54, 57, and 60 h and double insemination at 54 and 60 h were 76, 72, 47 and 72% after receiving PMSG, compared to 17, 22, 47 and 43%, respectively, without PMSG (P<0.01). Lambing rates were higher with PMSG-treated ewes (67, 67, 37 and 61% at 54, 57 and double inseminations at 54 and 60 h, respectively) than without PMSG (11, 11, 26 and 33%, respectively, P<0.01). When inseminated at 60 h post-progestogen removal, fertility decreased in PMSG-treated ewes (47% vs 72% at 57h, P<0.01).
(Olivera-Muzante *et al.* 2011)	Cervical insemination	Fresh Chilled	Non-return rate at 21 days post-AI Transabdominal ultrasound	Synchrovine (two doses of PGF2α 7 days apart), P4-eCG.	42, 48 and 54 h post-progesterone removal	Ewes inseminated at 42, 48, or 54 h had similar non-return rates (0.51, 0.46, 0.57), Fertility (0.27, 0.31, 0.26), and Fecundity (0.29, 0.32, 0.27), but was lower (P<0.05) than P4-eCG group inseminated at 54 h (0.61, 0.48, 0.52, respectively). Ewes inseminated at 42 h yielded lower (P<0.05) NRR21d, Fertility and Fecundity (0.28, 0.06, 0.06) compared to 48 (0.43, 0.24, 0.24) and 54 h (0.44, 0.22, 0.23, respectively). Ewes inseminated at 51 and 57 h had similar NRR21d (0.16 vs 0.20), Fertility (0.12 vs 0.14), and Fecundity (0.12 vs 0.15), respectively, but this was lower (P<0.05) than P4-eCG treated ewes at 54h (0.34, 0.28, and 0.33, respectively). Ewes inseminated at 51 and 57 h had similar NRR21d (0.51 vs 0.58),

Author	AI Technique	Semen Type	Fertility Measure	Hormones Administered	Timing of AI	Correlation to Fertility Measure
						Fertility (0.43 vs 0.51), and Fecundity (0.45 vs 0.56), but lower (P<0.05) results compared to P4-eCG treated ewes (0.75, 0.71, and 0.88, respectively)
(Bruno-Galarraga *et al.* 2021)	Single natural mating with ewe	Fresh	Ultrasonographic scanning 35 days after CIDR removal	400IU eCG, 500IU hCG	24 h after CIDR removal	Pregnancy was significantly higher in ewes synchronised with eCG than hCG (69.2% and 38.5%, respectively, P<0.05).
(Menchaca *et al.* 2004)	Cervical insemination	Fresh	Transrectal ultrasound 30 days after insemination	PGF2α	42, 48 and 54 h post-onset of oestrus.	The pregnancy rate of ewes inseminated at 42 h was similar to those inseminated at 48h but greater than those inseminated at 54h (56/152, 31/120, 37/164; respectively, P=0.09, <0.05).
(Findlater *et al.* 1991)	Intrauterine insemination	Frozen and fresh	Number of lambs born 24-day non-return rate	PMSG	54, 60 and 72 h (frozen) and 56 h (fresh) post-sponge removal.	Fresh semen had significantly higher conception rates (90%) than frozen-thawed at all insemination time points with frozen-thawed semen (65, 63, 46%, P<0.05).
(Maxwell 1986b)	Laparoscopic intrauterine	Frozen	Ewes marked by rams with crayon harness	intravaginal progestagen-impregnated	48, 60, 72 and 78 h	The percentage of ewes detected in oestrus at 12, 24, 36, 48, 60 and 72 h after sponge removal was 0.3%, 0.6%, 5.2%, 36.7%, 20.0% and 3.1%, respectively.

Author	AI Technique	Semen Type	Fertility Measure	Hormones Administered	Timing of AI	Correlation to Fertility Measure
			Lambing by observations	sponges, PMSG	post-sponge removal	Time to ovulation for teased ewes was 55.8h compared to unteased ewes, 59.7 h after sponge removal. Ovulation rates for teased and unteased ewes were 1.8 ±0.34 and 1.6 ±0.29, respectively. Ewes lambing, lambs born per ewe and number of lambs born per ewe at inseminations times of 48, 60, 72 and 78h were 45.9, 57.7 and 1.25; 55.1, 72.0 and 1.31; 57.4, 80.9 and 1.41; and 39.3, 60.7 and 1.54 (P<0.05).
(King *et al.* 2004)	Cervical and laparoscopic intrauterine insemination	Frozen	Lambs per ewe lambing	10IU oxytocin	54h (intrauterine), 48, 52, 55, 56, 60 h (cervical) post-sponge removal	Litter sizes were not affected by oxytocin (1.91 and 1.51 compared to those receiving oxytocin, 1.83 and 1.41); however, they were affected by insemination type (laparoscopic versus cervical, P<0.02).
(Scudamore *et al.* 1993)	Intrauterine insemination	Fresh	Ova were recovered by the laparoscopic procedure	CIDR, FGA, FGA + redispersed, FGA + redispersed +	48h after the last p-FSH injection	Progesterone + FGA pessary gave the highest ovulation and ovum recovery rates (P<0.05). PGF2α treated ewes, despite having a higher proportion of ova suitable for transfer, yielded a lower number of ova (P<0.05).

Author	AI Technique	Semen Type	Fertility Measure	Hormones Administered	Timing of AI	Correlation to Fertility Measure
				progesterone, PGF2α		

1.5. ENVIRONMENTAL FACTORS THAT MAY INFLUENCE FERTILITY FOLLOWING AI

With Australia facing a warming climate, the impact of changing environmental conditions and the lengthening of seasons has become a predominant area of focus for sheep fertility research. Sheep are sensitive to acute environmental changes, including, feed availability, temperature, rainfall, humidity, evaporation, and sunlight hours. Several of these factors have been studied during natural breeding programs, yet a limited number focus on effects during AI. Reproductive outcomes focus on the impact of egg and embryo quality, sperm transport and implantation. While the effect of heat stress has been examined extensively on the fertility of ewes and semen production in rams (reviewed by (Romo-Barron *et al.* 2019; van Wettere *et al.* 2021). The question still remains: which is the most vulnerable stage of the reproductive cycle, i.e. folliculogenesis, ovulation, fertilisation or implantation during AI?

Spring lambing is Australia's predominant reproduction management choice, which coincides with optimal conditions during gestation and lambing. As producers seek to accelerate ewe productivity by lambing every eight months, breeding can occur in anovulatory and transition seasons, increasing the prevalence of heat stress (van Wettere *et al.* 2021). In 1964, Dutt exposed ewes to heat (32 °C) in environmentally controlled rooms over the oestrous cycle, during breeding and 1 day following mating. Heat treated ewes showed increased morphological abnormalities of the ova, increased embryo mortality and significantly lower fertilisation rates (Dutt 1964). In a paddock, (Lindsay *et al.* 1975) measured fertility and lambing decrease by 2.7% and 3.5%, respectively when temperatures were over 32.0 °C per each consecutive day. This has not yet been considered for an artificial breeding program and would help educate growers and lead to better fertility results for industry.

Depending on climatic rainfall patterns, an increase in heat and humidity can either bring extended periods of drought and poor crop yield, reducing the quantity and quality of

feed and contributing nutritional stress to production systems (Seo 2010). Alternatively, it can lead to increased periods of rainfall and thunderstorms. While not in Australia, (Đuričić *et al.* 2019) measured that the sexual activity of sheep peaked during wet seasons and measured fecundity to be greatest during winter months. Similarly, (Abecia *et al.* 2016) found lambing rates to AI to be positively correlated to annual rainfall. Over recent years, Australia has experienced an extended period of La Nina, bringing intense rainfall and flooding. Acute flooding can reduce access to grazing, altering the ewe's nutritional status and negatively impact of stress and elevated cortisol on reproductive performance. To date, there are no studies which have examined the influence of excessive rainfall and flooding on fertility following AI.

Other environmental factors to be considered following AI include humidity, sunlight, and evaporation. Previous reports on pelibuey and blackbelly sheep in Mexico suggest that relative humidity may contribute to reduced oestrus activity (Galina *et al.* 1996). More recently, (Abecia *et al.* 2016) demonstrated that temperature, solar radiation, wind speed, precipitation, and humidity significantly affected pregnancy rates of sheep following AI. As of current, the impact of such parameters is yet to be quantified and explored within the unique Australian conditions. Nonetheless, these initial studies raise concern for industry, considering the negative effect of these environmental conditions on the welfare, productivity, and reproductive performance of ewes.

1.6. CONCLUDING REMARKS AND OBJECTIVES OF THE CURRENT STUDY

Within the Australian sheep industry, producers strive to accelerate genetic gain and improve reproductive efficiency through the dissemination of elite genetics using reproductive technologies and artificial breeding programs, however, variation in the success rate of laparoscopic artificial insemination amongst programs and sires have likely hindered the rate of adoption throughout the industry and ultimately the rate of genetic gain for the sheep industry. While extensive literature has focused efforts to understand the cause of such variable

fertility, the degree to which various factors interact and influence AI success in sheep remains unknown. This limitation is also likely confounded by a lack of feedback on pregnancy results to artificial breeding companies that can consolidate data across the industry for analysis.

This review has explored the historical and contemporary technical protocols for artificial laparoscopic intrauterine insemination in sheep. It has also extensively detailed the various factors related to the ewe, sire or semen, and environment which may contribute to the success of any AI program. While several studies have reported correlations with fertility, in particularly surrounding *in vitro* sperm quality, contradictions have arisen when different measures of fertility are used to assess success of multiple factors have not been considered in the same study. For example, the fertility information used is often assumed or estimated rather than reported on the same batch or sample of semen tested, reducing the scope of the conclusions drawn. Those studies completed in sheep have also focused on either cervical AI or field fertility following a natural breeding program. Thus, results cannot be used interchangeably with laparoscopic AI. Finally, where studies are located internationally, the environmental context in which the experiment is conducted is vastly different to the Australian continent and climate. This, therefore, warrants a thorough investigation of factors affecting the success of laparoscopic artificial insemination in sheep, considering multiple sires, multiple programs over several years, and measuring *in vitro* semen assessment parameters on the same batch of semen used for insemination.

Having a better understanding of how sperm traits alter fertility in the ewe following AI would enable industry tore-affirm standards of semen assessment, providing the opportunity to potentially screen males for semen factors known to influence the success of laparoscopic AI. This could contribute to a reduction in the variation seen in fertility following AI, increasing adoption of the technology, accelerating the rate of genetic gain for the industry. Ultimately,

producing more lambs, meat and fibre from superior sires, leading to a more profitable Australian sheep industry.

The work contained within this thesis hypothesised that a combination of male and female fertility factors would interact together to significantly influence the probability of pregnancy occurring in a ewe following artificial laparoscopic intrauterine insemination. This hypothesis will be examined using the following objectives;

I. To characterise key female reproductive traits that influence the likelihood of pregnancy occurring following laparoscopic intrauterine insemination in sheep.

II. To develop a comprehensive multivariate binomial logistical regression model that integrates male, female and environmental factors to estimate pregnancy likelihood following artificial laparoscopic intrauterine insemination in sheep.

III. To assess and validate the predictive accuracy of the multivariate fertility model in field conditions, comparing its ability to distinguish pregnancy from non-pregnant ewes against ultrasound diagnostics.

IV. To recommend practical guidelines or standards for the assessment and artificial insemination of frozen-thawed semen in sheep, incorporating optimal male and female fertility traits to maximise pregnancy rates.

V. To investigate the effect of different *in vitro* processing techniques, such as freezing concentration and thawing, on the post-thaw quality of frozen-thawed ram spermatozoa, suggesting recommendations for industry use.

Chapter 2. Uterine tone influences fertility of Merino ewes following laparoscopic artificial insemination.

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2.1. ABSTRACT

Artificial insemination plays a critical role in facilitating rapid genetic and production gains within the sheep industry. However, variable rates of AI success remain a concern for the industry and a barrier to adoption. Furthermore, the degree to which female factors influence the success of intrauterine laparoscopic AI rather than natural mating remains unknown. As such, this study investigates the effect of several factors collected during the time of AI, on the success of intrauterine laparoscopic AI. Data sets was generously donated by artificial breeding companies and stud breeders during routine commercial AI operations. AI data was collected over 3 breeding seasons during commercial AI programs (N=24 programs) using Merino ewes (N=24,700). Sire ID (N=253), time of AI following progesterone removal (approx. 43-59h post-removal), uterine tone and intra-abdominal fat (both scored 1-5) as well as age of the ewe were all recorded at the time of AI. Transcutaneous ultrasound subsequently determined pregnancy rate approximately 55 days post-AI. A multivariate regression analysis was performed and revealed pregnancy success to increase when semen was inseminated into a ewe with a uterine tone score of 4 or 5 (P<0.001). The remaining factors fell short of significance within the multivariate model. An interclass coefficient variation matrix was also used to

determine the proportion of variation contributed to AI success by random factors allocated in the model; site, sire, AI date and breeding season (45.99%, 29.94%, 15.15% and 8.92%, respectively). These results highlight the influence of uterine tone on ewe fertility following laparoscopic AI, but also that program location and the sire used can further modify this influence on pregnancy rate. These factors must now be considered in combination with semen factors per individual sire used during AI to ascertain the contribution of several factors to the success of laparoscopic AI in Australia.

2.2. INTRODUCTION

Artificial Insemination is an artificial reproductive technology used to increase the number of lambs born to elite flocks and to accelerate genetic gain across the Australian sheep industry. Due to the complex structure of the ewes' cervix, frozen-thawed semen of adequate quality must be deposited into the uterus via laparoscope (Killen and Caffery 1982) to aid successful fertilisation. Historically, laparoscopic AI within the sheep industry has always been considered reasonably successful, with the general understanding that approximately 70% of ewes inseminated with frozen-thawed semen should fall pregnant (Donovan *et al.* 2004a; Geenty *et al.* 2014). Despite this understanding, reports of poor or variable success rates place AI at risk of waning adoption, threatening the rate of genetic gain for the industry. Previous studies have shown the success rate of laparoscopic AI to vary between commercial programs in different locations (Maxwell and Watson 1996; Luther *et al.* 2007; Geenty *et al.* 2014), between programs on the same site over several breeding years, the sires used (Eppleston and Maxwell 1995; Hill *et al.* 1998; Del Olmo *et al.* 2013; Gibbons *et al.* 2019) as well as the various AI technicians and protocols (Ybañez *et al.* 2017).Fertility results following laparoscopic AI have even been recorded to reach lows of 12% (Hill *et al.* 1998). Should the cause of this variation be determined, the success and adoption rate of AI could increase, increasing genetic progress for the Australian sheep industry.

Several factors have been identified to contribute to female fertility following laparoscopic intrauterine AI. Female characteristics such as; age (Shorten *et al.* 2013), breed (merino) (Narayan *et al.* 2021), body condition score (Shorten *et al.* 2013), intra-abdominal fat, uterine tone and method of oestrus synchronisation (Fukui *et al.* 1999; Vilariño *et al.* 2010) and time of insemination (Langford 1982; Walker *et al.* 1989; Olivera-Muzante *et al.* 2011) have all been explored. Yet, the degree to which these factors influence the success of AI in sheep and how they interact remains to be discovered. Primarily due to variation in the measure of fertility reported across literature, such as scanning rate, return to oestrus rate and/or lambing survival, most recently reviewed by (Spanner *et al.* 2024a). Initial research suggests that ewe fertility is linked to increasing age from two to five years old (Mulvaney 2011; Shorten *et al.* 2013; Narayan *et al.* 2021). While most of the research aligns with this relationship, research from (Kleemann and Walker 2005), also found no influence of ewe age on AI success. While a rising plane of nutrition (Bennett *et al.* 1964; Coop and Clark 1969; Edey 1970) and body condition score of 2.5-3.5 has been reported optimal for ewe fertility (Cranston *et al.* 2011; van Burgel *et al.* 2011), its correlation to intra-abdominal fat or the layer of fat surrounding the abdominal cavity during AI is yet to be reported. Excessive fat mobilisation can cause fatal ketosis and limit the availability of free space in the abdomen, reducing the ability of the rumen to expand when ingesting the high-fibre, high-calorie diets recommended for conception and pregnancy (Kerr 2018). Additionally, in preparation for insemination, the use of a consistent and accurate synchronisation protocol involving intravaginal progesterone administration (CIDR) (EAZI-BREED CIDR Sheep and Goat Device, Zoetis, Australia) (Walker *et al.* 1989; Fukui *et al.* 1999; Swelum *et al.* 2015), and the distribution of eCG (Langford 1982; Walker *et al.* 1986; Bruno-Galarraga *et al.* 2021) has been intensively studied throughout literature (Spanner *et al.* 2024a) as has the impact of insemination time post-CIDR removal (Langford 1982; Menchaca *et al.* 2004; Olivera-Muzante *et al.* 2011) to coincide with time of ovulation,

(Walker *et al.* 1989; Vilariño *et al.* 2010). Ultimately, the progression of this work has helped develop the optimal synchronisation protocol to better improve chances of ovulation, fertilisation, and pregnancy success. Therefore, current Australian practice within the Australian sheep and goat industry is to administer a single dose of eCG following the removal of CIDR inserted for 12-14 days (Evans and Maxwell 1987) with insemination to occur 47 to 55h post-CIDR removal. Whilst this is recommended by the manufacturers, inevitably variability in AI time and response to exogenous hormones exists throughout industry, thus making it inextricably important to know the impact of alternative insemination times and their impact on AI success. An ewe's response to the synchronisation process and therefore ability to enter oestrus and ovulate is difficult to assess without subjectively observing the tone of the uterus and/or gross morphological structures of the ovaries with a laparoscopic. A tone score of one represents a uterus which is flaccid and pale and suggests there is limited or no response to the synchronisation process. A uterine tone of five represents a turgid and pink uterus and a strong response to the synchronisation process (Spanner *et al.* 2024a). The question remains on the relationship between uterine tone, time of ovulation and the most effective time of semen deposition to coincide with the descending oocyte. Whilst previous literature has attempted to correlate uterine tone with pregnancy success following insemination in mares (Hayes and Ginther 1986; Griffin *et al.* 1992; Bonafos *et al.* 1994; Bonafos *et al.* 1995) and dairy cows (Loeffler *et al.* 1999), a study of this nature is yet to exist for merino sheep. Understanding this relationship will help establish a more accurate and precise time of insemination based on quality of the semen and length of survival in the female, increasing the chances of successful pregnancy.

As previously mentioned, variation in how fertility is measured or defined across studies and species and the limited sample size makes it harder to reach significance and draw conclusions. Furthermore, it is well known that reproductive traits are lowly heritable

(Fossceco and Notter 1995). This puts even more pressure on external factors (nutritional status, oestrus synchronisation, time of insemination) to influence fertility. As such, the current study aims to build a multivariate regression model to assess the impact of female parameters recorded at the time of laparoscopic AI on successful pregnancy. The conclusion of this research will help establish breeding standards for ewes across the sheep industry, allowing breeders to select ewes with high fertility potential for artificial breeding programs. Additionally, this research could lead to improved breeding efficiency by reducing reproductive variability, an increase in the number of lambs born to elite flocks and an acceleration of genetic gain across the Australian sheep industry.

2.3. METHODS AND MATERIALS

2.3.1. Ethics and Animals

Data was generously donated by artificial breeding companies and stud breeders during routine commercial AI operations. As such, no ethics was required.

2.3.2. Breeding seasons and Locations of Animals

Data was collated across 3 breeding seasons. During the 2020-2021 breeding season, data on 4263 Merino ewes was recorded across 4 sites, located in the Central and South Wheat Belt of Western Australia and the Riverina district of NSW. Data collection for AI began in November 2020 and finished in February 2021.

During the 2021-2022 breeding season, data on 8253 Merino ewes was recorded across 9 sites located in the Central and South Wheat Belt of Western Australia, the Central North region of Victoria, Murray Land Yorke Peninsula of South Australia and the Central West, Tablelands and Northwest slopes and plains of NSW. Data collection for AI began in November 2021 and finished in April 2022.

Finally, during the 2022-23 breeding season, data was collected on 12,184 Merino ewes across 11 sites located in the Central and South Wheat Belt of WA, Central North of Victoria,

Murray Land Yorke Peninsula of South Australia and the Central West, Tablelands and Northwest Slopes and plains of NSW. Data collection for AI began in November 2022 and finished in April 2023.

The management of ewes and rams was as per the requirements for each site. This inevitably varies between sites and breeding programs; however, each follows industry practice.

2.3.3. Experimental Design

Merino ewes (N=24,700, split across three breeding seasons and 24 commercial AI programs conducted in farms located in NSW, VIC, SA and WA) were synchronised for oestrus and assessed for uterine tone, intra-abdominal fat, age and time of insemination post-CIDR removal as part of routine artificial insemination protocols. As per industry standards, ejaculates were collected by either artificial vagina or electroejaculation from Merino sires (N=253) and inseminated either fresh (FR; N=11) or frozen-thawed (FZ; N=242). Ewe parameters, including season, site, sire and type of semen used, were recorded during AI. Each ewe underwent pregnancy scanning approximately 55 days post-AI using standard industry practice.

2.3.4. Sire, Semen Collection Type and Dose at Insemination

Ejaculates were collected either by artificial vagina or electroejaculation from Merino rams and processed for either immediate insemination (fresh (FR); N=3,475 ewes) or frozen as per the protocol followed by each artificial breeding company. Fresh semen was collected via electroejaculation and inseminated within 30-60mins (one batch of 30-40 ewes). Semen was thawed on the day of AI by an artificial breeding company and later inseminated (frozen-thawed (FZ); N=21,225 ewes).

Following industry protocols, synchronised ewes (0.3g progesterone CIDR; Zoetis, Australia and eCG; Minitube, VIC, Australia) received approximately 0.2mL of semen per uterine horn or a minimum of 2×10^6 motile spermatozoa.

2.3.5. *Assessment of Ewe Age*

The colour of the ear tag located in the left ear of the ewe indicated the year of birth for each year. Each colour represents a year of drop (Table 2.1). This was then subtracted from the current year (either 2020, 2021, 2022 or 2023) of AI to determine the age of each ewe at AI.

Table 2.1: Year of drop coloured electric ear tag identification for sheep (modified from (Meat and Livestock Australia 2011)).

Year of Drop	Colour Ear Tag
2017	White
2018	Orange
2019	Green
2020	Purple
2021	Yellow

2.3.6. *Assessment of AI Time Post CIDR Removal*

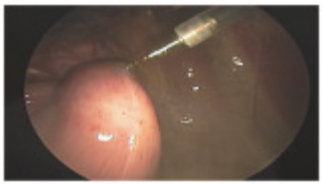
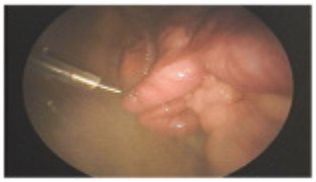
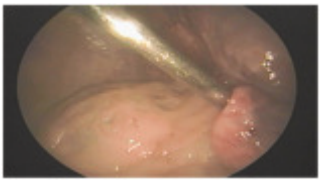
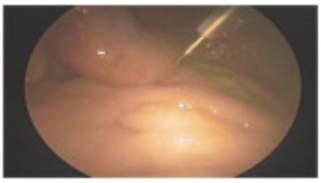
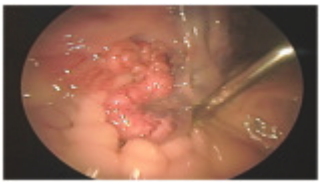
As per normal industry procedure, during synchronisation, each ewe was assigned a CIDR pull group to ensure ewes were inseminated within the optimal time frame. At the time of insemination in the cradle, the eID tag of each ewe was scanned using a Tru-Test XRS2 (Tru-Test Datamars, Australia) giving a time stamp for data collection in the cradle. To determine the time of AI post-CIDR removal, this was subtracted from the end time of the CIDR pull group each ewe was allocated. This was standardised across each CIDR pull group across all programs. Data was presented as hh:mm:ss post-CIDR pull.

2.3.7. *Assessment of Intra-abdominal Fat Score*

During laparoscopic AI, the internal fat covering the abdominal organs was visualised and subjectively assessed by the technicians performing the insemination, scoring the ewe between one (little to no fat present) to five (high abundance of fat present). The assessment of intra-abdominal fat was standardised across AI programs using the five-score system (Table 2.2).

Images were taken prior to AI, and agreements were made between assessors, prior to assessment.

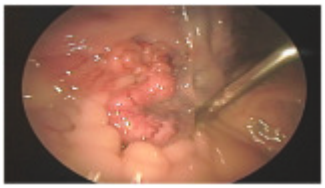
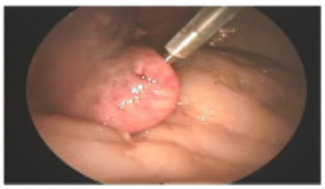
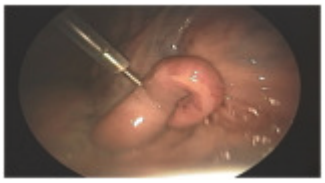
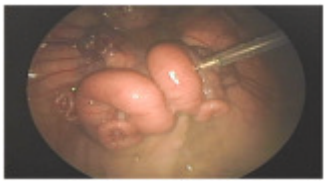
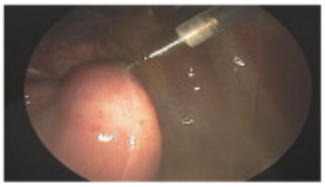
Table 2.2: The standardisation of intra-abdominal fat scores assessed at AI by inseminator, scored one to five.

Score	Figure	Visual Description
1		Little to no fat is present within the abdominal cavity.
2		A small amount of fat lining the abdominal region of the ewe.
3		A moderate amount of fat lining the abdominal region of the ewe.
4		Large masses of internal fat covering the abdominal region of the ewe.
5		Excessive amount of fat around the abdominal region of the ewe.

2.3.8. Assessment of Uterine Tone Score

At the same time as the intra-abdominal fat assessment, the tone of the uterus was scored as a subjective observation by the technicians and recorded as a value between one (pale, flaccid uterus) to five (bright pink turgid uterus), as seen in Table 2.3. The assessment of uterine tone was standardised across AI programs using the five-score system (Table 2.3). Images were taken prior to AI, and agreements were made between assessors, prior to assessment.

Table 2.3: The standardisation of uterine tone scores assessed at AI by inseminator, scored one to five.

Score	Figure	Visual Description
1		Flaccid uterine horns.
2		Soft uterine horns.
3		Moderate swelling of uterine horns.
4		The greater amount of swelling and some resistance to penetration during insemination.
5		Swollen/turgid uterine horns. Resistance to penetration during insemination.

2.3.9. Pregnancy Scanning

Depending on the AI program and site, the pregnancy result per ewe was determined approximately 55 days post-insemination. Ewes were fasted 24h prior to scanning. A real-time cutaneous ultrasound (oviscan 6 with a 3.5 MHz probe) was used to scan each ewe to determine the presence of fetuses and their number. Pregnancy from AI was recorded as either 1 (pregnant) or 0 (empty), while the number of fetuses observed was recorded as the exact number.

2.3.10. Statistical Analysis

Ewe ID was matched between AI and pregnancy datasets to create one Masterfile. Data was then cleaned to remove ewes without pregnancy data. Ewes were kept in the data set for analysis if they maintained a sire ID, site location, AI date, and at least one female fertility parameter.

Overall pregnancy data was assessed to determine average pregnancy and reproductive rates. AI success was determined by calculating the number of ewes pregnant over the total number of ewes inseminated. Reproductive rate was determined by calculating the number of offspring (fetal number) over the total number of ewes inseminated. Descriptive statistics were included to evaluate the number of ewes inseminated and the percentage pregnant for each factor level recorded, Site, Sire ID and Breeding Season. All values included mean ± standard error of the mean and were de-identified for anonymity.

A binomial linear mixed model regression (REML) was performed using R Studio (Version 2023.09.1+494) to determine the effect of female fertility traits on AI success. An intra-class coefficient correlation (ICC) analysis was performed to determine the proportion of variability contributed by individual factors to develop the random model (Breeding season, site, AI date and sire).

A preliminary univariate analysis using the above random model was first conducted to assess the effect of each individual factor on pregnancy rates. Factors that returned a *P-valueP-value* of <0.2 were subsequently used to build the multivariate model considering the random effects (Breeding season, Site, AI date and Sire). The REML model was refined by the backwards selection process, eliminating fixed effect variables and interactions ($P>0.05$). The final multivariable model included only factors at contribute to AI success ($P<0.05$).

For any significant fixed effects, an Emmeans pairwise comparison was performed to assess the significant difference between groups within the variable. Groups were considered different to each other if the comparison returned a *P-value* of <0.05.

A Cramer's V Association Matrix was also performed to determine any associations or correlations of factors to pregnancy success.

2.4. RESULTS

2.4.1. Overall Descriptive Statistics of Dataset

Across the 3 breeding seasons, data was collected from 24 700 ewes and 253 rams from 24 sites. The pregnancy rate to AI ranged from 50.68% to 84.75%, averaging 65.50%. The reproductive rate averaged 143.21%. Table 2.4 displays the mean ± SEM and range for each female factor recorded during AI. Supplementary File 1 describes the percentage of ewes pregnant for each level within ewe fertility factor.

Table 2.4: Descriptive statistics of factors recorded during AI over the entire dataset (N = 24,700) including mean (±SEM) and range for each factor

Ewe Fertility Factor	Mean ± SEM	Range
Age of ewe at AI (years)	3.18 (±0.01)	1 to 13
Uterine tone score	3.22 (±0.01)	1 to 5
Visual Intra-abdominal fat score	3.06 (±0.01)	1 to 5
Time of AI post-CIDR removal (hh:mm:ss)	51:48:46 (±0:00:42)	43:49:26–59:30:45
Sperm Type	n/a	Frozen (FZ) or fresh (FR)

2.4.2. Contribution of Variation Caused by Random Terms

The ICC analysis was performed on the random model to assess the proportion of total variance contributed by random terms. The proportion of variation in pregnancy success attributed to breeding seasons, site, AI date, and sire was 8.92%, 45.99%, 15.15%, and 29.92%, respectively. The level of significance was not performed on these variations.

2.4.2.1. The Proportion of Variation in Pregnancy Success Contributed by Site and Breeding Season

Across the 24 sites used within this analysis, the pregnancy rate ranged from 50.68% to 84.75%, averaging at 65.50% ewe pregnancy to AI (Figure 2.1). Throughout the 2021, 2022 and 2023 breeding seasons the average percentage of ewes pregnant varied between 64.20, 69.78 and 63.05%, respectively.

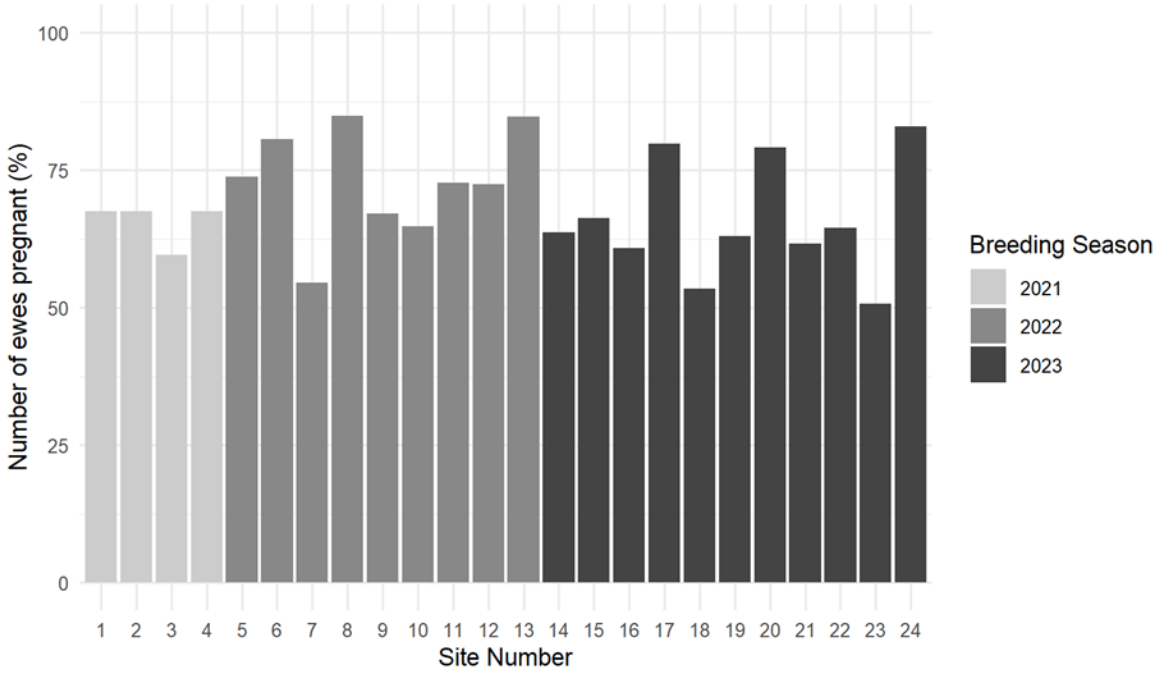


Figure 2.1: Variation in pregnancy rates for each site (deidentified, N = 24) recorded during the 2021, 2022 and 2023 breeding seasons.

2.4.2.2. The Proportion of Variation in Pregnancy Success Contributed by Sire

From the multivariable model, 29.94% of the variation detected between the random terms was contributed by the sire used in the program. Across 243 sires, the pregnancy rate ranged from 16.17% to 93.75% (Figure 2.2).

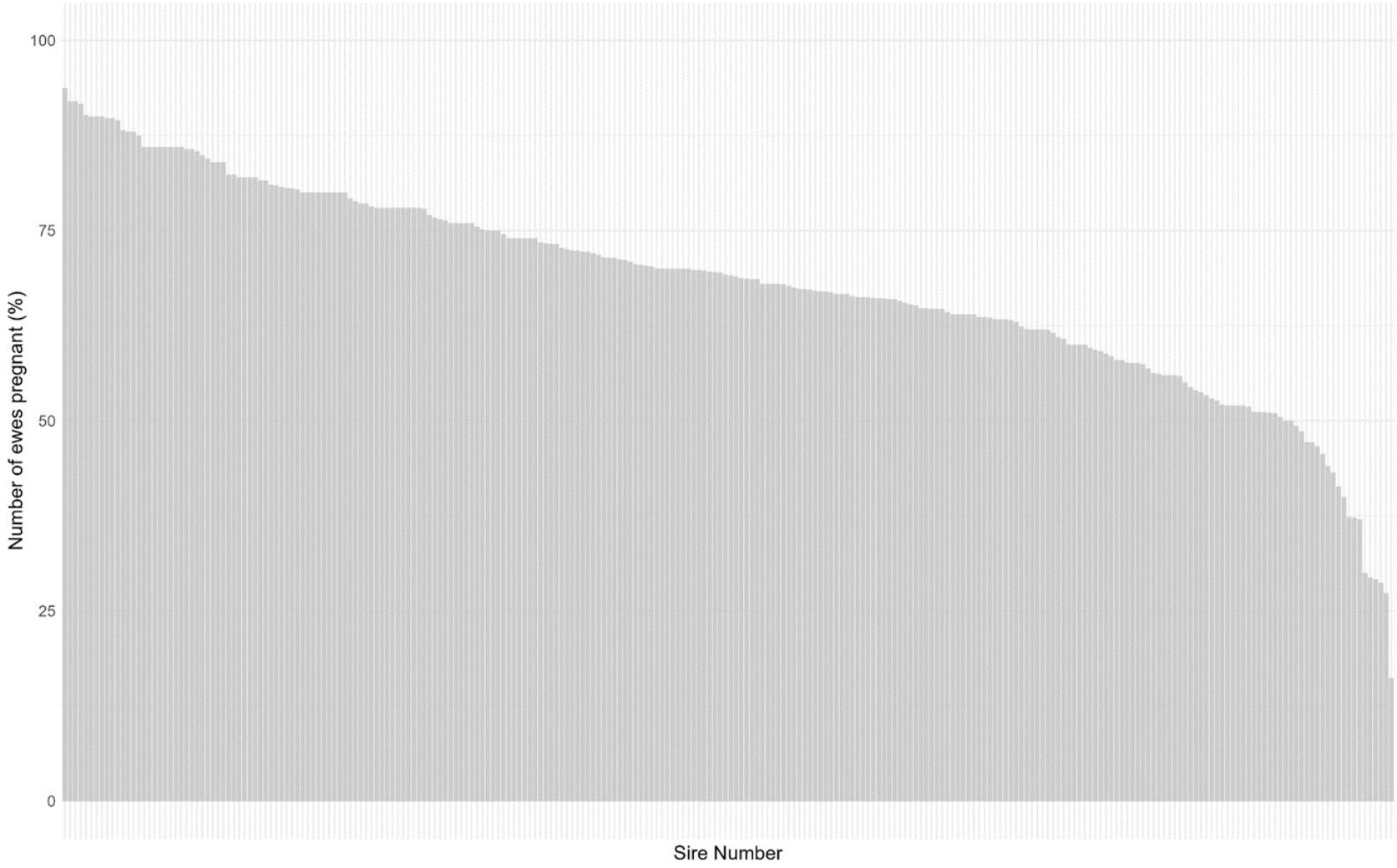


Figure 2.2: Pregnancy rate for each sire (deidentified) throughout the three breeding seasons, N = 253. Number of ewes inseminated per sire ranged from 7 to 426, average = 75.21±3.24 ewes.

2.4.3. The Impact of Uterine Tone on Pregnancy Success

When run in a univariate model, age, uterine tone and intra-abdominal fat returned a significant *P-value* (Supplementary File 2). Yet, when considered in the same multivariable model, only **Uterine Tone** remained significant (Supplementary File 2). There were no interactions between variables.

Ewes inseminated with a uterine tone score of 1+2 on the day of insemination recorded a considerably lower pregnancy rate when compared to ewes who scored 3 and 4+5 (57.88%, 65.21%, 70.29%, respectively, $P<0.05$, Figure 5). Similarly, ewes that scored a uterine tone of 3 recorded a notably lower pregnancy rate compared to ewes with a uterine tone score of 4+5 ($P<0.05$, Figure 2.3).

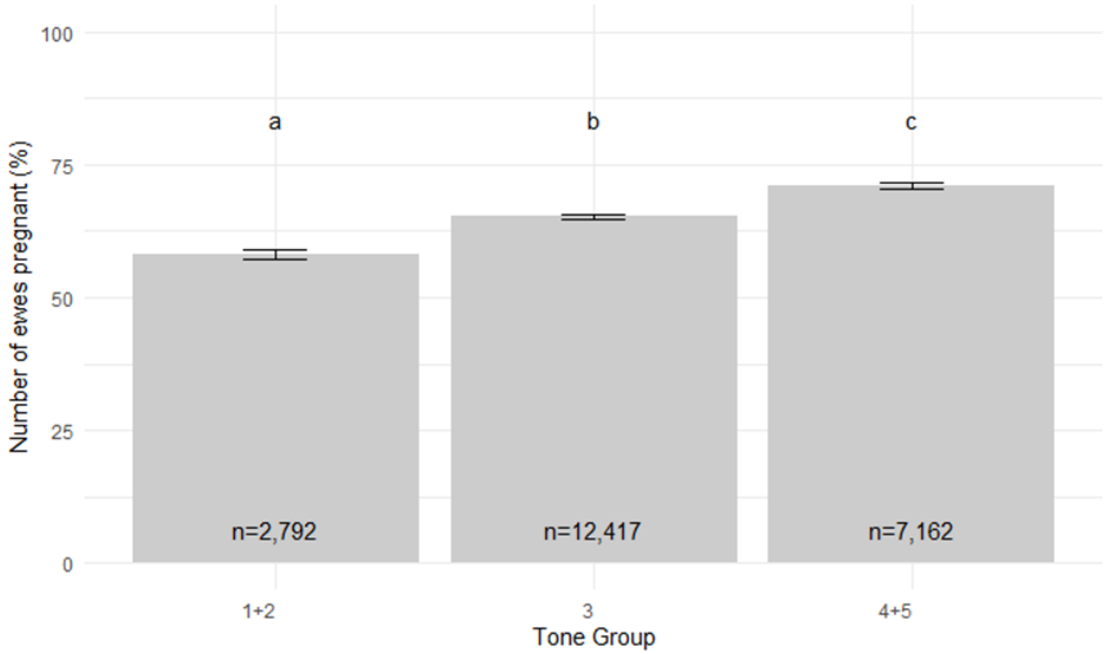


Figure 2.3. Pregnancy rate by tone group ±SEM. Columns that differ in subscript indicate a significant difference between tone groups (*P*<0.05). Labels within columns indicate sample size per factor group.

2.4.4. Cramers Association Between Individual Factors and Pregnancy Rate

In Table 2.5, the Cramers Association statistical test demonstrates the relationship between categorical variables and binomial outcomes and their reciprocal *P-valueP-value*

(P<0.05 threshold of significance). All factors returned a significant result with the exception of sperm type.

Table 2.5. Listed are the Cramer's V Association Matrix for each variable to the success of pregnancy.

Factor	Chi-squared test (P value)	Cramer's V
Age grouped	<0.0001	0.0075
Tone grouped	<0.0001	0.017
Fat grouped	<0.0001	0.017
AI time post-CIDR pull grouped	0.00044	0.0061
FR or FZ	0.4892	0.00086

2.5. DISCUSSION

This study is the largest collection of AI data that combines records from three breeding seasons across 24 commercial sites into one model, determining the impact of female traits on the success of laparoscopic artificial insemination programs in sheep. While other studies have reported higher numbers of ewes within a fertility data set (Hill *et al.* 1998), this study is unique in that it considers the fertility of each ewe individually, rather than across the mob or flock. The average pregnancy rate across the 24,700 ewes was 65.50%, followed by a reproductive rate of 143.21%. The ICC statistical analysis also demonstrated a significant proportion of variation contributed by the random terms of the model, that is, breeding season (8.92%), site (45.99%), AI date (15.15%) and sire (29.94%). This suggests that ultimately the ability to accurately predict the success of AI in sheep is challenging and that future work is needed to develop strategies to best control and mitigate variation caused by the environment (site, season) and sire (male factors). Nevertheless, when this variation was accounted for, uterine tone, subjectively measured at the time of AI, was found to influence the likelihood of an ewe falling pregnant following intrauterine laparoscopic AI.

The present study revealed a clear positive linear relationship between the uterine tone of ewes at AI and the likelihood of pregnancy in sheep. Ewes which scored a uterine tone of 1+2, 3 and 4+5, resulted in a pregnancy probability of 58.88%, 65.21% and 70.29%, respectively (Figure 3). Observing the uterine tone of ewes or other females in different livestock species during insemination has been linked to visually assessing the stage of oestrus (Hayes and Ginther 1986; Griffin *et al.* 1992; Bonafos *et al.* 1994; Bonafos *et al.* 1995; Sathe 2018) as well as the success of synchronisation using exogenous hormones across a mob or group of females (Hawken *et al.* 2005; Martinez *et al.* 2015). As a result of these findings, it could be suggested that a more stringent assessment of uterine tone and, therefore, selection of ewes during laparoscopic AI could help reduce the variability in pregnancy observed and improve the overall success rate.

Limited studies have reported the correlation between uterine tone and fertility in other species. One study in sheep reported no impact of uterine tone on fertility following laparoscopic (Duran 2018), contrary to that reported in the current study. Importantly though, here the authors used a three-score system to classify the uterine tone of ewes (0- no response; 1-medium response and 2-adequate response), compared to the five-score system used in the current study, additionally the former study was limited by sample size (N=462 ewes). The authors also mention a different result may have been recorded if all tone scores (0,1 and 2) were observed within the same study group or experiment. The sample size of the current study was over 24,000 ewes and all five tone scores were recorded within the study, agreeing with this hypothesis.

Understanding the relationship between uterine tone, stage of oestrus and fertility is important in order to maximise the use of uterine tone as a predictor of AI fertility. Changes in the structural nature of the female uterus or development of uterine tone is caused by rhythmic hormonal changes during oestrus and has been measured in the ewe (Ford 1982; Johnson *et al.*

1997; Reynolds *et al.* 1998; Zhang *et al.* 1999), mare (Hayes and Ginther 1986; Griffin *et al.* 1992; Bonafos *et al.* 1994), cow (Bonafos *et al.* 1995), dog (Barrau *et al.* 1975), cat (Bareither and Verhage 1980) and human (Kunz *et al.* 1998; Wildt *et al.* 1998). In sheep undergoing oestrous synchronisation, the 12-day administration of intravaginal progesterone, will increase the expression of progesterone in the blood, mimicking the natural function of a corpus luteum (Evans and Maxwell 1987). Once removed, the oestrogen: progesterone ratio increases, bringing with it a wave of follicular development and an increase in oestrogen levels, initiating oestrus 2 days later (Maxwell and Barnes 1986). Coinciding with a peak in oestrogen, a surge in luteinising hormone causes ovulation, and an eventual decrease in the ratio of oestrogen: progesterone, signifying the start of the luteal phase (Blavy *et al.* 2016; Newcombe *et al.* 2023). A previous study by (Murray *et al.* 1994) applied an oestrogen treatment to ovariectomised ewes, and after 2 days of treatment, saw an increase in the height of epithelial uterine gland cells, maturing protein-synthesising organelles such as Golgi bodies as well as an expansion of the rough endoplasmic reticulum. This was later confirmed by (Reynolds *et al.* 1998) who measured a 2.3-fold and 3.3-fold increase in uterine weight 24 and 48h following the onset of oestrus, respectively. Others have shown that an increase in the ratio of oestrogen: progesterone leads to an increase in flow of blood through uterine vasculature (Ford 1982) largely on day 2 of oestrus, decreasing again between day 2-8 (Johnson *et al.* 1997). An increase in blood flow would certainly aid pre-ovulatory myometrial cervix contractions which occur 1-2 days prior to oestrus in sheep, rising in amplitude from 7-45 mm Hg during oestrus (Polovtseva 1940). It could therefore be concluded that increasing levels of oestrogen, such as that during early oestrus, causes hypertrophy and contractions of the uterine epithelium in sheep. It also suggests that the greater the uterine tone, the greater the level of ooestrogen, signifying that ovulation is imminent. Similar conclusions have also been drawn for the cow, where uterine tone was reported to increase during oestrus, decreasing following ovulation (Bonafos *et al.* 1995). This

physiological uterine response is known to be beneficial in sperm transport, with contractions helping to propel spermatozoa towards the oviduct and site of fertilisation (Hawk and Conley 1974; Hawk and Cooper 1977). Up until now, the results presented in this paper are the first to show the relationship between increased uterine tone on sperm fertilisation following AI and later embryo development and/or implantation. Therefore, it could now be recognised that greater oestrogen concentration and uterine tone in sheep is an indicator of successful oestrus synchronisation, onset of ovulation and optimal time of insemination, promoting sperm and oocyte survival in the female tract.

While fresh spermatozoa remains fertile in the female reproductive tract for up to 50 h oocytes only remain viable for fertilisation between 6-8h after ovulation (Hunter and Nichol 1993). Given the difference in length of viability, it remains essential for the spermatozoa to be present before ovulation. It would be of interest to map the sequence of events following deposition of semen in ewe with a uterine tone of four or five, her subsequent time of ovulation and success of fertilisation. This would enable a more accurate window of insemination to be established aiding industry should uterine tone or sperm quality be sub-optimal.

The outcome of the final model considers the influence of uterine tone alongside the influence of random effects, including breeding season, Site, AI date and sire. The calculation of the intraclass correlation coefficient (ICC) of ewe pregnancy rate served as a valuable metric to gauge the appropriateness of random terms within the model (Liljequist *et al.* 2019). In other words, those factors returning the highest ICC justified its inclusion in the random model. (Liljequist *et al.* 2019). Within the current study, the proportion of variability caused by the breeding season, site, sire and AI date in the final model was 8.92%, 45.99%, 29.94% and 15.15%, respectively. A similar concept was explored by (Abdalla *et al.* 2019), when they investigated factors affecting bull fertility. By modelling the effect of individual sires as a random effect, this considered the unobserved effects that occur due to data clustering. It is

also estimated as a 'distributed' value rather than a 'fixed' value. Subsequently, this can control for the variation unable to be explained by the fixed effects in the final model (Abdalla *et al.* 2019).

The current results suggest that regardless of the uterine tone of the ewes, the pregnancy outcome will be further affected depending on the sire used. The fertility of each sire used in the study (N=253) is represented in Figure 2.2, with an average pregnancy success of 68.13 ± 0.85%, ranging from 16.17 to 93.75%. This result remains consistent with other reports in species, including beef cattle (Kasimanickam *et al.* 2008; Schenatto *et al.* 2016), dairy cattle (Abdalla *et al.* 2019), stallions (Suliman *et al.* 2020) and boars (Huang *et al.* 2023), which show variation in fertility rates between males and even across individual ejaculates. The heterozygosity between sires is hypothesised to be related to their differences in semen quality (Gillan *et al.* 2008; Beilby *et al.* 2009; Love 2011; Kumaresan *et al.* 2017; Macías *et al.* 2020; Vallet-Buisan *et al.* 2023). Whilst male predictor variables were not considered within this current model, subsequent research combining male, and female parameters will enable an investigation into how uterine tone influences fertility when exposed to samples with variable quality. This would then determine whether *in vitro* semen factors contribute to variation in fertility exhibited across male ejaculates and inseminate doses. Previous studies have shown potential predictors of ram fertility to be linked to the concentration of sperm frozen and subsequently inseminated (Visser and Salamon 1974b; Alvarez *et al.* 2012), motility, velocity and other kinematic parameters (Del Olmo *et al.* 2013) as well as morphology (Almadaly *et al.* 2016; Almadaly *et al.* 2019) when addressed individually. Yet, it is now essential to combine these parameters together in the one advanced *in vitro* semen assessment, analysing the same sample of semen used to inseminate ewes in an AI program. Other advanced semen characteristics also need to be considered including but not limited to DNA integrity (Nordstoga *et al.* 2013), acrosome integrity (O' Meara *et al.* 2008), cell viability (Santolaria *et*

al. 2015), membrane fluidity (Pérez-Pé *et al.* 2002; O' Meara *et al.* 2008) and the level of oxidative stress within the cell. Future studies will assess these factors together with female fertility parameters to accurately assess pregnancy probability following intrauterine laparoscopic AI.

The impact of the site, AI date, and breeding season also remain key contributors to variation, regardless of ewe uterine tone. As represented in Figure 3, there are significant differences in pregnancy rates exhibited across sites and breeding seasons around Australia. Collectively, program location and date as well as breeding season contributed 62.06% of the variation in AI pregnancy success. Despite following best industry practices and all assessors being experienced, given the subjective nature of *in vitro* sperm processing, freezing and thawing, it is inevitable that each program and thaw day will contribute variation. Indeed, this has been shown in other parts of the world (Ybañez *et al.* 2017). Yet, this only reinforces the need to conduct further investigation into the standardisation of key semen processing factors for the sheep industry, streamlining protocols for artificial breeding companies and reducing potential variation on AI success. Furthermore, AI programs can be influenced by elements such as temperature and rainfall (Abecia *et al.* 2016; Đuričić *et al.* 2019), nutrition (Abecia *et al.* 2006) and animal management (Henry *et al.* 2018). It is clear through previous literature that these effects remain predominant in the success of any breeding program whether natural or artificial. Changes in atmospheric levels of carbon dioxide, higher temperatures, variability of precipitation and increases in extreme weather events (Henry *et al.* 2018), could also affect the consistency of AI success. Primarily, due to the inability to control and maintain optimal environmental conditions for both ewes and rams to be most productive. Changes in environmental conditions can also have direct impacts on animal physiology, behaviour, production and welfare (Henry *et al.* 2018), further affecting reproduction and pregnancy success. As such, future studies will need to consider the environmental conditions during the

time of AI, alongside, male, and female factors. In doing so, this aims to further develop our understanding of the optimal conditions for intrauterine laparoscopic AI and how factors can be used to predict pregnancy success. Ultimately, this will allow producers to best support and mitigate varying conditions to ensure reliable and consistent AI results.

2.6. CONCLUSION

In summary, the results from the present study indicate that when female factors or factors recorded at the time of AI are considered, the uterine tone of ewes influences the likelihood of successful pregnancy. Furthermore, a significant amount of variation was attributed to both the site and sire used. To better understand how these factors are related, future models must also assess site (or environmental factors) and *in vitro* semen factors from the sires used during AI to accurately explain the variation witnessed across AI breeding programs. Assessing the impact of *in vitro* semen qualities of samples used during AI programs, such as sample volume, sperm motility, concentration, morphology, cell permeability, viability, acrosome integrity and oxidative cell stress on sire fertility, will help to develop a better understanding of what contributes to a successful AI program. It will also help reinforce recommendations for semen processing and, with future work, establish a set of semen standards that could be used to screen and assess a sire's fertility prior to AI. Overall, the results provide strong evidence of the importance of assessing uterine tone at the time of insemination as an indicator of oestrus synchronisation, the onset of ovulation and optimal timing of semen deposition. These results will have an ongoing impact on artificial breeding companies and stud breeders alike, reducing the variability in AI success rates and increasing the number of lambs born to elite flocks across the Australian Merino industry.

Chapter 3. A multivariate model for the prediction of pregnancy following laparoscopic artificial insemination of sheep

The experiments described herein have been published as: Spanner, E.A., de Graaf, S.P., Rickard, J.P., 2024. A multivariate model for the prediction of pregnancy following laparoscopic artificial insemination of sheep. *Scientific Reports* 14, 27556. doi: 10.1038/s41598-024-79253-x

3.1. ABSTRACT

The causes of variation in the success of laparoscopic artificial insemination in sheep are not well understood. As such, this study incorporated the contributions of multiple male and female factors relevant to the success of AI into a comprehensive prediction model for pregnancy success. Data from Merino ewes (N=30,254) including age, uterine tone (1; pale/flaccid-5; turgid/pink), intra-abdominal fat (1; little to no fat present-5; high fat), time of insemination and sire used, were recorded during AI. A subset of semen per sire (N=388) was thawed and assessed for volume, subjective motility, sperm concentration, and morphology. Sperm motility (CASA), viability and acrosome integrity (FITC-PNA/PI), membrane fluidity (M540/Yo-Pro), mitochondrial superoxide production (Mitosox Red/Sytox Green), lipid peroxidation (Bodipy C11), level of intracellular reactive oxygen species (H2DCFDA) and DNA fragmentation (Acridine Orange) were also assessed 0, 3 and 6 h post-thaw. Logistic binomial regression revealed sperm concentration (P<0.001), CASA parameters at 0 h (PCA3; P=0.03), viable acrosome intact sperm at 6 h (P=0.02), abnormal morphology (P<0.001), uterine tone (P<0.001) and intra-abdominal fat (P=0.03) of ewes influenced likelihood of

pregnancy. Results generated will help standardise the pre-screening and selection of semen and ewes prior to artificial breeding programs, reducing variation in the success of sheep AI.

3.2. INTRODUCTION

The efficient and sustainable production of sheep requires consistent genetic improvement, most rapidly achieved through the application of assisted reproductive technologies, such as laparoscopic AI. It is generally accepted that 70% of ewes inseminated via laparoscopic AI should fall pregnant (Geenty *et al.* 2014), yet variation in success is apparent between geographical regions, across breeding seasons, sires and even ejaculates of the same sire (Eppleston and Maxwell 1995; Hill *et al.* 1998; Del Olmo *et al.* 2013; Gibbons *et al.* 2019). This uncertainty surrounding the reliability of AI outcomes has contributed to waning adoption and subsequent negative flow on effects to the rate of genetic and production gains to the national flock. Identifying specific female and male fertility factors, particularly *in vitro* semen characteristics which are linked to pregnancy success following AI, would enable producers and breeding companies to screen sires and frozen samples prior to breeding programs, eliminating samples likely to give sub-optimal results. This would help reduce variability in program success and give the industry greater confidence in the application of AI.

The ability to predict the success of AI based on a sire's semen characteristics or ewes' condition has long been sought. While several factors are known to influence fertility both during natural and AI (Visser and Salamon 1974a; Visser and Salamon 1974b; Langford 1982; Ainsworth and Downey 1986; Maxwell 1986b; Maxwell and Barnes 1986; Walker *et al.* 1986; Walker *et al.* 1989; Findlater *et al.* 1991; Scudamore *et al.* 1993; Hill *et al.* 1998; Naqvi and Gulyani 1998; Fukui *et al.* 1999; Pérez-Pé *et al.* 2002; King *et al.* 2004; Menchaca *et al.* 2004; O' Meara *et al.* 2008; Vilariño *et al.* 2010; Olivera-Muzante *et al.* 2011; Alvarez *et al.* 2012; Del Olmo *et al.* 2013; Nordstoga *et al.* 2013; Santolaria *et al.* 2015; Swelum *et al.* 2015; Almadaly *et al.* 2016; Almadaly *et al.* 2019; Macías *et al.* 2020; Riesco *et al.* 2020; Bruno-

Galarraga *et al.* 2021; Hitit *et al.* 2021; Walker *et al.* 2023a), correlations to fertility outcomes in sheep have been largely contradictory and fail to consider multiple male *and* female factors in the same study, comparing within the individual ewe rather than flock average. Our previous work (Spanner *et al.* 2024c) analysed data collected on ewes during AI and showed uterine tone (considered a proxy for the physiological response of ewes to the oestrous synchronisation protocol) to be an important indicator of AI success. Ewes, which scored a uterine tone of 4 or 5 at the time of AI, recorded a 12.41% increase in pregnancy rate compared to ewes, which scored a uterine tone of 1 or 2 ($P<0.05$). Similarly, ewes that scored a uterine tone of 3 recorded notably lower pregnancy rates compared to ewes with a uterine tone score of 4 or 5 ($P<0.05$). It is therefore now imperative to assess the influence of ewe factors, like uterine tone, on fertility in conjunction with the *in vitro* characteristics of the semen used for AI in the same model.

Today, with the advance of objective semen assessment techniques, there is a wide variety of semen parameters known to collectively define a 'fertile' spermatozoon (Spanner *et al.* 2024a). Previous research on bulls (Januskauskas *et al.* 2000; Gillan *et al.* 2008; Sellem *et al.* 2015), rams (Palacín *et al.* 2013; Santolaria *et al.* 2015; Vašíček *et al.* 2022), stallions (Morrell *et al.* 2008; Love 2011), and boars (Didion *et al.* 2009) have all identified a correlation between sperm motility and velocity parameters (Bailey *et al.* 1994; Holt *et al.* 1997; Januskauskas *et al.* 2003; Palacín *et al.* 2013), as well as sperm morphology (Kruger *et al.* 1988; Phillips *et al.* 2004; Perry 2021) with pregnancy success. Additional research has also looked at the concentration at which sperm is frozen prior to AI, as a proxy for insemination dose (Visser and Salamon 1974b; Alvarez *et al.* 2012) having an effect on pregnancy success. However, the use of flow cytometry and intracellular fluorochromes now enables us to study alterations in sperm membrane phospholipids (Thundathil *et al.* 2000; Almadaly *et al.* 2021), cell viability, acrosome integrity (O' Meara *et al.* 2008; Vicente-Fiel *et al.* 2014), DNA fragmentation

(Evenson and Jost 2000), and measures of excessive reactive oxygen species (Peris *et al.* 2007; Vašíček *et al.* 2022) and mitochondrial function (Kumaresan *et al.* 2017). While certain studies have adeptly assessed the influence of these specific semen characteristics on ram fertility following AI (Sánchez-Partida 1999; Pérez-Pé *et al.* 2002; Papadopoulos *et al.* 2005; O' Meara *et al.* 2008; Vicente-Fiel *et al.* 2014), limited standardisation in their application across studies has contributed to contradictions in their described effect on fertility. Notably, stemming from the multitude of tests available for a single trait, a lack of direct comparison between samples inseminated and analysed, as well as reduced sample sizes, have limited the likelihood of successful fertility prediction (Spanner *et al.* 2024a).

As such, the present study sought to determine the influence of female data collected during AI and *in vitro* semen assessment characteristics post-thaw on the likelihood or probability of pregnancy occurring following laparoscopic AI in sheep. Results will lead to a better understanding of which *in vitro* semen traits correlate to fertility and, therefore, the fertility potential of a particular frozen-thawed sample. The design of a model to predict AI would also facilitate the identification of accurate standards for the sheep artificial breeding community. When optimal semen is combined with a fertile, well-conditioned ewe, the likelihood of pregnancy should increase, reducing the variability of AI programs and reproductive failure.

3.3. METHODS AND MATERIALS

3.3.1. Ethics and Animals

The data used in this research was generously donated by artificial breeding companies and stud breeders during routine commercial AI operations. Animals are not directly involved in this study, as such, no additional ethical approval was required. All procedures were conducted with consultation from the University of Sydney Animal Ethics Committee.

The management of ewes and rams adhered to the standard industry practices and requirements for each site, and all methods complied with relevant guidelines and regulations. All methods are reported in accordance with the ARRIVE guidelines.

3.3.2. Breeding Seasons and Location of the Animals

AI data was collected in Australia between November and April during 2020-21 (N=9,817 ewes, 123 sires, 10 sites), 2021-22 (N=8,253 ewes, 116 rams, 9 sites) and 2022-23 (N=12,184 ewes, 149 rams, 11 sites). Sites were located in the Central and South Wheat Belt of Western Australia (Mediterranean climate: hot, dry summers and mild, wet winters), Central North of Victoria (Temperate climate: cool to mild winters and warm to hot summers), Murray Land Yorke Peninsula of South Australia (Mediterranean climate: dry, hot summers and mild, rainy winters) and the Central West (Temperate climate: warm summers and cool winters), Tablelands (Oceanic climate: cooler with higher rainfall) and Northwest Slopes and plains (Subtropical climate: hot summers and mild winters) of NSW. Animals were selected and managed for artificial breeding programs as per individual commercial stud preferences.

3.3.3. Experimental Design

Merino ewes (N = 30,254, split across three breeding seasons and 30 commercial AI programs conducted on farms located in NSW, VIC, SA and WA) were synchronised for oestrus and assessed for uterine tone, intra-abdominal fat, age, PMSG dose and time of insemination post-CIDR removal as part of routine AI protocols, as per the previous study (Spanner *et al.* 2024c). Following industry standards, ejaculates from Merino sires (N=388) were collected and immediately laparoscopically inseminated (fresh; N=29 ejaculates) or frozen (N=359) as either pellets (N = 239) or straws (N= 120), thawed and then laparoscopically inseminated into ewes. Parameters including season, day, site, sire and type of semen used, uterine tone, and intra-abdominal fat were recorded during AI. Synchronised ewes (0.3g progesterone CIDR; Zoetis, Australia and eCG; Minitube, VIC, Australia) received approximately 0.2mL of semen per

uterine horn. Each ewe underwent pregnancy scanning approximately 55 days post-AI using standard industry practice. Approximately 2 pellets or 5 straws per batch per sire used for insemination were sent to The University of Sydney, for advanced *in vitro* semen assessment. Individual ewe pregnancy was match to semen assessed *in vitro* at a sire level.

3.3.4. Assessment of Ewe Factors

3.3.4.1. Assessment of ewe age

The colour of the ear tag located in the left ear of each ewe indicated the year of birth. Each colour represents a year of drop (Table 2.1). This was then subtracted from the current year (2020, 2021, 2022, or 2023) of AI to determine the age of each ewe at AI.

3.3.4.2. Assessment of intra-abdominal fat score

During laparoscopic AI, the internal fat covering the abdominal organs was visualised and subjectively assessed by the technicians performing the insemination, scoring the ewe between one (little to no fat present) to five (high abundance of fat present) (Table 2.2) as per the previous study (Spanner *et al.* 2024c).

3.3.4.3. Assessment of uterine tone score

At the same time as the intra-abdominal fat assessment, the tone of the uterus was scored as a subjective observation by the technicians and recorded as a value between one (pale, flaccid uterus) to five (bright pink turgid uterus) (Table 2.3) as per the previous study (Spanner *et al.* 2024c).

3.3.4.4. Assessment of AI time post-CIDR removal

As per the previous study (Spanner *et al.* 2024c), during oestrous synchronisation, each ewe was assigned a CIDR pull group to ensure ewes were inseminated within the optimal time frame. At the time of insemination in the cradle, the eID tag of each ewe was scanned using a Tru-Test XRS2 (*Tru-Test Datamars, Australia*) giving a time stamp for data collection in the

cradle. To determine the time of AI post-CIDR removal, this was subtracted from the end time of the CIDR pull group each ewe was allocated. This was standardised across each CIDR pull group across all programs. Data was presented as hh:mm:ss post-CIDR pull.

3.3.5. Advanced in vitro Assessment of Sperm Characteristics Post-thaw

A subset of the semen used from each sire was stored and thawed within the same breeding season and the AI program. Pellets (n=2) were thawed in a glass thawing tube for 2 minutes in a 37ºC water bath with agitation, while straws (n=4 straws) were thawed for 30 seconds in a 37ºC water bath with agitation. The total volume per sample was recorded by suspending the sample in a pipette prior to being diluted 1:0.5 with PBS + 0.3% BSA (Phosphate Buffed Saline + 0.3% Bovine Serum Albumin; pH 7.4, osmolarity 297). This was then held at 37ºC over a 6 h incubation period. Following an initial assessment of sperm concentration (described below; 3.3.5.1), an aliquot of each sample was taken at 0, 3 and 6 h post-thaw and further diluted to 50×10^6 sperm/mL with PBS + 0.3% BSA.

3.3.5.1. Assessment of sperm concentration, subjective motility, and percentage of abnormal morphology post-thaw

The concentration of each frozen sample was determined using a NucleoCounter SP-100 (ChemoMetec) immediately post-thaw. 50 µL of semen was diluted with S100 reagent (ChemoMetec) and analysed according to the manufacturer's instructions. Concentration recordings were taken twice (within 10 %) and the average was used for further calculations.

Subjective motility was first assessed after the initial 1:0.5 dilution with PBS + 0.3 % BSA as well as following dilution of the sample to 50×10^6 sperm/mL at each time point. The percentage of motile spermatozoa was subjectively assessed using a phase-contrast microscope (x100) described by (Evans and Maxwell 1987). Samples (6 µL) were placed on slides and enclosed using a 22 × 22 mm coverslip warmed to 37 ºC. Values were obtained to the nearest 5% by examining five fields of each sample (kept on a heated slide and coverslip at 37ºC).

After thawing, 10 µL of semen was fixed with 190 µL (1+10 dilution) in 3 % NaCl. Within a 24h timeframe, the percentage of abnormal spermatozoa was subjectively assessed using a phase-contrast microscope (x400). Capturing a minimum of 200 cells, the results were converted into the percentage of the sample containing spermatozoa with abnormal morphology. Morphological defects include head defects (detached heads, acrosomes reacted, amorphic heads), damaged midpieces (proximal droplets, bent midpieces) and tail abnormalities (distal reflex, coiled tails, broken tails). As such, this was a measure of the proportion of abnormal spermatozoa.

3.3.5.2. Assessment of sperm motility and kinetics analyses using a computer-assisted sperm analysis (CASA)

Sperm motility was measured using the computer-assisted sperm analysis (HT CASA IVOS II (Animal Breeder) Version 1.13.7; Hamilton-Thorne, USA) using the appropriate settings for ram spermatozoa (this includes, amongst others; head size 10–42 µm^2, progressive motility thresholds of straightness 80 % and average path velocity 75 µm/s). Samples were further diluted to a concentration of 25×10^6 sperm/mL with PBS+ 0.3% BSA before 6 µL was placed on slides warmed to 37 ºC (Cell Vu; Millennium Sciences, Mulgrave, Victoria, Australia) and enclosed with a 22 × 22 mm coverslip. For each sample, eight fields of video recordings were recorded, capturing a minimum of 200 cells (frame rate 60 Hz). Motility and kinematic parameters were subsequently calculated including total and progressive motility, ALH, BCF, LIN, STR, VAP, VCL, VSL, and WOB.

3.3.5.3. Flow cytometric analysis

Samples were assessed for a range of membrane and metabolic indicators at a final concentration of 10×10^6 sperm/mL following staining with various fluorochromes. Using the CytoFLEX (CytoFLEX and CytExport 2.0 Software Beckman Coulter; USA), three lasers were employed; 50 mW 488 nm, 50 nW 638 nm and 80 mW 405 nm. All samples were stained

with the DNA probe Hoechst 33342 (final concentration of 1µg/mL), which has a fluorescence detection filter of 450/45 BP, to gate any possible debris in the sample. Sperm cells were isolated from total events based on 488 nm forward and side scatter profiles. For each of the below variables, 10,000 sperm cells were analysed, and a minimum of 1,000 Hoechst 33342 positive events were required to obtain valid results.

Assessment of sperm acrosome integrity and viability

Sample preparation for sperm viability and acrosome integrity was performed as previously described (Pool *et al*. 2020), by staining a combination of Propidium Iodine (PI, final concentration 6μM) and Fluorescein isothiocyanate peanut agglutinin (FITC-PNA, final concentration 0.4μg/mL) for 10 minutes at 37 ^{0}C. PI and FITC-PNA fluorescence detection was on 690/50, and 525/40 nm bandpass (BP) filters, respectively. Cells were considered viable with intact acrosomes if cells were both PI and FITC-PNA negative.

Assessment of sperm membrane lipid fluidity

Changes in lipid fluidity within the membrane of viable spermatozoa were assessed using a staining combination of both merocyanine 540 (M540, final concentration 0.83 μM) and Yo-Pro (final concentration 25 nM) for 10 minutes at 37 ^{0}C. The fluorescence of M540 and Yo-Pro was detected on a BP filter of 585/42 nm and 525/40 nm, respectively. A sperm population was considered viable if it was recorded as Yo-Pro negative. The median value for M540 fluorescence of this viable population was used to determine the relative membrane lipid fluidity. Results with a greater mean value corresponded to greater lipid destruction on the membrane, thus, greater membrane fluidity.

Assessment of mitochondrial superoxide production

Mitochondrial superoxide production was assessed using a dual stain combination of Mitosox Red (final concentration 2.5 µM) and Sytox Green (final concentration 30 nM) for 20 minutes at 37 ºC. Mitosox Red fluorescence was detected at 585/42 and Sytox Green fluorescence on

525/40 BP filters. A sperm population of Sytox Green negative, “live”, was used to determine the median Mitsox Red fluorescence value relative to the amount of mitochondrial superoxide production. A positive control was created by combining each sample with 5 μM hydrogen peroxide to stimulate mitochondrial superoxide production. The positive control was used to assess the effectiveness of the stain and determine the appropriate gating of stained populations.

Assessment of lipid peroxidation

Lipid peroxidation of the sperm membrane was assessed using Bodipy C11 (581/591). Samples were aliquoted and stained with the Bodipy C11 probe (final concentration 10 μM) at 37 ºC for the entirety of the 6 h assessment. At each time point, the samples had a timed incubation for 30 minutes. Once staining was complete, samples were centrifuged for 10 minutes at 800 ×g. After removing the supernatant, each pellet was resuspended in a PBS + 0.3 % BSA buffer and counterstained with PI (final concentration 6 μM) for 10 minutes at 37 ºC before running on the CytoFlex. The detection of lipid peroxidation was measured by both 585/42 and 525/40 BP filters. The live population was first gated and used to determine the percentage of cells with positive Bodipy C11 fluorescence. This indicated the relative change in lipid peroxidation of a given sample. A positive control was made up by combining aliquots of all samples and incubated with 5 μM hydrogen peroxide to induce greater lipid peroxidation. The positive control was used to assess the effectiveness of the stain and determine the appropriate gating of stained populations.

Assessment of intracellular reactive oxygen species (ROS)

To determine the relative amount of oxygen species (ROS) within a cell, a staining combination of dichlorodihydrofluorescein diacetate acetyl ester (H2DCFDA final concentration 5 μM) and PI (final concentration 6 μM) was used. Samples were aliquoted and stained with the H2DCFDA at 37 ºC for the entirety of the 6 h assessment. At each time point, the samples had a timed incubation of 1h before being centrifuged for 10 minutes at 800 ×g. After removing

the supernatant, pellets are resuspended in a PBS + 0.3 % BSA buffer and counterstained with PI (final concentration 6 μM) for 10 minutes at 37 ºC before running on the CytoFlex. The H2DCFDA fluorescence was determined on the 525/40 BP filter. The H2DCFDA fluorescences in live cells (PI negative) was used to measure intracellular ROS production. A positive control was made up by combining aliquots of all samples and incubated with 5 μM hydrogen peroxide to induce greater ROS. The positive control was used to assess the effectiveness of the stain and determine the appropriate gating of stained populations.

Assessment of DNA integrity

At 0 and 6 h post-thaw, 40 μL of each sample (50×10^6 sperm/mL) was aliquoted for DNA assessment. Each sample was washed by resuspending the sample in 2 mL of PBS+ 0.3 % BSA and centrifuging for 10 minutes at 800 ×g. The supernatant was removed before resuspension and repeat centrifugation After the final spin, the supernatant was removed, and pellets (final concentration approximately 2×10^6 sperm) were snap-frozen in liquid nitrogen for 30 seconds before being stored at -80 ºC until assessment.

DNA fragmentation was measured using flow cytometry on a (Cytek Aroura 3L; Sydney Flow Cytometry) after staining with Acridine Orange (AO), as described by Evenson and Jost (2000), with some minor changes. In summary, snap frozen samples were diluted to a concentration of 2×10^6 sperm/mL with a TNE buffer (0.15 M NaCl, 0.01 M Tris HCl, 1 mM disodium EDTA pH 7.4). A 100 μL aliquot of the sample was taken and diluted with 200 μL of Acid Detergent Solution (0.08 N HCl, 0.15 M NaCl, 0.1 % TritonX 100 pH 1.2), which was gently mixed by swirling in hand for 30 seconds. Once mixed, samples were stained with 600 μL of Acridine Orange (final concentration 6μg/mL) for 3 minutes before being assessed using flow cytometry. Green (B2) and Red (V11) fluorescence were detected using the 528/21 and 644/27 BP filters, respectively. The flow rate was set to slow, and a minimum of 1,000 cells were recorded per sample. DNA fragmentation was determined by the relative amount of

single-stranded DNA (ssDNA) in proportion to the total amount of spermatozoa (dsDNA + ssDNA) and indicated by the amount of red fluorescence regarding the total amount of fluorescence.

3.3.6. Measure of Fertility Determined by Ultrasound

Depending on the AI program and site, the pregnancy status per ewe was determined approximately 55 days post-insemination. Ewes were fasted 24h prior to scanning. A real-time cutaneous ultrasound (Oviscan 6 with a 3.5 MHz probe) was used to scan each ewe to determine the presence of fetuses and their number. As per the previous study (Spanner *et al.* 2024c), pregnancy from AI was recorded as either 1 (pregnant) or 0 (empty), while the number of fetuses observed was recorded as the exact number.

3.3.7. Statistical Analysis

Ewe ID was matched between AI and pregnancy datasets while sire ID was matched between AI and *in vitro* semen analysis datasets to create one Masterfile. All data was therefore compared within the individual ewe. Data was then cleaned to remove ewes without pregnancy data. All statistical analyses were performed on R Studio (Version 2023.09.1+494).

In accordance with the previous study (Spanner *et al.* 2024c), the overall pregnancy data was assessed to determine average pregnancy and reproductive rates. AI success was determined by calculating the number of ewes pregnant over the total number of ewes inseminated. The reproductive rate was determined by calculating the number of offspring (fetal number) over the total number of ewes inseminated. Descriptive statistics were performed to evaluate the number of ewes inseminated and the percentage pregnant for each categorical factor level recorded, as well as site, sire ID and breeding season. All values included mean ± standard error of the mean (SEM) and were de-identified for anonymity.

At 0, 3 and 6 h post-thaw, the relationships between *in vitro* semen traits were assessed by Spearman rank correlation. As the CASA measurements were highly correlated, measures

of total motility, progressive motility, ALH, BCF, LIN, STR, VAP, VCL, VSL, and WOB were combined using a Principal Component Analysis (PCA, Table 3.1) to reduce multiple testing bias and collinearity. The first 3 principal components were significant (eigenvalues >1), and together accounted for 92 % of the variation in the data (Table 3.1). PCA1, accounted for 54.92 % of the variation and was interpreted as a composite measure of sperm velocity, with positive loadings (<±0.30) from WOB, VSL, VAP, STR, LIN and BCF (Table 3.1). PCA2, accounting for 24.73 % of the variation, had a strong positive loading of VCL, VAP and ALH (Table 3.1), and again is interpreted as a measure of sperm velocity. PCA3 contributed 12.90 % of the variation and had a strong negative loading from Total Motility and Progressive Motility (-0.70 and -0.53, respectively), interpreted as a measure of sperm motility. The significant components were subsequently used in the regression analyses.

Table 3.1. Eigenvalues and variances explained by the first 3 PCAs at 0 h post-thaw, along with the loading of each measurement within the PCA for CASA motility and velocity traits.

	PC1	PC2	PC3
Eigenvalue	5.49	2.47	1.29
Variance (%)	54.92	24.73	12.90
Total Motility	0.21	0.21	-0.70
Progressive Motility	0.29	0.23	-0.53
ALH	-0.29	0.45	0.01
BCF	0.32	0.05	0.05
LIN	0.39	-0.21	0.09
STR	0.38	-0.19	0.11
VAP	0.32	0.37	0.26
VCL	0.03	0.60	0.26

	PC1	PC2	PC3
VSL	0.36	0.28	0.26
WOB	0.39	-0.20	0.08

ALH, amplitude of lateral head displacement; BCF, beat-cross frequency; LIN, linearity; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble; PCA, principal component.

A logistical binomial regression analysis was used to examine the influence of female and male fertility traits on the probability of pregnancy post-AI. Additionally, an intraclass correlation coefficient (ICC) analysis was conducted to explain the proportion of variance attributed to individual factors within the random model, encompassing Site, Sire ID including those that were Frozen-thawed, and Thaw Day. A preliminary univariate analysis was then run to determine the impact of each individual factor on pregnancy achievement. The logistical binominal regression model was refined by the backward selection process, eliminating non-significant fixed effect variables and interactions ($P>0.05$). The final multivariable model included only significant factors and interactions ($P<0.05$). For all variables within the model, an odds ratio was performed to determine the likelihood of change in pregnancy following a single unit change in the factor whilst keeping the model consistent. This included the odds ratio percentage change and 95 % CL.

For any fixed categorical effects, an Emmeans pairwise comparison was performed to assess the significant difference between groups within the variable. Groups were considered significantly different to each other if the comparison returned a *P-value* of <0.05. All values are reported with mean ±SEM.

3.4. RESULTS

3.4.1. Overall Descriptive Statistics of the Dataset

Data was collected from 30,254 ewes and 388 rams from 30 sites. Table 3.2 shows the total number of ewes and sires included in the dataset and the resultant fertility following laparoscopic AI across the three breeding seasons.

Table 3.2. Overall Pregnancy data across all three breeding seasons. Calculated as the proportion of ewes pregnant compared to the total number of ewes inseminated per breeding seasons.

Breeding Season	Number of Sites	Total Ewes in the Program	Total Sires Used in the Program	Ewes Pregnant to AI (%)	Total Reproductive Rate (%)
2020-21	10	9,817	123	68.07	105.35
2021-22	9	9,253	116	69.78	119.27
2022-23	11	12,184	149	63.05	90.03
Total	30	30,254	388	66.51	102.97

Table 3.3 displays the mean ±SEM and range for each fertility factor recorded. For each *in vitro* semen parameter, it tracks the change of each trait 0, 3, and 6 h post-thaw. Additionally, Supplementary File 3.1 describes the percentage of ewes' pregnancy for each level within the categorical factors recorded in the data set.

Table 3.3. Descriptive statistics of factors recorded at AI, as well as *in vitro* semen parameters recorded 0, 3 and 6 h post-thaw, including mean (±SEM) and range for each factor.

Factor	Variables	0 h			3 h			6 h		
		Mean (± SEM)	Min	Max	Mean (± SEM)	Min	Max	Mean (± SEM)	Min	Max
Initial Assessment	Volume (µL)	509.14 ± 0.80	160	800						
	Motility (%)	57.37 ± 0.08	20	85						
	Concentration ($\times 10^6$)	569.75 ± 2.06	81.29	2283.75						
	Morphology (%)	15.46 ± 0.06	2.5	70						
	Package									
Subjective Motility	50×10^6 sperm/mL (%)	48.99 ± 0.10	10	80	40.03 ± 0.10	0	70	33.47 ± 0.10	0.1	78.4
	25×10^6 sperm/mL (%)	41.43 ± 0.12	0	85	32.17 ± 0.11	0	75	26.36 ± 0.12	0	67.7
CASA	Total motility (%)	40.77 ± 0.12	5.8	89.5	32.75 ± 0.11	1.8	87.1	29.35 ± 0.12	0	75
	Progressive motility (%)	30.21 ± 0.10	2.3	79.8	22.11 ± 0.10	0.3	73.3	20.26 ± 0.10	0	70
	ALH (µm)	6.01 ± 0.01	3.29	9.58	8.41 ± 0.07	0.40	73.19	6.79 ± 0.04	0.50	43.75

	BCF (Hz)	38.78 ± 0.03	20.97	45.67	39.62 ± 0.07	5.81	95.51	38.92 ± 0.05	6.24	74.28
	LIN (%)	63.23 ± 0.06	34.58	87.14	60.46 ± 0.08	30.49	150.09	63.26 ± 0.07	19.03	94.47
	STR (%)	90.23 ± 0.03	70.47	97.79	89.52 ± 0.10	36.97	209.53	88.99 ± 0.06	39.60	125.64
	VAP (µm/s)	117.08 ± 0.13	64.74	188.15	114.11 ± 0.12	58.56	205.17	107.11 ± 0.20	8.82	192.69
	VCL (µm/s)	179.00 ± 0.16	114.55	262.26	180.74 ± 0.24	14.42	268.76	161.05 ± 0.31	11.05	271.42
	VSL (µm/s)	106.68 ± 0.14	44.87	180.25	100.75 ± 0.17	0.8	205.29	96.75 ± 0.21	8.47	254.15
	WOB (%)	68.59 ± 0.05	47.94	89.89	66.50 ± 0.06	0.3	112.60	67.68 ± 0.08	3.4	116.16
Flow Cytometry	Acrosome Integrity and Viability (%)	20.35 ± 0.06	0.20	60.00	15.58 ± 0.05	0.20	50.1	11.74 ± 0.04	0	34.64
	Membrane Fluidity	56685.55 ± 380.32	8035.60	186186.70	58271.32 ± 382.78	4647.7	246915.6	60655.29 ± 389.77	10338.1	192183.6
	Mitochondrial Superoxide	4616.44 ± 23.24	1450.30	19392	6168.49 ± 46.86	1351.2	55111	9223.27 ± 80.17	1671.1	71570.9
	Lipid Peroxidation	398.23 ± 15.43	-4049.3	8905.5	3494.14 ± 55.25	-3525.8	55870.6	3733.28 ± 39.93	-5114.1	31011.3

	Intracellular Reactive Oxygen Species	983.23 ± 2.07	101.8	2234.1	1149.75 ± 2.87	123.9	3956.8	1289.26 ± 3.35	477.3	4496.3
	DNA Integrity (%)	3.16 ± 0.02	0.53	18.16				6.56 ± 0.05	0.29	51.69
Factors collected at AI	Tone	3.22 ± 0.005	1	5						
	Fat	3.06 ± 0.005	1	5						
	Age	3.18 ± 0.009	1	13						
	Time of AI Post-CIDR Pull	51:48:46 ± 0:00:42	43:49:26	59:30:45						

ALH, amplitude of lateral head displacement; BCF, beat-cross frequency; LIN, linearity; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble; PCA, principal component.

3.4.2. Contribution of Variation Caused by Random Terms

The ICC analysis was performed on the random model to assess the proportion of total variance contributed by random terms. The proportion of variation in pregnancy success attributed to Site, Sire frozen-thawed, and Thaw Day was 53.57 %, 22.02 %, and 24.42 %, respectively. The level of significance was not assessed on these variations.

3.4.2.1. The proportion of variation in pregnancy success contributed by site

From the multivariable model, the site where data was collected contributed 53.57 % of the variation detected between the random terms. With 30 sites across the 3 breeding seasons, the pregnancy rate ranged from 49.89 % to 89.02 % (Figure 3.1).

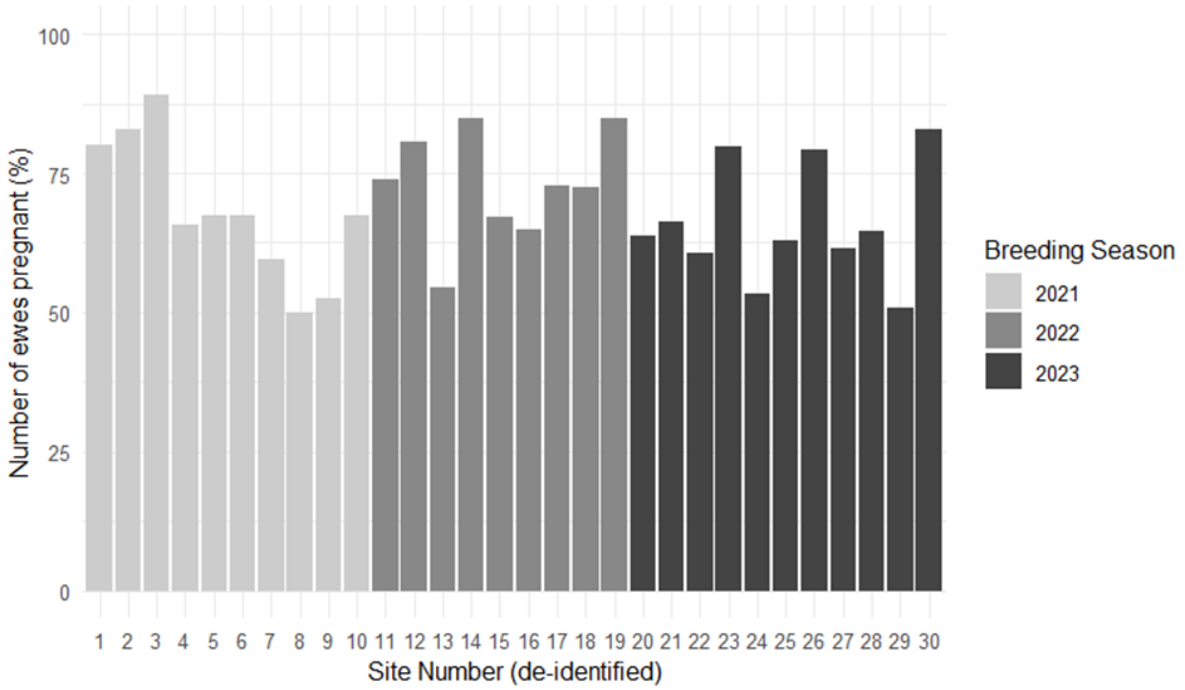


Figure 3.1: Variation in pregnancy rates for each site (deidentified, N=30) recorded during the 2021, 2022 and 2023 breeding seasons. Calculated as the proportion of ewes pregnant compared to the total number of ewes inseminated per site.

3.4.2.2. The proportion of variation in pregnancy success contributed by sire

From the multivariable model, 24.42 % of the variation detected between the random terms was contributed by the sires with frozen-thawed semen used for AI. Across the 388 sires, the pregnancy rate ranged from 15.88 % to 96.67 %, averaging 68.54 % (Figure 3.2).

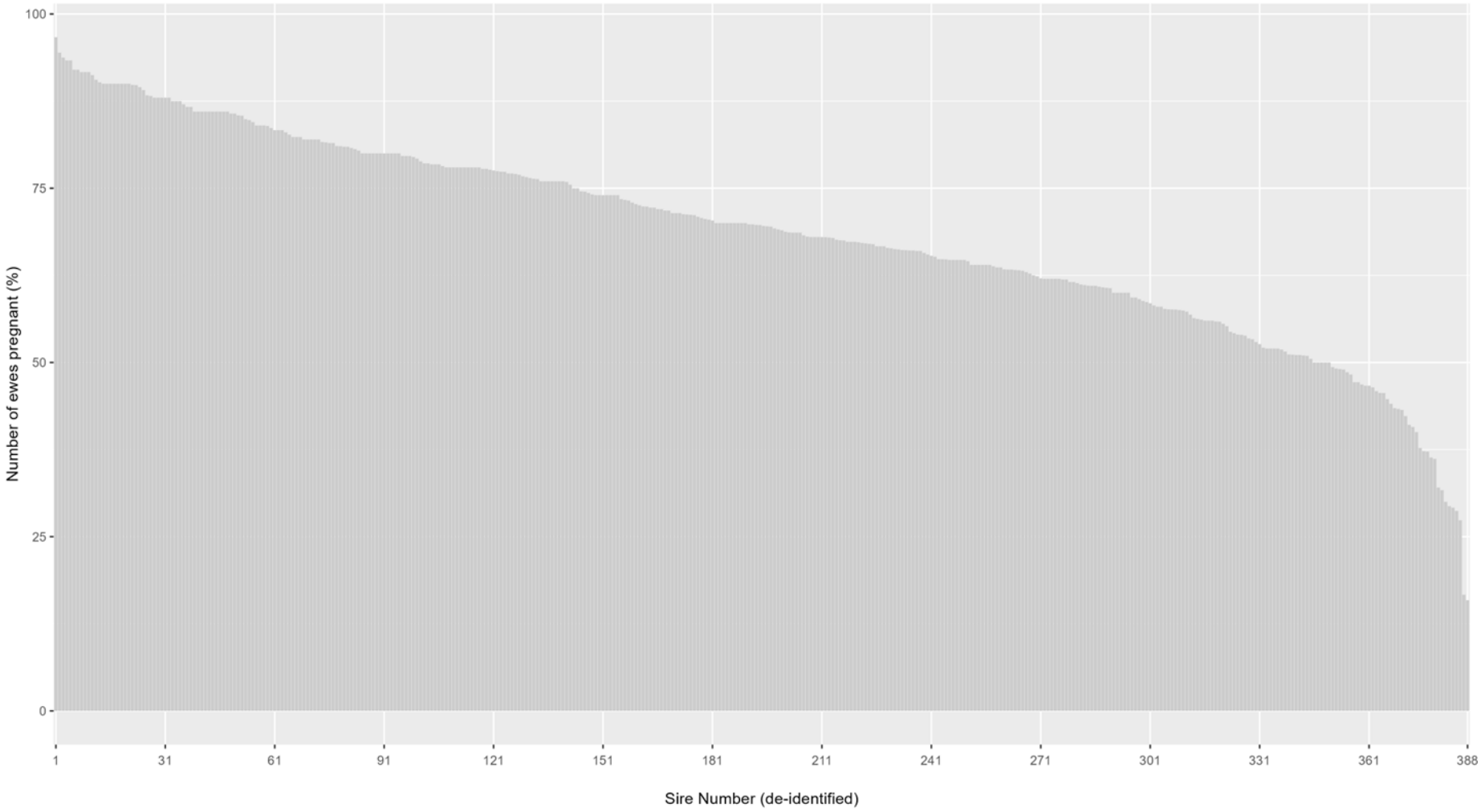


Figure 3.2: Pregnancy rate for each sire (deidentified) throughout the three breeding seasons, n=388. Calculated as the proportion of ewes pregnant compared to the total number of ewes inseminated per sire. The number of ewes inseminated per sire ranged from 7 to 361, with an average of 69.74 ± 2.15 ewes.

3.4.3. Factors Within the Model Found to Influence Pregnancy Following Laparoscopic AI of Sheep

Despite 33 factors returning significant *P-value*s when considered in a univariate logistic model (data not shown), only 7 remained significant when included in the same binomial logistic regression model. Sperm freezing concentration ($\times 10^6$ sperm/mL), the percent of morphologically abnormal spermatozoa, the proportion of viable spermatozoa with intact acrosomes at 6 h post-thaw, CASA at 0 h post-thaw, uterine tone and intra-abdominal Fat of ewes, were found to significantly ($P<0.001$, $P<0.001$, $P=0.021$, $P=0.033$, $P<0.001$, $P=0.047$, respectively, to influence the likelihood of pregnancy. There were no significant interactions between variables ($P>0.05$).

3.4.3.1. The impact of the number of sperm frozen on the probability of successful pregnancy

The average freezing concentration for a pellet and straw was 722×10^6 ±15.27 sperm/mL, and 270.75×10^6 ±16.47 sperm/mL, respectively. Freezing concentration ranged from 81.29 to 2283.75×10^6 sperm/mL and averaged 569.75 ±2.06×10^6 sperm/mL. Notably, there was no significant interaction observed between freezing concentration and package type within the model ($P>0.05$). Therefore, freezing concentration was considered across package types. Following an odds ratio calculation, it was determined that an additional 100×10^6 sperm/mL frozen in either a pellet or straw corresponded to a 5.09 % increase in pregnancy probability (OR = 1.05, 95 % CI: 1.05 to 1.05, Figure 3.3).

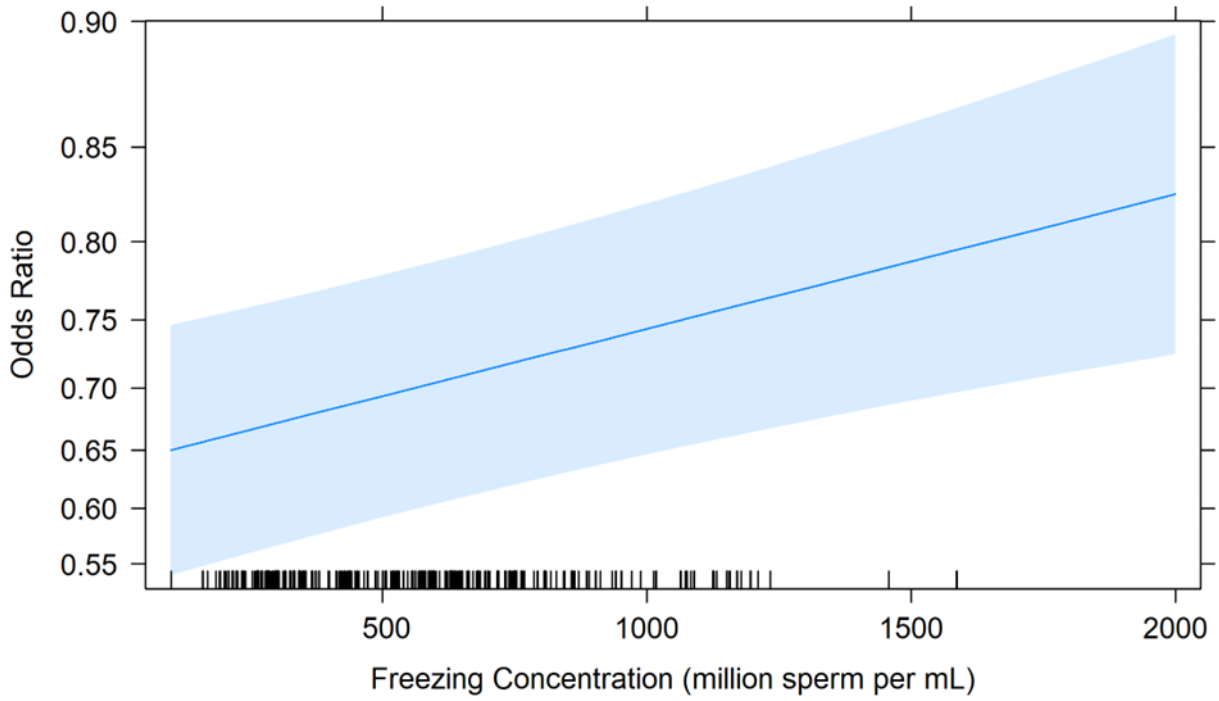


Figure 3.3: The Odds Ratio plot shows the relationship between an increase in freezing concentration per pellet or straw on the predicted probability of a ewe falling pregnant if she was laparoscopically inseminated with that sample. The predicted probability was generated from the model in RStudio, with 95 % Confidence Interval (shaded blue area). Black markers along the x-axis indicate the spread of raw data per individual sire within the model. The blue line indicates the odds ratio of the sample at the given freezing concentration.

3.4.3.2. The impact of abnormal sperm morphology on the probability of successful pregnancy following laparoscopic AI of sheep

The average number of morphologically abnormal spermatozoa per frozen-thawed sires sample was 15.46 ± 0.06 %, ranging from 2.5 to 70 % abnormal spermatozoa. The odds of a 1 % increase in abnormal spermatozoa corresponded to a 1.07 % decrease (OR = 0.99, 95 % CL: 0.98 to 1.00, Figure 3.4) in the probability of a ewe being pregnant.

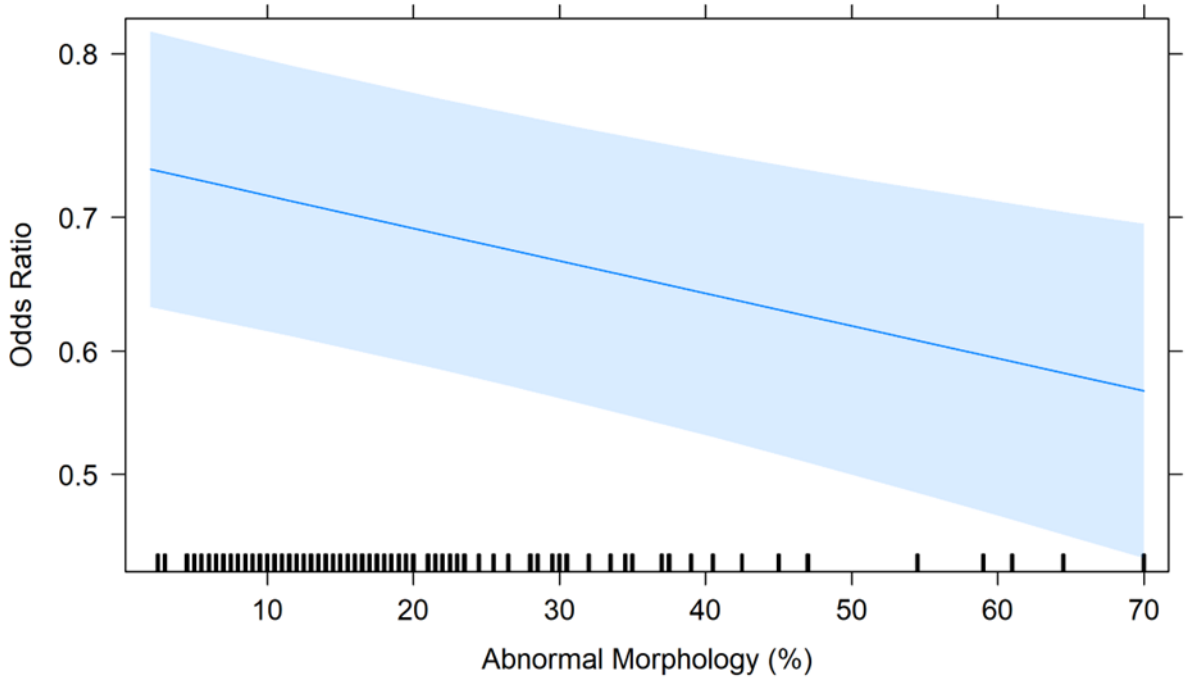


Figure 3.4: The Odds Ratio plot shows the relationship between an increase in the number of abnormal spermatozoa on the predicted probability of an ewe falling pregnant if she was laparoscopically inseminated with that sample. The predicted probability was generated from the model in RStudio, with 95 % Confidence Interval (shaded blue area). Black markers along the x-axis indicate the spread of raw data per individual sire within the model. The blue line indicates the odds ratio of the sample at the given abnormal morphology percent.

3.4.3.3. The impact of sperm viability and acrosome integrity on the probability of successful pregnancy following laparoscopic AI of sheep

At 6 h post-thaw, the average percentage of acrosome intact and viable spermatozoa for frozen-thawed sires was 11.74 % ± 0.04 %, ranging from 0 to 34.64 %. An analysis of the data revealed that the odds of a 1 % increase in the number of viable sperm with intact acrosomes corresponded to a 1.01 % increase (OR = 1.01, 95 % CI: 0.34 to 2.56) in the probability of ewes being pregnancy (Figure 3.5).

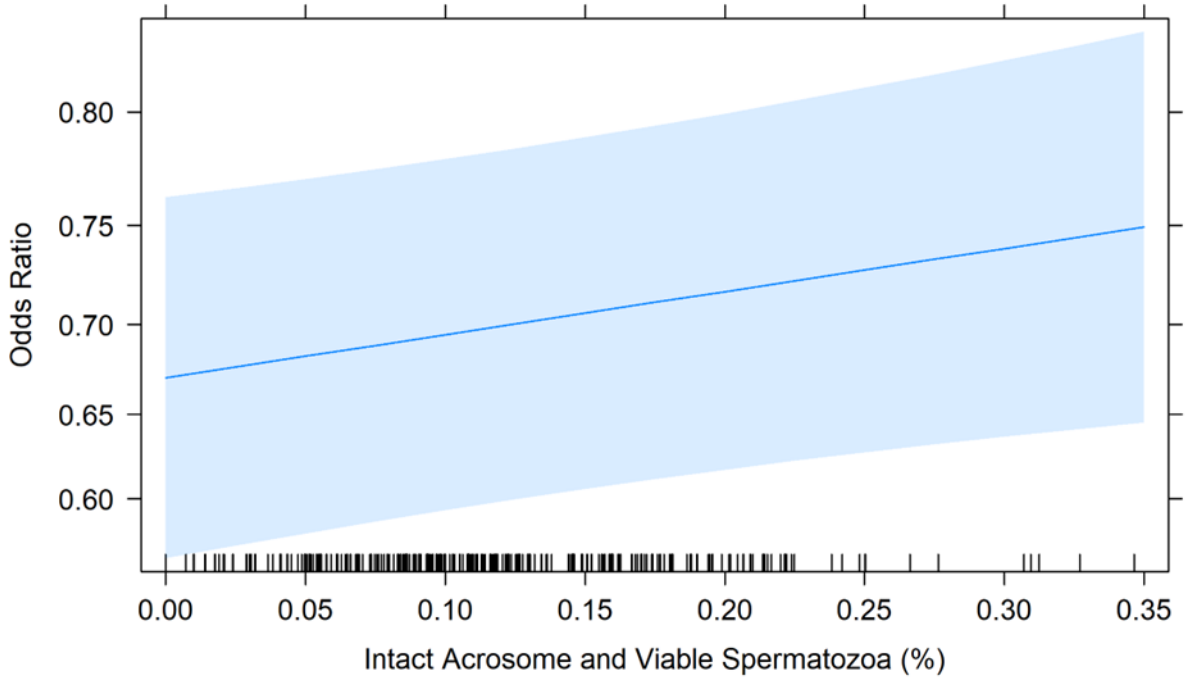


Figure 3.5: The Odds Ratio plot showing the effect of the percentage of Acrosome Intact and Viable spermatozoa on the predicted probability of an ewe falling pregnant if she was laparoscopically inseminated with that sample. The predicted probability was generated from the model in RStudio, with 95 % Confidence Interval (shaded blue area). Black markers along the x-axis indicate the spread of raw data per individual sire within the model. The blue line indicates the odds ratio of the sample at the given percentage of viable spermatozoa with intact acrosomes.

3.4.3.4. The impact of CASA motility and velocity traits (CAS PCA3) on the probability of successful pregnancy following laparoscopic AI of sheep

As seen in Supplementary File 3.2, there is a clear and strong correlation between CASA traits at 0, 3 and 6 h post-thaw. Following the formation of the PCA variable (Table 3.1), Although PCA1 and PCA2 explained a significant amount of variation, only PCA3 remained significant in the final model (P=0.033). PCA3 had a strong negative loading from Total Motility and Progressive Motility (-0.70 and -0.53, respectively), which is interpreted as a measure of sperm motility.

At 0 h post-thaw, the average total motility for frozen-thawed sires was 40.77 % ± 0.12 %, ranging from 5.8 to 89.5 %. The average CASA progressive motility for frozen-thawed sires was 30.21 ±0.10 %, ranging from 2.3 to 79.8 %. The odds ratio analysis of CASA PCA3 indicated an inverse association with pregnancy outcomes, with the odds of pregnancy decreasing by 0.37 % for every standard deviation away from the average for motility (OR = 1.00, 95 % Cl: 1.00 to 0.99, Figure 3.6). This infers that reduced sperm motility characteristics, as represented by higher CASA PCA3 scores, are associated with reduced odds of pregnancy.

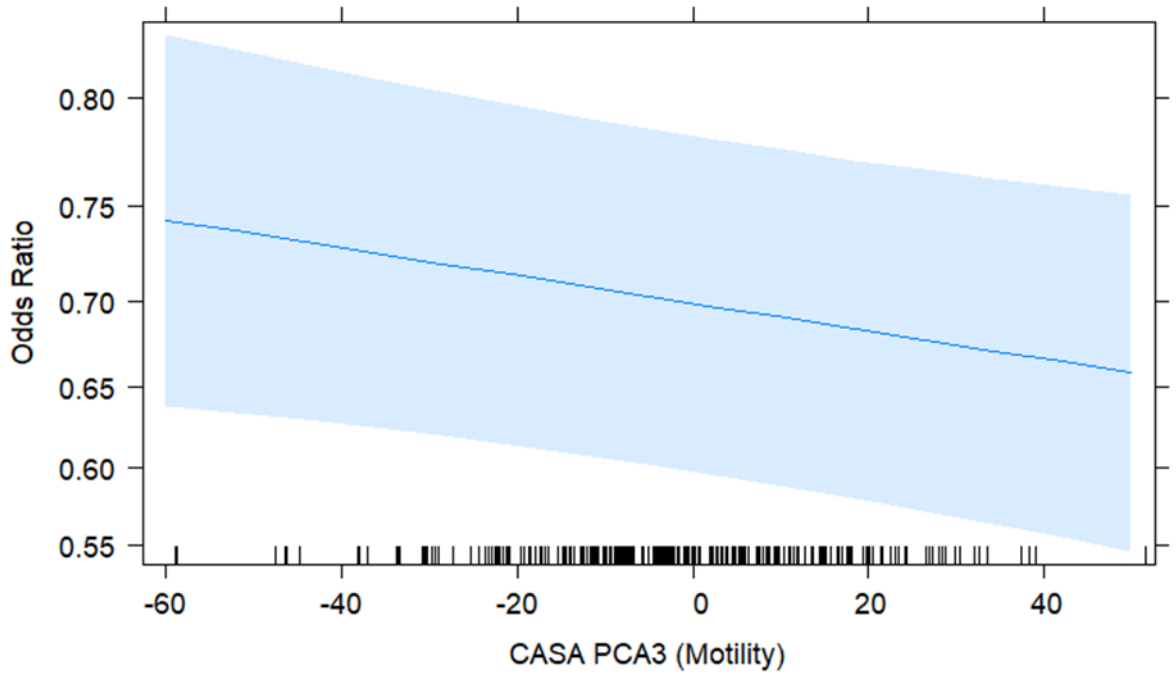


Figure 3.6: The Odds Ratio plot shows the relationship between the CASA PC3 at 0 h post-thaw on the predicted probability of an ewe falling pregnant if she was laparoscopically inseminated with that sample. The PC3 is centred around the mean value of 0, and each individual point is represented by its deviation (distance) from this mean, measured in standard deviations. The predicted probability was generated from the model in RStudio, with 95 % Confidence Interval (shaded blue area). Black markers along the x-axis indicate the spread

of each PC3 point calculated by the PCA. The blue line indicates the odds ratio of the sample at the given abnormal morphology percent.

3.4.3.5. The impact of uterine tone on the probability of successful pregnancy following laparoscopic AI of sheep

Within the model, an increase in uterine tone had a positive effect on the probability of pregnancy rate. The average uterine tone score was 3.22 ±0.0005, ranging from 1 to 5. As seen in Supplementary File 4, a uterine tone score of 1+2 (57.88 %, N=2780 ewes) scored a significantly lower AI success rate than a uterine tone score of 3 (65.20 %, N=12387 ewes) and 4+5 (70.29 %, N=7086 ewes) (P<0.0001, <0.001, respectively). Additionally, ewes with a uterine tone score of 3 recorded significantly lower probability of pregnancy than ewes which scored a uterine tone score of 4+5 (P=0.0091).

The odds ratio of achieving a successful pregnancy was 5.55 % higher for ewes that scored a uterine tone score of "3" compared to a uterine tone score of "1+2" (OR = 1.06, 95 % Cl: 0.59 to 1.88, Figure 3.7). Similarly, the odds of AI pregnancy were 7.21 % higher for the category "4+5" compared to "1+2" (OR = 1.07, 95 % Cl: 0.44 to 2.60, Figure 3.7).

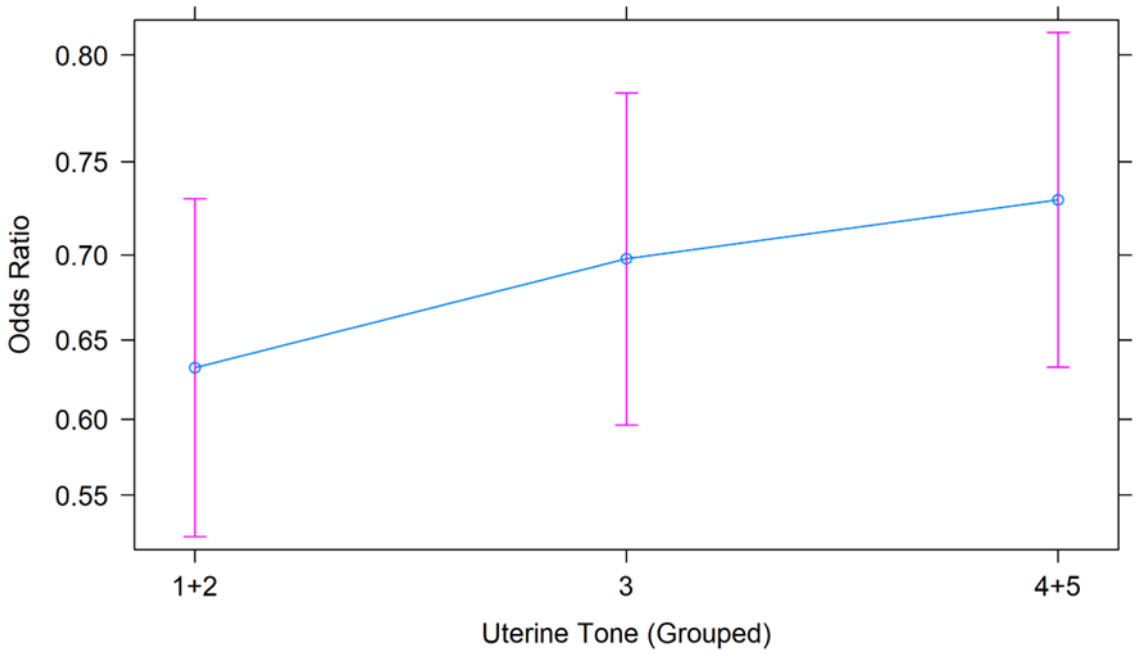


Figure 3.7: The Odds Ratio plot shows the relationship between the uterine tone groups on the predicted probability of an ewe falling pregnant if she was laparoscopically inseminated. The predicted probability was generated from the model in RStudio, with 95 % Confidence Interval (pink error bars). The blue dots indicate the odds ratio of the ewe with the given uterine tone score.

3.4.3.6. The impact of intra-abdominal fat on the probability of successful pregnancy following laparoscopic AI of sheep

The model also calculated that an increase in intra-abdominal fat had a positive effect on the probability of pregnancy rate. The average intra-abdominal fat score was 3.06 ±0.005, ranging from 1 to 5. As seen in Supplementary File 4, an intra-abdominal fat score of 1+2 (59.36 %, N=3895 ewes) scored a significantly lower AI success rate than an intra-abdominal fat score of 4+5 (71.94 %, N=4748 ewes, *P*=0.038,). There was no significant difference in pregnancy rate between ewes which scored an intra-abdominal fat score of 3 (65.60 %, N=13385 ewes) and 1+2 or 3 to 4+5 (*P*>0.05).

The odds ratio of achieving a successful pregnancy was 5.37 % higher for ewes, with an intra-abdominal fat score of "3" compared to an intra-abdominal fat score of "1+2" (OR = 1.05, 95 % Cl: 0.89 to 1.25, Figure 3.8). Similarly, the odds of successful pregnancy were 6.81 % higher for the intra-abdominal fat category "4+5" compared to "1+2" (OR = 1.07, 95 % Cl: 0.78 to 1.47, Figure 3.8).

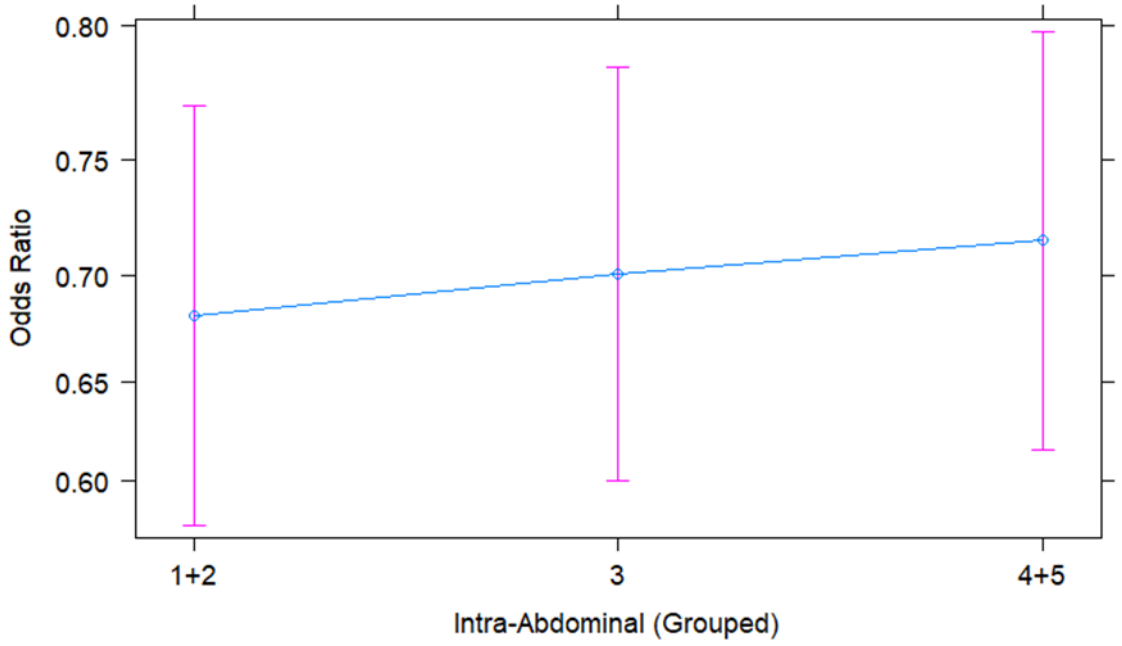


Figure 3.8: The Odds Ratio plot shows the relationship between the intra-abdominal fat groups on the predicted probability of an ewe falling pregnant if she was laparoscopically inseminated. The predicted probability was generated from the model in RStudio, with 95 % Confidence Interval (pink error bars). The blue dots indicate the odds ratio of the ewe with the given intra-abdominal fat score.

3.5. DISCUSSION

This study investigated the impact of female factors recorded during AI and male *in vitro* semen traits assessed post-thaw on the probability of pregnancy occurring in sheep. This study has considered male and female fertility traits simultaneously, comparing the pregnancy success of the individual ewe rather than across flock averages. The resultant fertility model can now be used to explain the variation detected in pregnancy success following laparoscopic AI. An increase in freezing concentration (and thus sperm per insemination dose), percentage of viable, acrosome intact spermatozoa and motility kinematics will result in a positive linear increase in the probability of pregnancy occurring in a ewe. In addition, ewes with a uterine tone and intra-abdominal fat score of 4 or 5 are more likely to result in pregnancy than ewes with a score of 1 or 2. Finally, a decrease in the percentage of spermatozoa in a sample with

morphological abnormalities will increase pregnancy probability. However, it is still important to consider the overarching variation contributed by the environment, site or location where AI occurred, as these factors will further modify the influence of the predictive factors mentioned above. The results of the above study demonstrate that the fertility of sheep following laparoscopic AI cannot be predicted off a single parameter and further shows the importance of considering multiple fertility factors with confounding effects in concert. While it is useful to critically analyse previous studies which have referred to the role or impact of these factors individually on sheep fertility (discussed below), the model will only successfully predict fertility if all are considered in unison. Nevertheless, the identification of predictive *in vitro* semen traits can now be used to pre-screen and select sires or frozen semen samples prior to use in a breeding program. This will help to improve the success rates of artificial breeding programs, offering producers an effective tool to increase genetic and production gains in a challenging industry.

The impact of the number of sperm frozen on the probability of successful pregnancy

The concentration at which sperm is frozen has the potential to impact cryo-survival post-thaw as well as subsequent insemination dose. In the current study, the number of sperm frozen in either a pellet or straw was found to influence the likelihood of successful pregnancy following laparoscopic AI. For every additional 100×10^6 sperm/mL frozen, the probability of achieving pregnancy increased by 5.09 % (Figure 3.3). However, there was no interaction with package type. While there is currently no agreed-upon standard for the industry, it is generally assumed that a pellet should be frozen between 600-800×10^6 sperm/mL and used to inseminate approximately 3 ewes. A straw should be frozen at 200-300×10^6 sperm/mL and equate to 1 dose per ewe (Evans and Maxwell 1987). Pleasingly, in the current study, the average concentration of pellets and straws was $722\times10^6 \pm 15.27$ and $270.75\times10^6 \pm 16.47$ sperm/mL,

respectively, suggesting the data collected accurately reflected current protocols used throughout the sheep artificial breeding industry in Australia.

The authors interpret the above result as the number of spermatozoa frozen as a proxy for the insemination dose. For laparoscopic AI, it is recommended that each ewe should receive approximately 25×10^6 motile spermatozoa or 12.5×10^6 motile spermatozoa/horn (Maxwell 1986a). Assessing the sperm concentration post-thaw ensures a more accurate insemination dose. The recommended dose in sheep has remained largely unchanged since the mid-80s, when it first emerged as a reproductive tool for sheep (Killen and Caffery 1982). Previous studies have demonstrated that an increase in motile spermatozoa dose from 0.5 to 50×10^6 sperm resulted in lambing rates of 27 % to 62 % (Maxwell 1986a). Which then later, a sperm dose of 20×10^6 sperm/mL achieved a rate of 76.8 % (Maxwell 1986b). In contrast, other studies have also found no difference in conception rates when doses were reduced from 52.2×10^6 to 13×10^6 sperm/mL (Findlater *et al.* 1991; Eppleston *et al.* 1994). The lack of significant difference in these studies may be related to the number of ewes used per treatment structure, which limits statistical power, or the compounding effects of sperm type (fresh, liquid stored, or frozen) or diluents used, ultimately making it difficult to compare results across studies.

It is important to emphasise that while higher numbers of sperm frozen theoretically offer increased insemination doses, it is important to find the balance between optimal freezing conditions to ensure sperm survival and effective insemination doses. This concept has been studied abundantly in previous literature (Crockett *et al.* 2001; D'Alessandro *et al.* 2001; Nascimento *et al.* 2008; Alvarez *et al.* 2012) across livestock species. Studies (D'Alessandro *et al.* 2001; Alvarez *et al.* 2012) found that freezing at concentrations above 600×10^6 sperm/mL reduced sperm viability, acrosome integrity and motility. Attributed to the excessive build-up of free radicals, it is proposed to cause changes in the sperm: cryoprotective agents (Palacios Angola *et al.* 1992). The higher the amount of cryoprotectant per sperm cell, the higher the

percentage of microdomains (unfrozen water channels), leading to better quality post-thaw (Nascimento *et al.* 2008). Alternate studies (D'Alessandro *et al.* 2001) reported lambing rates of 57.1 % when sperm was frozen at 800×10^6 sperm/mL and inseminated at 160×10^6 spermatozoa. This was compared to 81.2 % when sperm was frozen at 200×10^6 sperm/mL and inseminated at 40×10^6 spermatozoa. Standardising doses prior to insemination recorded similar results to 25 million sperm (Alvarez *et al.* 2012). Sperm frozen at 200 and 400×10^6 sperm/mL recorded a higher lambing rate of 57.5 % compared to sperm frozen at 800×10^6 sperm/mL, which returned a lambing rate of 45.5 %.

In any event, the concentration at which sperm is frozen directly impacts insemination dose, which can then further alter pregnancy results. Semen must be frozen at an appropriate concentration to mitigate the impacts of freeze-thaw damage and optimise the number of sperm per ewe per insemination dose. With an increase in the accuracy and number of technologies currently available on the market that can objectively measure sperm concentration, it should be easier to ensure samples are frozen at accurate concentrations, regardless of package type. Further studies must now focus on establishing thresholds that could be used as standards in industry, increasing the chances of pregnancy success.

<u>The impact of the percentage of abnormal spermatozoa on the probability of successful pregnancy</u>

The assessment of sperm morphology is common practice during routine basic semen assessment for a number of species, including stallions (Morrell *et al.* 2008; Love 2011), bulls (Gillan *et al.* 2008) and boars (Kondracki *et al.* 2020), yet its correlation with the fertility of frozen-thawed ram spermatozoa has been contradictory (Mickelsen *et al.* 1981; O' Meara *et al.* 2008; Almadaly *et al.* 2016). In the current study, results reported that for every 1 % increase in the percentage of abnormal spermatozoa within a frozen sample, a 1.07 % decrease in the probability of a ewe falling pregnant would be observed (Figure 3.4). In our study, sperm

morphology was classified as either abnormal or normal with this approach aligning with the methods and results reported by previous ram studies (Almadaly *et al.* 2021). In this study, a significant difference in the percentage of abnormal ram spermatozoa was reported between groups that exhibited high and low fertility (4.46 ±0.30 % compared to 13.46 ±1.37 %, respectively). Our current study builds upon these results by directly comparing the morphology of samples inseminated per ewe rather than the fertility average of a group of individuals.

As reviewed (Spanner *et al.* 2024a), the morphology of an individual spermatozoon is an important indicator of its fertilising potential and has been proven in a number of species including; deer (Malo *et al.* 2005), bull (McGowan *et al.* 2002; Gillan *et al.* 2008) and stallions (Morrell *et al.* 2008; Love 2011) and rams (Almadaly *et al.* 2016; Almadaly *et al.* 2019). At least in the cattle industry, standards to measure bull sperm morphology are frequently used to assess and grade the quality of bull samples (Perry 2021). To date, nothing of this detail exists for the sheep industry. Thus, the results of the current study are a positive step forward for the industry, providing a comprehensive data set that accurately reflects a negative relationship between increasing morphology abnormalities and sheep fertility following AI. Even more so, the results provide evidence that even basic morphology assessment is a key parameter that should be considered when assessing the quality of ram samples during fertility assessment.

<u>The influence of the percentage of viable, acrosome intact spermatozoa on the probability of successful pregnancy</u>

The current model revealed that for every 1 % increase in the number of viable sperm with intact acrosomes at 6 h post-thaw, a 1.01 % increase in the probability of pregnancy occurred (Figure 3.5). A viable, acrosome intact spermatozoon is essential for the normal functioning of the cell, implying that sperm are capable of transitioning to the site of fertilisation, fusing with

the zona pellucida, undergoing the acrosome reaction (Abou-Haila and Tulsiani 2000; Januskauskas *et al.* 2000) and achieving successful fertilisation (Januskauskas *et al.* 2000).

The relationship between sperm viability and fertility following insemination has been extensively proven across multiple species, recording a correlation 'R' score of R=0.32, 0.64, 0.64, 0.05, 0.68 and 0.28 in bulls (Januskauskas *et al.* 2000; Gillan *et al.* 2008; Sellem *et al.* 2015), dairy bulls (Alm *et al.* 2001), stallions (Wilhelm *et al.* 1996) and boars (Christensen *et al.* 2004), respectively. These studies agreed with the current study, that the greater the proportion of viable sperm and acrosomal integrity, the greater the probability of pregnancy. Of the literature above, only (Sellem *et al.* 2015) successfully measured the viability of bull sperm at 0 and 4h post-thaw. However, as the current study measured viability at 6 h, this could more closely imitate the environment sperm are exposed to following deposition within the female tract. In general, sperm are inseminated just prior to a ewe ovulating; therefore, they are required to survive for up to 6-12h before interacting with an oocyte. For laparoscopic AI, measuring the level of viability or live: dead with intact acrosomes at 6 h would ensure the population of sperm deposited in the uterus was capable of achieving fertilisation after incubation at 37ºC (artificially post-thaw in a water bath or *in vivo* within the reproductive tract).

Opposing this trend, (O' Meara *et al.* 2008) measured no importance or significance of ram sperm viability to pregnancy rates. In contrast to the current study, however, this study compared sperm viability to previously recorded fertility following cervical AI with a very low number of samples (O' Meara *et al.* 2008). Nonetheless, these results have the potential to establish industry standards, which would be crucial for enhancing reproductive outcomes.

<u>The impact of motility and velocity traits assessed using CASA (PCA3) on the probability of successful pregnancy</u>

The use of a principal component analysis (PCA) for CASA variables underscores the positive impact of sperm motility and kinetic traits on pregnancy likelihood while considering the extreme correlation between factors. Of all the PCAs considered within the current dataset, PCA3 exhibited the greatest influence on pregnancy, primarily driven by total and progressive motility (-0.70 and -0.53, respectively). Analysis of the odds ratios revealed a 0.37 % increase in the odds of pregnancy occurring for every negative standard deviation away from the average (Figure 3.6). This indicates that as total and progressive motility values increase, the PCA3 loadings or value decreases. Thus, as PCA3 values decline, the likelihood of pregnancy occurring increases. In other words, the model makes biological sense given that a sample with high total and progressive motility is also likely to exhibit efficient metabolism of substrates and be more capable of achieving fertilisation (Martínez-Pastor *et al.* 2009; Aitken *et al.* 2012) so the probability of achieving pregnancy increases.

Extensive research in several species has focused on the relationship between motility as determined by CASA and fertility. Studies across livestock species, including bulls (Januskauskas *et al.* 2000; Gillan *et al.* 2008; Kumar *et al.* 2016), stallions (Love 2011), deer (Malo *et al.* 2005), rats (Gaddum-Rosse 1981), humans (Holt *et al.* 1989), salmon (Gage *et al.* 2004) and rams (Del Olmo *et al.* 2013), have all shown similar results to the current study where, as total sperm motility and average path velocity increase, the likelihood of pregnancy also increases (Januskauskas *et al.* 2000; Malo *et al.* 2005; Love 2011; Kumar *et al.* 2016). Previous research (Del Olmo *et al.* 2013) has demonstrated the relationship between motility assessed by CASA and fertility of cryopreserved ram sperm. They saw a direct relationship between an increase in the average-path velocity (VAP), the curvilinear velocity (VCL) and the head beat-cross frequency (BCF), correlated with the percentage of ewes pregnant following AI (R^2 = 0.678, 0.745, 0.852, respectively). This is not always the case in all literature, and some studies (O' Meara *et al.* 2008; García-Álvarez *et al.* 2010), have reported

a lack of correlation between kinematic parameters and fertility following laparoscopic AI in sheep. The contradictory results in previous ram studies are unsurprising, given the different protocols and diluents used for assessment, making it difficult to compare across studies. Suggesting the need for standardisation across the industry.

The Impact of Uterine Tone on the Probability of Successful Pregnancy

The present study revealed a clear linear relationship between the uterine tone of ewes at the time of AI and the resultant pregnancy. Pregnancy rates increased from groups 1+2 (58.13 %), 3 (65.30 %) and 4+5 (71.04 %; $P<0.05$, Figure 3.7). Changes in uterine tone have been previously studied in mares (Hayes and Ginther 1986; Griffin *et al.* 1992; Bonafos *et al.* 1994) and dairy cows (Loeffler *et al.* 1999), and more recently in ewes (Spanner *et al.* 2024c). The relationship between uterine tone, sperm quality and fertility is hypothesised to be related to the response of the ewe to oestrous synchronisation (Langford 1982), coinciding the deposition of semen artificially with subsequent ovulation in the ewe (Evans 1988). An increase in uterine tone corresponds to a surge in ooestrogen levels, causing epithelial uterine gland cells and rough endoplasmic reticulum to expand (Akbulut and Çelik 2021) increasing blood flow to the area (Spanner *et al*. 2024c). This results in an increase in hypertrophy and contractions, aiding sperm transport from the cervix to the oviduct (Hawk and Conley 1974; Hawk and Cooper 1977). It also implies ovulation is imminent, signposting optimal insemination time and increased chances of fertilisation (Spanner *et al.* 2024c) should sperm quality be appropriate. Further studies could track changes in the hormone profile of ewes corresponding to alterations of uterine tone and follicular development via laparoscope to pinpoint the exact time between a uterine tone score 4 or 5 and ovulation. Overlaid with the ability of different sperm types to survive incubation, this would enable us to accurately predict the optimum time of insemination in relation to uterine tone, increasing pregnancy success after AI. For now, the results above suggest that uterine tone could be a useful tool for screening ewes prior to insemination.

Observing the tone of ewes subjectively (once standardised) and excluding those ewes with a uterine tone lower than 3.5 prior to AI could contribute to less variable pregnancy rates across the industry.

The Impact of Intra-abdominal Fat on the Probability of Successful Pregnancy

Similar trends were also observed in the intra-abdominal fat level of ewes undergoing AI. The study revealed a clear linear relationship between intra-abdominal fat and AI success in sheep, showing an increase in pregnancy rate from groups 1+2 (59.77 %), 3 (65.70 %) and 4+5 (72.56 %, Figure 3.8). From these findings, the probability of a ewe with an intra-abdominal fat score of 4 or 5 was 6.81 % higher than ewes', which scored an intra-abdominal fat score of "1+2". The inclusion of intra-abdominal fat in a fertility model is a novel parameter yet to be fully explored in livestock species. Previous research has focused heavily on the use of a ewes' body condition score (BCS) as the gold standard for pre-breeding soundness or fertility assessments (Spanner *et al.* 2024a) given it identifies a ewes' nutritional status, health, and reproductive potential.

Studies in sheep have successfully linked BCS scores to fertility success (Kenyon *et al.* 2004; Corner-Thomas *et al.* 2015; Silva *et al.* 2016), yet limited studies have linked internal fat levels with fertility (Kenyon *et al.* 2014). A small proportion of papers have demonstrated a significant correlation between BCS, internal fat deposits (Bravo *et al.* 1997) and ultrasound subcutaneous fat depth within the abdominal region of the ewe (Silva *et al.* 2016). Studies have recommended maintaining a BCS 3 – 3.5 will help optimise reproductive performance (conception rate, little size, weaning rate and oestrus cycles to conception) and flock profitability (Vatankhah *et al.* 2012). The current study saw an increase in the likelihood of pregnancy when ewes had an intra-abdominal fat score of 4 or 5. This suggests that while BCS and internal fat score are likely correlated to some degree, the scale used to assess internal fat score means at the upper limits, intra-abdominal fat score should still be considered

individually and likely acts as a more accurate predictor for pregnancy following AI. To maximise fertility potential, it is recommended that ewes with an intra-abdominal score below 3 at the time of AI should be excluded from insemination, when matched with the correct sperm type of adequate quality.

3.6. CONCLUSION

This study revealed the link between *in vitro* semen parameters, ewe traits, and pregnancy following laparoscopic AI, increasing the potential of artificial breeding programs to reduce breeding inefficiencies and improve sheep reproductive potential. This model must now be validated with unseen data to establish thresholds for each variable, allowing the pre-screening of rams, their semen and ewes prior to an AI breeding program. The application of these results in the industry could result in a reduction in variable results, restoring industry confidence and increasing the adoption of artificial reproductive technologies within the sheep industry.

Chapter 4. The validation and application of an ovine fertility model using standardised *in vitro* thresholds to predict the likelihood of pregnancy

The experiments described herein are under review for publication as: Spanner, E.A., de Graaf, S.P., Rickard, J.P., 2024. The validation and application of an ovine fertility model using standardised *in vitro* thresholds to predict the likelihood of pregnancy. *Under review for publication to Theriogenology.*

4.1. ABSTRACT

Deciphering a ram or ewe's reproductive potential is crucial to ensure high reproductive performance and maximise production outcomes. This study validates the accuracy of an ovine fertility model created to predict the likelihood of pregnancy occurring following laparoscopic artificial insemination and proposes *in vitro* semen standards to improve pregnancy outcomes. Semen from Merino sires (N=26) was inseminated into synchronised Merino ewes (N=1269) across three breeding seasons (2021–2023). Uterine tone and intra-abdominal fat of ewes were scored at AI, while the freezing concentration, abnormal sperm, acrosome viability (6 h) and CASA motility and velocity traits (0 h) of semen inseminated were assessed post-thaw (6 h; 37 ºC). Pregnancy predictions were compared with ultrasound-confirmed pregnancies ~55 days post-AI, using discrimination and calibration tests to correctly assess its ability to classify pregnant and non-pregnant ewes. The model demonstrated high accuracy (77 %), precision (96 %) and recall (76 %) but lower specificity (33 %). It recorded an F1-score of 0.85, with an Area Under the Curve (AUC) of 0.62. There was no statistical difference between predicted and actual pregnancy results (P=0.184) despite an error value of 26 %. A cutting point split data

for each *in vitro* semen predictor and calculated the average pregnancy rate above and below this point. The cutting point with the greatest difference between pregnancy rates was chosen as the semen threshold. When entered into the model, these thresholds returned a cumulative pregnancy probability of 64.3 %. These standards could be used to screen semen before AI, reducing the variability of laparoscopic AI programs for the industry.

4.2. INTRODUCTION

Artificial insemination is a reproductive technique used within the artificial breeding industry to enhance rapid genetic gain by selectively pairing superior rams and ewes. It is well-known that numerous factors can influence the fertility of both the ram and ewe (Spanner *et al.* 2024a), however, until recently, the industry lacked a comprehensive statistical analysis of fertility trends in AI specific to individual ewes. Moreover, the shortage of studies that integrate both male and female factors has led to a lack of standardised methods for assessing *in vitro* semen quality and female fertility traits. When examining reproductive potential, many previous studies focused on predefined fertility outcomes, such as the percentage of females pregnant or overall herd pregnancy rates (Spanner *et al.* 2024a), yet identifying the likelihood of pregnancy at the individual ewe level is crucial for improving economic efficiency, reproductive success, and genetic gain within a production system. By screening semen from sires and removing ewes unlikely to conceive, pregnancy success will increase, reproductive performance will be enhanced, and confidence surrounding the use of laparoscopic AI technology will rise, increasing application across the industry.

To address these above-mentioned shortcomings, (Spanner *et al.* 2024b) collected data from 30,254 ewes at the time of insemination during 30 programs between 2021-2023. Semen from the 388 sires used in these programs was also thawed and *in vitro* sperm characteristics were assessed. A predictive model was created that combined both male and female fertility traits to determine the likelihood of pregnancy occurring following laparoscopic AI. The model

identified higher sperm freezing concentration, reduced morphology abnormalities, greater motility and velocity kinematics and percentage of viable, acrosome intact sperm alongside higher scores of uterine tone and intra-abdominal fat increased the probability of pregnancy occurring (Spanner *et al.* 2024b). To date, the model proposed by (Spanner *et al.* 2024b), collated the largest and most comprehensive data set that combines female and male fertility traits with pregnancy data at an individual ewe level and provided significant potential for its application within the industry to establish standards for semen processing and assessment. The model created now requires validation with unseen data to assess its reliability and accuracy in predicting the pregnancy of ewes.

The creation and use of accurate predictive modelling can be a helpful decision-making tool to measure fertility potential. In addition to (Spanner *et al.* 2024b), other research in dairy cattle (Caraviello *et al.* 2006; Shahinfar *et al.* 2014; Hempstalk *et al.* 2015; Rutten *et al.* 2016; Fenlon *et al.* 2017), beef cattle (Urioste *et al.* 2007; Naya *et al.* 2017) and pigs (Perez-Enciso *et al.* 1993) have explored such modelling to predict conception, herd productivity, AI success, first-service outcomes, and litter size. However, until each model is validated, the reliability and impact of the model lie in question (Rutten *et al.* 2016). Validating such statistical models requires the model to predict correct pregnancy outcomes compared to actual pregnancy results. Previous studies have varied in their approaches to model validation. By segregating data into testing and validation data sets, machine learning can utilise a 5-fold or a 10-fold cross-validation process to assess the model's validity (Caraviello *et al.* 2006; Hempstalk *et al.* 2015). Alternative regression models can use calibration methodology to test the accuracy of predicted probability, including bootstrapping (Rutten *et al.* 2016), Hosmer-Lemeshow tests and Mean Absolute Calibration Error (MACE) (Fenlon *et al.* 2017) statistics. Additionally, discrimination evaluations (including precision, recall and the F-measure) have been used to

assess the model's ability to distinguish between positive and negative outcomes (Fenlon *et al.* 2017).

Model accuracy is often reported as the area under the curve (AUC) defined by the model's ability to classify instances correctly. Previous studies examining AUC values from fertility models have ranged from 0.62 (Fenlon *et al.* 2017) to 0.76 (Shahinfar *et al.* 2014), where an AUC of 1 indicates perfect classification accuracy, while an AUC score of 0.5 suggests the model performs no better than random guessing. When using only logistic regressions to validate fertility models, (Hempstalk *et al.* 2015) and (Fenlon *et al.* 2017) conducted research on dairy cows and reported an AUC of 0.66 and 0.62, respectively. Highlighting these values is relevant, as they demonstrate that logistic regression, while effective, may yield a moderate predictive power, underscoring the challenges in achieving high accuracy in fertility predictions and providing a meaningful benchmark for evaluating our own model's performance.

We can apply this process to validate the initial fertility study performed by (Spanner *et al.* 2024b). It is hypothesised that a pregnancy probability threshold between 0.5 and 0.6 will effectively distinguish between pregnant and non-pregnant ewes. By incorporating a higher threshold and accounting for variability in the data set, the model is expected to identify a larger proportion of pregnant ewes than not pregnant. The null hypothesis states no significant difference will exist between the model's predicted pregnancies and actual pregnancies detected via ultrasound.

Regarding *in vitro* semen traits, the relationship between freezing concentration and pregnancy rate is expected to plateau at excessively high concentrations. Morphology is anticipated to optimise at around 15 % abnormalities, consistent with existing findings, while viability scores at 6 h post-thaw are predicted to fall below 10 %. However, due to the

complexity of the PCA scoring system, standardising motility remains a challenge that will require further refinement.

Validation of the model and establishment of standards will help cement its application in the artificial breeding industry as a useful tool to screen ewes and semen from sires prior to AI breeding programs, reducing variability and improving confidence in ARTs within the sheep industry.

4.2.1. Ethics and Animals

The data used in this research was generously donated by artificial breeding companies and stud breeders during routine commercial AI operations. Animals are not directly involved in this study, as such, no additional ethical approval was required. The management of ewes and rams adhered to the standard industry practices and requirements for each site, and all methods complied with relevant guidelines and regulations.

4.2.2. Experimental Plan

To validate the fertility model, new and unseen data was collected from Merino ewes (N=1269), which were synchronised for oestrus and assessed for uterine tone and intra-abdominal fat as part of routine laparoscopic AI protocols. As per industry standards, ejaculates from sires (N=26) were collected, prepared, and thawed (Evans and Maxwell 1987) as either pellets (N=24; 2 pellets/glass tube; 2 minutes vigorous shaking at 37 ºC) or straws (N=2; 2 straws/glass tube; 30 sec vigorous shaking at 37 ºC) and inseminated into ewes (roughly 50 ewes/sire). Pregnancy scanning per ewe occurred 55 days post-insemination. The remaining frozen pellets and straws were sent to the University of Sydney for *in vitro* semen assessment where pellets (2 pellets/glass tube; 2 minutes vigorous shaking at 37 ºC) and straws (2 straws/glass tube; 30 seconds vigorous shaking at 37 ºC) were thawed (Evans and Maxwell 1987), incubated for 6 h at 37 ºC and assessed for freezing concentration, percent abnormal morphology, viability, acrosome integrity and motility kinematics. Data per ewe was entered

into the fertility model (Equation 1 and Supplementary File 4.1) to predict the probability of pregnancy occurring.

$$Fertility\ model = logit\left(P(AI\ Pregnancy)\right)$$
$$= \hat{\beta}0 + \hat{\beta}1 * Acrosome\ and\ Sperm\ Viability\ (6hr) - \hat{\beta}2$$
$$* Abnormal\ Morphology + \hat{\beta}3 * Freezing\ Concentration - \hat{\beta}4$$
$$* CASA\ PCA3\ (0hr) + \hat{\beta}5 * Uterine\ Tone + \hat{\beta}6 * Intra$$
$$- abdominal\ fat + \mu_{site} + \mu_{sire} + \mu_{thaw} + E$$

These results were then compared to the actual pregnancy diagnosis per ewe determined via transcutaneous ultrasound. Subsequent model discrimination and calibration analyses occurred prior to establishing standardised cutting points for *in vitro* semen traits to maximise pregnancy.

4.2.3. Collection of Data

4.2.3.1. Assessment of Uterine Tone and Intra-abdominal Fat

At the time of AI, the tone of the uterus was scored as a subjective observation by the technician and recorded as a value between 1 (pale, flaccid uterus) to 5 (bright pink turgid uterus), as per (Spanner *et al.* 2024c). Simultaneously, the internal fat covering the abdominal organs was visualised and subjectively assessed by the technician performing the insemination, scoring the ewe between 1 (little to no fat present) to 5 (high abundance of fat present), as per (Spanner *et al.* 2024c).

4.2.3.2. Evaluation of Sperm Freezing Concentration

The concentration of each frozen sample was determined using a NucleoCounter SP-100 (ChemoMetec) immediately post-thaw. An aliquot of 50 μL of semen was diluted with S100 reagent (ChemoMetec) and analysed according to the manufacturer's instructions.

Concentration recordings were taken twice (within 10 %) and the average was used for further calculations.

4.2.3.3. Assessment of Abnormal Sperm Morphology

After thawing, 10 μL of semen was fixed with 190 μL (1+10 dilution) in 3 % NaCl. The percentage of abnormal spermatozoa was subjectively assessed using a phase-contrast microscope (x400 magnification). Capturing a minimum of 200 cells, the results were converted into the percentage of the sample containing spermatozoa with abnormal morphology. As such, this was a measure of the proportion of abnormal spermatozoa.

4.2.3.4. Assessment of Sperm Motility and Other Kinematics Traits

Based on the model above, motility and sperm kinematics recorded at 0 h post-thaw had a significant influence on the likelihood of pregnancy occurring when considered with the other factors. In the current study, at 0 h post-thaw sperm motility was measured using the computer-assisted semen analysis system (*HT CASA IVOS II (Animal Breeder) Version 1.13.7; Hamilton-Thorne, Beverly, MA, USA*) at a setting appropriate for ram spermatozoa (this includes head size 10 – 42 μm^2, progressive motility thresholds of straightness 80 % and average path velocity 75 μm/s). Samples were diluted to a concentration of 25×10^6 sperm/mL before 6 μL was placed on slides pre-warmed to 37 ºC (Cell Vu; Millennium Sciences, Mulgrave, Victoria, Australia) and enclosed using a 22 × 22 mm coverslip. For each sample, eight fields of video recordings capturing a minimum of 200 cells (frame rate 60 Hz) was required. Motility and velocity parameters assessed included total and progressive motility, ALH, BCF, LIN, STR, VAP, VCL, VSL, and WOB.

4.2.3.5. Assessment of Viable Sperm with Intact Acrosomes

Based on the model above, the percentage of viable, acrosome intact sperm recorded at 6 h post-thaw had a significant influence on the likelihood of pregnancy occurring when considered with the other factors. In the current study, at 6 h post-thaw, the viability and

acrosome integrity of each sample was assessed using the CytoFLEX flow cytometer (Beckman Coulter, Lane Cover, Australia). Sperm viability and acrosome integrity were examined using Propidium Iodine (PI, final concentration 6 μM), DNA probe Hoechst 33342 (final concentration of 1 μg/mL) and fluorescein isothiocyanate peanut agglutinin (FITC-PNA, final concentration 0.4 μg/mL) for 10 minutes at 37 ºC (Final concentration of 10×10^6 sperm/mL). PI and FITC-PNA fluorescence detection was on 690/50, 450/45 and 525/40 nm bandpass (BP) filters, respectively. Sperm cells were isolated from total events based on 488 nm forward and side scatter profiles. For each variable, 10,000 sperm cells was analysed, and a minimum of 1,000 Hoechst 33342 positive events was required to obtain valid results using the CytExport 2.0 software (Beckman Coulter, Lane Cove, Australia). Cells were considered viable with intact acrosomes if cells were both PI and FITC-PNA negative (recorded at a percentage of the parent).

4.2.4. Measure of Actual Fertility

Pregnancy was determined approximately 55 days following insemination using real-time cutaneous ultrasound to detect the presence of fetuses and their number. A real-time cutaneous ultrasound (Oviscan 6 with a 3.5MHz probe) was used to scan each ewe to determine the presence of fetuses and their number. Pregnancy from AI was recorded as a value of 1 versus empty, recorded as 0. Further, the number of fetuses carried was recorded as the exact number. The pregnancy rate (PR) was calculated as the number of ewes pregnant at Day 50/number of ewes inseminated × 100. Furthermore, the reproductive rate (RR) was calculated for each sire by the number of fetuses at Day 50/number of ewes inseminated × 100.

4.2.5. Statistical Analysis

All statistical analysis was performed in R studio (Version 2023.09.1+494).

4.2.5.1. Calculation of Predicted Fertility

To calculate the probability of pregnancy, fertility data recorded on the unseen data was run through the model (Equation 1). This involved entering PCA-transformed variables derived from the model's PCA loadings (Table 3.1), along with values for acrosome and sperm viability (6 h), abnormal morphology, concentration, uterine tone and intra-abdominal fat. The model then generated probabilistic predictions of pregnancy outcomes, ranging from 0 to 1. Discriminate thresholds were used to evaluate the ability of the fertility model to distinguish between positive (1) and negative (0) insemination outcomes. A threshold was set at 0.56 to transform the predicted probabilities into binary insemination outcomes.

$$P_{AI\ Pregnancy}(\geq 0.56) = 1$$

$$P_{AI\ Pregnancy}(< 0.56) = 0$$

4.2.5.2. Evaluation of Model Performance

The measures of discrimination and calibration were used to evaluate the model predictions. Calculations were based on tabulating the predicted fertility results compared to the actual fertility results as the number of true-positive (TP), false-positive (FP), true-negative (TN), and false-negative (FN) scores following an AI breeding program.

Accuracy was used to evaluate the effectiveness of the binary classification model. It measures the overall correctness of the fertility model predictions for both pregnant and non-pregnant.

$$Accuracy = \frac{TP + TN}{TP + FP + TN + FN}$$

Precision was calculated to identify how many of the ewes predicted to be pregnant occurred to be pregnant in the data set. It measures the accuracy of positive predictions made by the fertility model.

$$Precision = \frac{TP}{TP + FP}$$

Specificity was calculated to identify how many of the ewes predicted not to be pregnant. It measures the accuracy of negative predictions made by the fertility model.

$$Specificity = \frac{TN}{TN + FN}$$

Recall (sensitivity) was calculated to identify the proportion actual number of ewe pregnant following AI that were identified by the fertility model. It measured the fertility model's ability to identify all relevant instances.

$$Recall\ (sensitivity) = \frac{TP}{TP + FN}$$

Precision and recall scores were combined to calculate the F-measure (F1-score), a measure of the fertility model's performance and effectiveness.

$$F1\ score = 2[\frac{Precision * Recall}{Precision + Recall}]$$

For the fertility model, a reviver operating characteristic (ROC) curve was created and graphed to demonstrate the tradeoff between FP and TP as the discrimination threshold was altered.

The area under the ROC curve (AUC) quantifies the fertility model's ability to distinguish between pregnant and not pregnant. AUC values range from 0 to 1 and indicate the probability that the fertility model will predict a greater amount of fertility results when compared to random assumption. Calibration tests included the Mean Absolute Calibration Error (MACE), which quantifies the average absolute difference between the predicted probabilities of pregnancy following AI and the observed proportions of pregnancies. This calculates the error of the fertility model's predictions.

4.2.6. Fertility Traits Standardisation

To explore the relationship between semen parameters and pregnancy rates, a sequence was generated to establish cutting points spanning the range of the given sperm trait. For each

cutting point, the data set was divided into two subsets: pregnancy rates below or equal to the cutting point and pregnancy rates above and equal to the cutting point. This was visualised by displaying the pregnancy rates for each cutting point above and below as a point for each cutting point, differentiated by colour. A rug plot was also included to show the distribution of the sperm trait.

The optimal cutting point was determined by the point with the largest difference in pregnancy rates between the subsets above and below the threshold. This excluded outliers, the last three extremity points within the data set for each semen trait. This approach aimed to pinpoint the critical threshold for each sperm trait that maximised the difference in pregnancy outcomes, thereby optimising pregnancy rates following artificial insemination. Graphed is the vertical dashed line that highlights the optimal cutting point for each sperm trait.

The positive predictive value (PPV) was used to determine the model's performance following the selection of the cutting point. PPV measures the proportion of true predictions amongst all the positive predictions within the fertility model.

$$PPV\ (\%) = \frac{TP}{TP + FP}$$

4.3. RESULTS

4.3.1. Performance of Fertility Model

When the pregnancy threshold was set at 0.56, Table 4.1 combines the discriminatory and calibration values obtained when predicted pregnancies were compared to actual pregnancies. Figure 4.1 shows the ROC curve plot for the model's prediction of pregnancy. The *P-value* obtained from the Chi-squared test (P=0.184) failed to reject the null hypothesis, indicating no association between predicted pregnancies and actual pregnancies. This confirms that the model's predictions for pregnancy do not deviate significantly from the observed pregnancies.

Table 4.1. Accuracy, Precision, specificity, Recall, F1-Score, the area under the curve (AUC), mean absolute calibration error (MACE) and chi-square results for the unseen validation data when using the fertility model.

Performance Variable	Fertility Model
Accuracy (%)	74
Precision (%)	96
Specificity (%)	33
Recall (sensitivity) (%)	76
F1 – Score	0.85
AUC	0.62
Mean absolute calibration error (%)	26
Chi-squared (*P-value*)	0.184

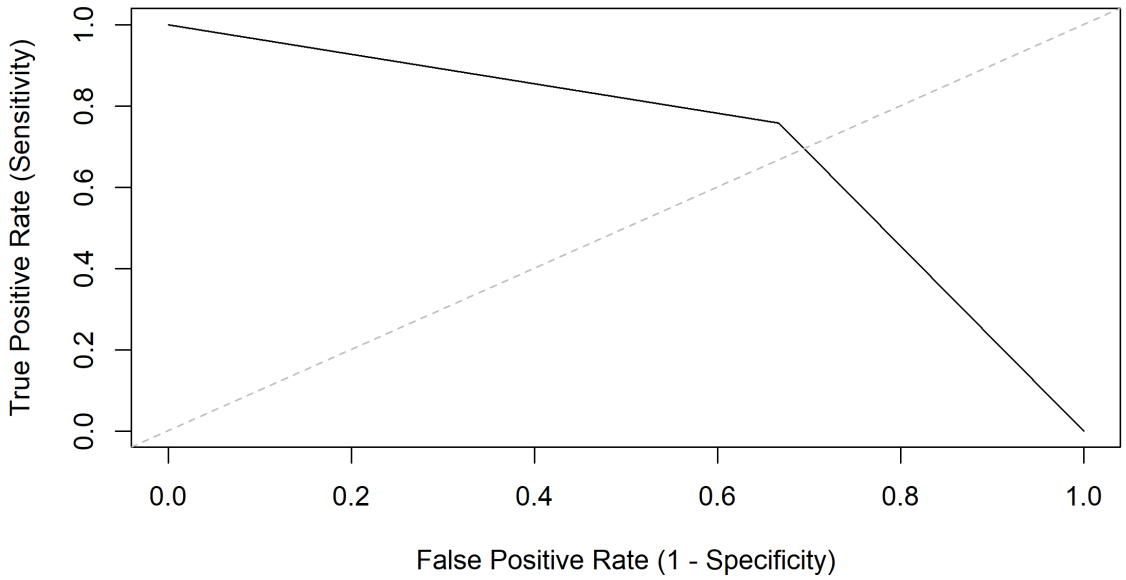


Figure 4.1: Receiver Operating Characteristic (ROC) Curve Analysis: ROC Curve Assessing the Diagnostic Accuracy of the Fertility Model to Predict Pregnancy Following AI. The degree of deviation from the diagonal line (random guess) provides insights into the discriminative power of the fertility prediction model.

4.3.2. Fertility Traits Standardisation

Table 4.2 identified the threshold for each *in vitro* semen traits, which showed the greatest difference between pregnancy rates calculated above and below the cutting point. Additionally, the positive predictive value for each *in vitro* trait was used to determine pregnancy successfully.

Table 4.2. The optimal threshold for each *in vitro* trait for which the pregnancy rate above and below the cutting point is greatest. The positive predictive value for each threshold.

Fertility Traits	Threshold	Pregnancy rate below threshold	Pregnancy rate above threshold	Positive Predictive Value (PPV)
Freezing Concentration ($\times 10^6$ sperm/mL)	680.85	0.73	0.82	0.30
Abnormal Sperm Morphology (%)	15.5	0.81	0.71	0.54
Viable sperm with Intact acrosomes 6 h (%)	6.32	0.62	0.79	0.84
CASA PCA 0 h	18.34	0.67	0.75	0.21

4.3.2.1. Cumulative Probability of Pregnancy

When each *in vitro* and female fertility trait threshold was entered into the fertility model, the cumulative probability of pregnancy occurring (P(Pregnancy)≥0.56) in an ewe was 0.643 or 64.3 %.

4.3.2.2. Freezing Concentration

Figure 4.2 demonstrates the pregnancy rate above and below each freezing concentration within the validation data set. A threshold value of 680.850×10^6 sperm/mL (indicated by the dotted line) was calculated as the maximum point of difference between pregnancy rates above and below the cutting point. Above the threshold, the pregnancy rate was 82 %, whereas below the threshold, it was 73 %.

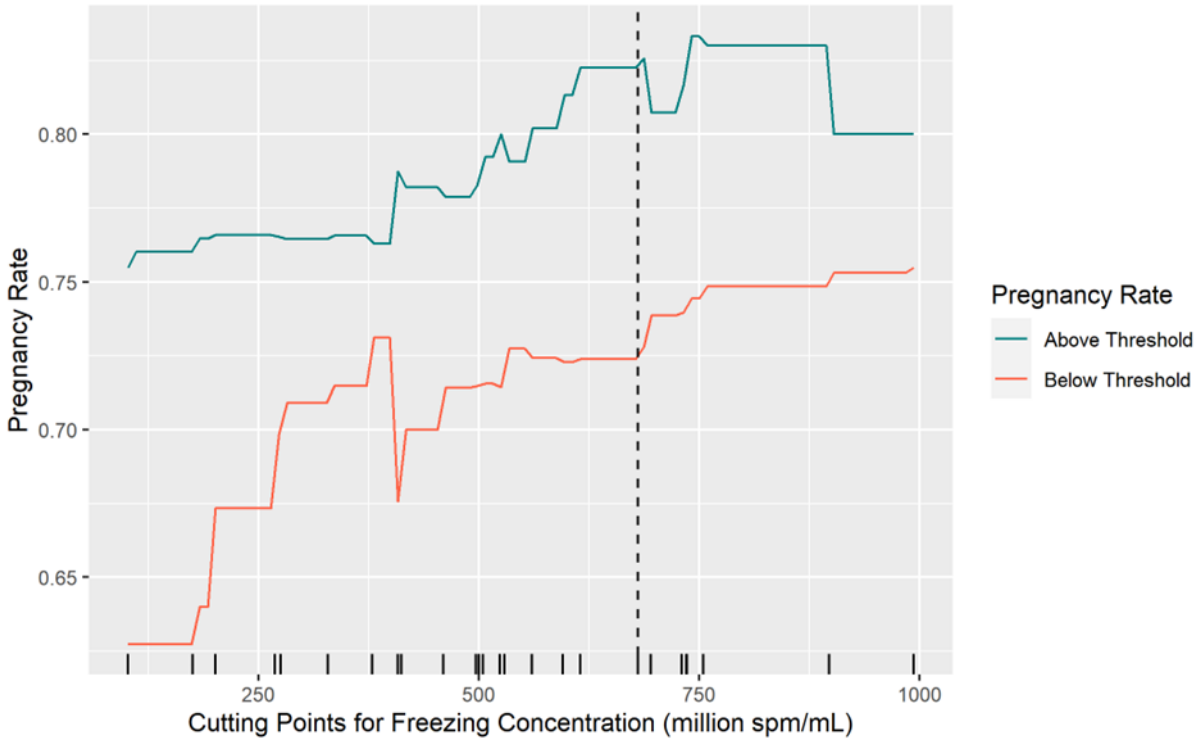


Figure 4.2: The relationship between freezing concentration (×10^6 sperm/mL) and pregnancy rates, distinguishing between rates above and below a chosen threshold. The dashed vertical line represents the optimal threshold selected at 680.85 million sperm/mL. The coloured lines indicate pregnancy rates above (blue) and below (orange) the threshold (cutting point). The rug plot displays the distribution of data points (cutting points) for the validation data set.

4.3.2.3. Abnormal Sperm Morphology

Figure 4.3 demonstrates the pregnancy rate above and below for each value of abnormal sperm morphology within the validation data set. A threshold value of 15.5 % (indicated by

the dotted line) was calculated as the maximum point of difference between pregnancy rates above and below the cutting point. Above the threshold, the pregnancy rate was 71 %, whereas below the threshold, it was 81 %.

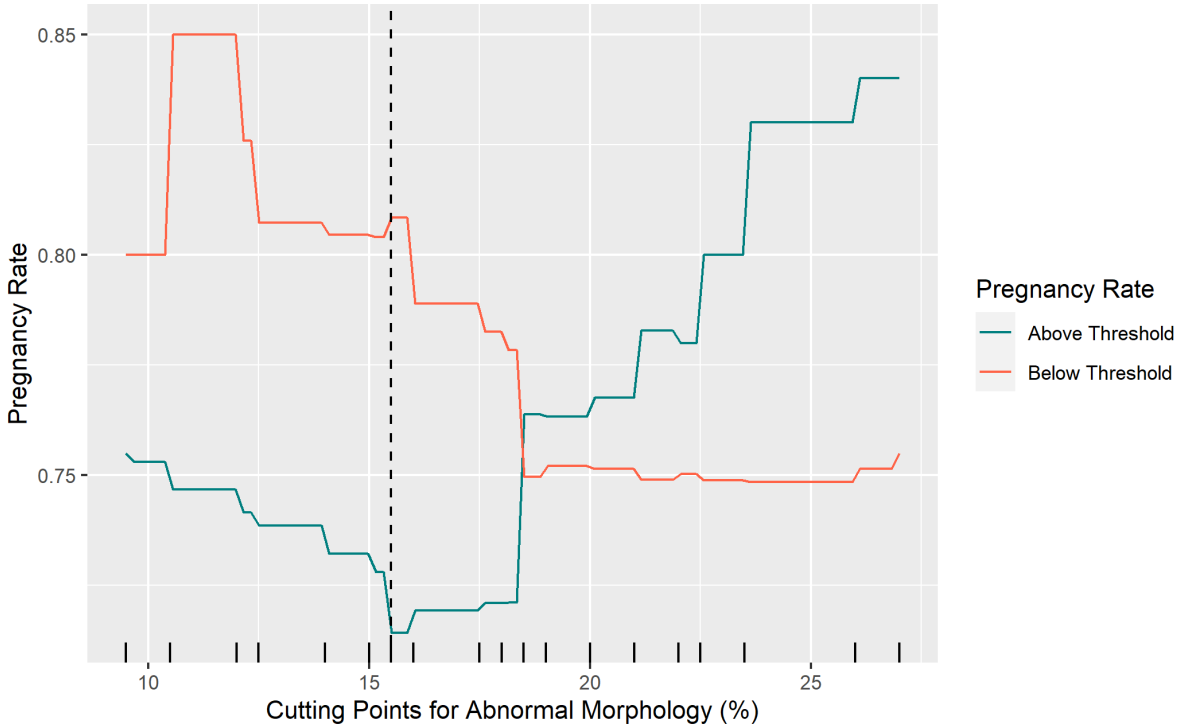


Figure 4.3: The relationship between abnormal sperm morphology (%) and pregnancy rates, distinguishing between rates above and below a chosen threshold. The dashed vertical line represents the optimal threshold selected at 15.5 %. The coloured lines indicate pregnancy rates above (blue) and below (orange) the threshold (cutting point). The rug plot displays the distribution of data points (cutting points) for the validation data set.

4.3.2.4. Viable Sperm with Intact Acrosomes (6 h post-thaw)

Figure 4.4 demonstrates the pregnancy rate above and below for each percentage of viable sperm with intact acrosomes within the validation data set. A threshold value of 6.31 % (indicated by the dotted line) was calculated as the maximum point of difference between pregnancy rates above and below the cutting point. Above the threshold, the pregnancy rate was 79 %, whereas below the threshold, it was 62 %.

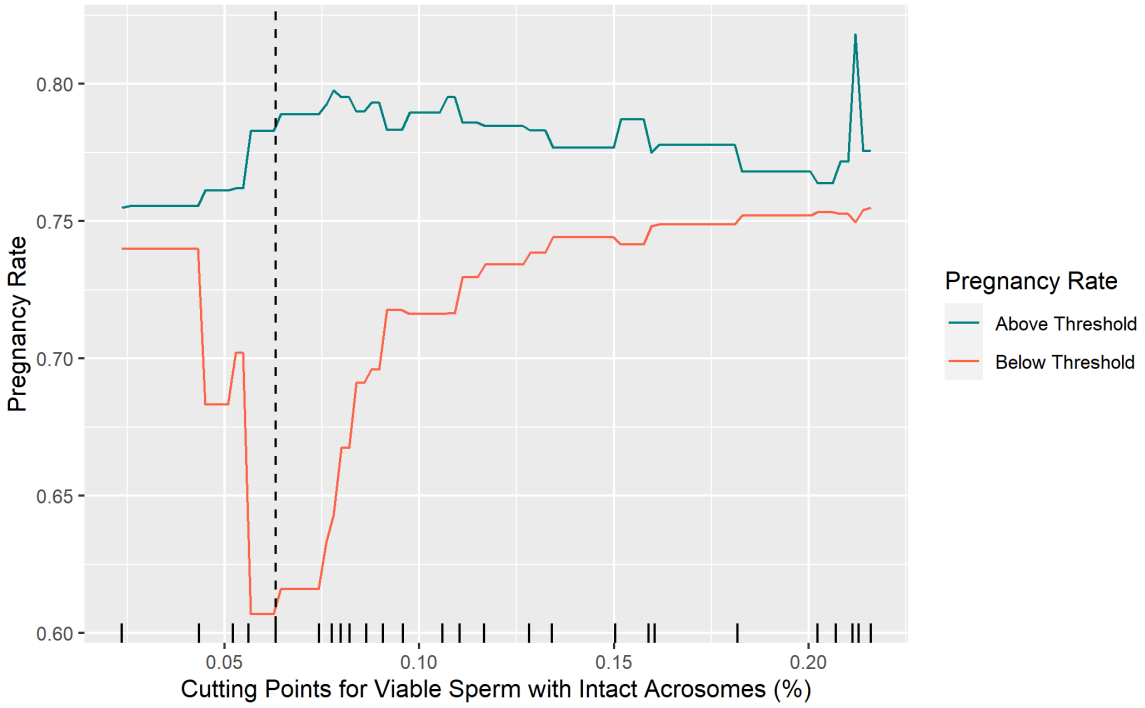


Figure 4.4: The relationship between viable sperm with intact acrosomes and pregnancy rates, distinguishing between rates above and below a chosen threshold. The dashed vertical line represents the optimal threshold selected at 6.32 %. The coloured lines indicate pregnancy rates above (blue) and below (orange) the threshold (cutting point). The rug plot displays the distribution of data points (cutting points) for the validation data set.

4.3.2.5. CASA PCA (0 h post-thaw)

Figure 4.5 demonstrates the pregnancy rate above and below for each value of the CASA PCA within the validation data set. A threshold value of 18.34 was calculated as the maximum point of difference between pregnancy rates above and below the cutting point. Above the threshold, the pregnancy rate was 75 %, whereas below the threshold, it was 67 %.

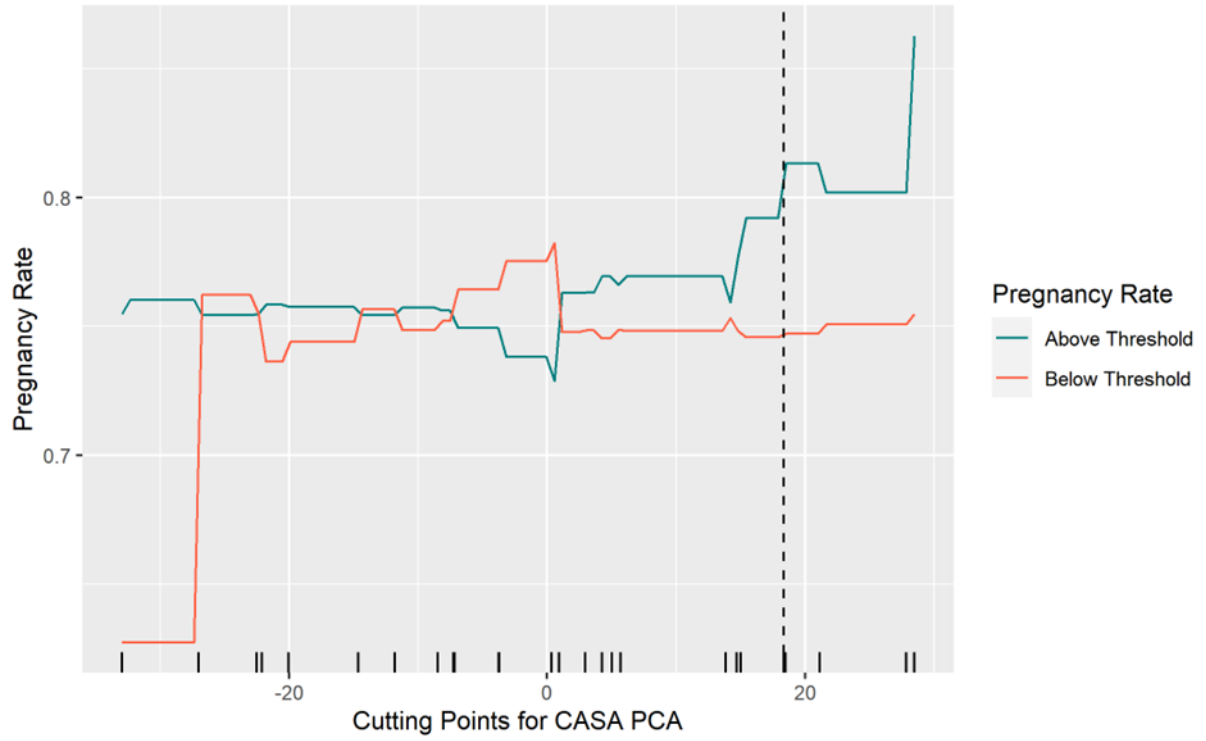


Figure 4.5: The relationship between values for the CSAA PCA and pregnancy rates, distinguishing between rates above and below a chosen threshold. The dashed vertical line represents the optimal threshold selected at -5.76. The coloured lines indicate pregnancy rates above (blue) and below (orange) the threshold (cutting point). The rug plot displays the distribution of data points (cutting points) for the validation data set.

4.4. DISCUSSION

In the current study, we have (i) successfully validated a fertility model by comparing the predicted probability of fertility to actual fertility results determined via ultrasound and (ii) suggested optimal thresholds for each of the *in vitro* semen parameters used in the model that could lead to greater fertility following laparoscopic AI in sheep. The model demonstrated high accuracy (74 %), excellent precision (96 %), lower specificity (33 %), strong recall (76 %), and an AUC of 0.62. There was no significant difference in the number of pregnancies predicted versus ultrasound detected (P=0.184), suggesting the predictive model is accurate and reliable. Thresholds for each predictor (freezing concentration; 680.85×10^6 sperm/mL, CASA PCA; 18.34, %viable; 6.31 and %abnormal; 15.5) were calculated and when combined in the model, the overall cumulative probability of pregnancy occurring was 64.3 %.

Validation of the fertility model

This chapter is the first to validate a predictive fertility model for use within an ovine AI breeding program. Discrimination allows for evaluating the model's ability to correctly classify pregnant and non-pregnant ewes, while calibration ensures that predicted probabilities align closely with observed outcomes (Caraviello *et al.* 2006; Shahinfar *et al.* 2014; Hempstalk *et al.* 2015; Rutten *et al.* 2016; Fenlon *et al.* 2017). This dual approach provided a rigorous assessment of the model's accuracy and reliability, particularly under practical sheep breeding conditions, where variation in fertility can be significant. To our knowledge, limited studies have used discriminatory methods to validate fertility models, with (Fenlon *et al.* 2017) investigating fertility in dairy cattle under field conditions. By applying similar approaches in our study, we aim to gain a greater insight into the model's predictive performance.

The model's accuracy in differentiating between pregnant and non-pregnant ewes was 74 %. In other words, the model correctly predicted 74 % of all pregnancy outcomes. More specifically, the model demonstrated a high precision of 96 %, accurately identifying 96 % of the positive pregnancy predictions. However, the model's specificity was comparatively lower at 33 %, only capable of identifying 33 % of the true negative cases. Thus, it can be understood that the model can better identify pregnant ewes than non-pregnant. The reason for this can stem from the pregnancy threshold of 0.56, whereby, variation will naturally remain around this threshold, affecting the balance between sensitivity and specificity. Adjusting the threshold could potentially improve the specificity score; however, this could come at the cost of reducing precision. Combining this information, the model recorded a recall (sensitivity) value of 76% and an F1-score of 0.85, indicating an overall very good performance by the model. With the completion of this study, it remains clear that no predictive model of pregnancy can solely discriminate the pregnancy outcome of an individual's pregnancy outcome. The study attempted to control these through using random effects, such as Site, Sire (frozen-thawed),

and Thaw Day. It is acknowledged, however, that many other influencing factors, including environment (Sawyer 1979; van Wettere *et al.* 2021), human error, and inseminator, could contribute to the accuracy of the model.

Combining all these results together, the AUC obtained from our fertility model was 0.62, indicating that on average, the model has a 62 % chance of correctly distinguishing between a randomly chosen pregnant or non-pregnant outcome. Although the fertility variables used in this model are specific to sheep, comparing the results to other species is important to gauge the performance and robustness of the model. The AUC value obtained from the current study matches the values obtained by (Hempstalk *et al.* 2015) and (Fenlon *et al.* 2017) in dairy bulls using logistic regression; however, lower than (Shahinfar *et al.* 2014) results using alternative machine learning algorithms (0.61 – 0.76 and 0.61 – 0.73 for primiparous and multiparous cows). Whilst the current model did not utilise machine learning, logistic regression models can more accurately predict pregnancy than other modelling methods (Hempstalk *et al.* 2015; Fenlon *et al.* 2017). Importantly, this is the first study to validate a fertility model in sheep using discriminatory techniques that statistically determine the model's ability to predict pregnant from non-pregnant ewes.

Finally, the MACE score obtained indicates that, on average, the model's predictions deviate from the actual pregnancy outcomes by 26 %. Proving our hypothesis correct, the chi-squared test found no significant difference between predicted and actual pregnancy outcomes (P=0.184). Despite the moderate percentage error value of 26 %, the result suggests that the fertility model's predictions align reasonably well with the actual outcomes.

Establishment of *in vitro* semen standards

Using this model, this chapter is also the first to identify and recommend thresholds for *in vitro* semen standards obtained from field fertility data. The thresholds or standards used resulted in

a cumulative pregnancy probability of 64 %. The model has, therefore, succeeded in differentiating two-thirds of the variation associated with AI breeding programs.

The model created by (Spanner *et al.* 2024b) reveals an intrinsic relationship between sperm freezing concentration and fertility, inferring that the number of sperm frozen acts as a proxy for the number of sperm inseminated. The trends in Figure 2.2 demonstrate a steady increase in pregnancy rates as concentration increases, plateauing at high concentrations. The critical threshold for sperm concentration of 680.85×10^6 sperm/mL proves the initial hypothesis correct. Sperm frozen at concentrations above this threshold yielded a pregnancy rate of 82 %, compared to 73 % for semen frozen below this concentration. In sheep, the standard practice involves freezing semen at higher concentrations if using pellets, which equates to three insemination doses, whereas straws are frozen at lower concentrations and used for a single dose (Evans and Maxwell 1987). The recommended insemination dose for laparoscopic AI is 25×10^6 motile spermatozoa (Evans and Maxwell 1987; Evans 1988; Salamon and Maxwell 1995a; Salamon and Maxwell 1995b; Salamon and Maxwell 2000). Research consistently shows that deviating from this recommendation negatively impacts pregnancy rates (Salamon *et al.* 1985; Maxwell 1986a; Casares *et al.* 1990; Findlater *et al.* 1991; Salamon and Maxwell 2000). (Maxwell 1986a) measured the percentage of ewes lambing after inseminations with 0.5, 5, 25, 50×10^6 spermatozoa to be 29.3, 26.8, 56.3, and 62.1 %, respectively. These results demonstrate that higher sperm numbers increase the likelihood of pregnancy. Within the current paper, freezing concentration has been set as a proxy to the insemination dose. Therefore, maintaining a freezing concentration equal to or above 680.85×10^6 sperm/mL will ensure the insemination dose contains enough motile spermatozoa to achieve pregnancies. This will enhance reproductive performance, reduce financial loss, and maximise productivity following AI across the industry.

The results of this chapter demonstrate a clear negative relationship between abnormal sperm morphology and the likelihood of pregnancy. Building on these initial findings by (Spanner *et al.* 2024b), the current study identified a threshold of 15.5 % abnormal sperm within a given sample. This study reports that any abnormal morphology above this threshold would contribute to a pregnancy rate of 71 %, compared to 81 % if abnormal morphology counts were below the threshold. The results indicate that higher sperm abnormalities correlate to decreased pregnancy rates, which has been widely discussed across livestock species, including stallions (Morrell *et al.* 2008; Love 2011), bulls (Gillan *et al.* 2008), rams (Mickelsen *et al.* 1981; O' Meara *et al.* 2008; Almadaly *et al.* 2016) and boars (Kondracki *et al.* 2020). However, many of the results remain inconsistent, and the ability to pinpoint an optimal threshold is limited. From our research, we can differentiate when the percentage of abnormal spermatozoa within a given sample will negatively affect pregnancy rates following AI. Moreover, with a positive predictive value of 54 %, the morphology assessment alone can correctly identify positive pregnancy cases over 50 % of the time. This is very important and a first for the industry, linking percentage abnormal to fertility outcomes. The results align with the current commercial semen recommendation for small ruminants of 15 % (Evans and Maxwell 1987). However, much detail lies in the question of reallocating abnormalities into subsections. Determining whether there are any relationships between specific abnormalities and fertility could help determine the minimum morphology requirements for successful AI (Spanner *et al.* 2024a). Whether classified by major to minor defects, morphological assessments lack uniformity, contributing to high variability among assessors and labs. Moreover, this is exacerbated by the lack of agreement on the classification system, which could contribute to the reduced PPV value obtained in our results.

Moving towards the intracellular components of spermatozoa, the fertility model developed by (Spanner *et al.* 2024b) identified sperm viability and acrosome integrity as two

critical indicators of fertility potential. These factors are crucial to the fertilisation process because sperm viability ensures survival after thawing, while acrosome integrity enables the sperm to penetrate the egg (Abou-Haila and Tulsiani 2000). Notably, these indicators were most significant at 6 h post-thaw, underscoring the need to assess sperm viability and acrosome integrity at this precise time point to gauge fertility potential accurately. With a positive predictive value of 84 %, a threshold of 6.32 % was established where values above this threshold contributed to a pregnancy rate of 79 % compared to 62 % when scored below this threshold. Successfully sitting within the range of the predicted hypothesis for this *in vitro* variable, a PPV of 84 % means that all the sperm samples measured could correctly identify 84 % of successful pregnancies. Unlike other studies, such as (Christensen *et al.* 2005), which qualified viability thresholds in dairy bulls, our work integrates sperm viability and acrosome integrity into a singular test. Studies across other livestock species, including bulls (Januskauskas *et al.* 2000; Gillan *et al.* 2008; Sellem *et al.* 2015), dairy bulls (Alm *et al.* 2001; Christensen *et al.* 2005), rams (O' Meara *et al.* 2008), stallions (Wilhelm *et al.* 1996) and boars (Christensen *et al.* 2004), show similar trends, in which greater fertility is related to higher sperm viability and acrosome integrity scores post-thaw. Unfortunately, these studies have not differentiated a threshold for optimal fertility. This is the first study within the sheep breeding industry to establish an optimal threshold for these *in vitro* measures, directly linking them to improved pregnancy rates in the field.

The final *in vitro* sperm trait assessed in the fertility model is the CASA Principal Component Analysis at 0 h post-thaw, where a critical threshold of 18.34 was identified. Pregnancy rates decreased to 67 % when CASA values fell below this threshold but rose to 75 % when above it. Whilst the units associated with the CASA PCA can be complex and hard to interpret, our work emphasises that total and progressive motility, as well as velocity traits, are essential factors in determining the fertility potential of sperm. Sperm motility is one of the

most important parameters of sperm quality (Van de Hoek *et al.* 2022), and the CASA objectively evaluates this by integrating motility with other kinematics parameters (Mortimer and Mortimer 2013). The CASA has been widely used in studies on bulls (Januskauskas *et al.* 2000; Gillan *et al.* 2008; Kumar *et al.* 2016), stallions (Love 2011), deer (Malo *et al.* 2005), humans (Holt *et al.* 1985), salmon (Gage *et al.* 2004) and rams (Del Olmo *et al.* 2013). Previous research has shown that increased motility and velocity correlate with increased pregnancy rates. However, identifying specific thresholds for each variable in CASA PCA is challenging due to the intricate nature of the PCA, which includes ten variables. As stated in the study's hypothesis, isolating the effect of one variable will fail to capture the total variability and interaction within the PCA framework, making the standardisation of the CASA difficult. Future research should aim to differentiate how each CASA variable within the PCA impacts pregnancy rates, moving beyond loading scores and correlations. Understanding the individual contribution of each variable will enhance the selection tool for rams, optimising breeding outcomes.

Implication for the industry

The implications of this research for the sheep industry are profound. This research has the potential to revolutionise artificial breeding of sheep, providing a fertility selection tool that can predict pregnancy outcomes and recommend accurate, data-driven *in vitro* fertility standards. This selection aid will enable breeders to select more fertile semen and ewes, maximise reproductive success, reduce financial losses and significantly boost overall productivity. The validated model can successfully identify pregnant from non-pregnant ewes accurately and precisely. In addition, the thresholds established can provide artificial breeding companies and stud breeders with realistic benchmarks for assessing sperm quality and the resultant fertility potential of a given sample, improving their decision-making process. Further research should focus on the intricate relationships between CASA traits, principal component

analysis, and their individual associations with fertility outcomes. This approach will provide a more comprehensive understanding of motility and kinetics, their influence on fertility and optimal thresholds to enhance reproductive success. Despite this, the standards or thresholds proposed in this paper must now be offered to the industry to ensure they can be realistically incorporated into semen processing protocols. Industry consultation and feedback are essential in order to create tangible outcomes and benefits for growers and the wider sheep industry.

4.5. CONCLUSION

This validated ovine fertility model can now be used as an effective decision-making tool for the sheep artificial breeding industry to select rams and ewes of high fertility potential. Through discrimination and calibration assessments, the current study examined the model's robustness and accuracy in predicting pregnancies in sheep following laparoscopic AI. The model demonstrated an exceptional ability to distinguish pregnant ewes. However, its ability to correctly differentiate non-pregnant ewes is more limited. This study successfully identified critical threshold points or standards for *in vitro* semen analysis linked to pregnancy success. This will allow rams, ejaculates and semen batches to be screened before use in artificial breeding programs to determine their fertility potential. Ultimately, this will result in more successful AI programs, reducing variation while accelerating production and genetic gain for the Australian sheep industry.

Chapter 5. The effect of freezing and thawing technique on the *in vitro* quality of ram semen in pellets

The experiments described herein are under review for publication as: Spanner, E.A., Rickard, J.P., de Graaf, S.P., 2024. The effect of freezing and thawing technique on the *in vitro* quality of ram semen in pellets. *Under review for publication to Animal Reproduction Science.*

5.1. ABSTRACT

The post-thaw quality of pellet-frozen ram semen was evaluated based on (i) sperm concentration, (ii) thawing diluent, and (iii) the number of pellets thawed simultaneously. Ejaculates from 3 Merino rams (n=3) were collected, with four replicates per ram (n=12) for each experiment. In Experiment 1, ejaculates were frozen at 200, 400, 600 or 800×10^6 sperm/mL. In Experiment 2, ejaculates were frozen at 600×10^6 sperm/mL and thawed in a dry test tube (control) or with 1+3 tris-citrate-fructose, tris-citrate-fructose+egg yolk, PBS+dye, PBS alone or IVF media (Emcare). In Experiment 3, groups of 1, 2 or 3 pellets were thawed in tris-citrate-fructose or a dry test tube (control). Post-thaw samples were tested for motility, acrosome and membrane integrity (FITC-PNA/PI) at 0, 3, and 6 h (all Experiments) and morphology at 0 h (Experiment 1). At 3 and 6 h post-thaw, motility was reduced at 200×10^6 sperm/mL compared to other concentrations ($P<0.05$). Across all time points, samples frozen at 800×10^6 sperm/mL ($P<0.05$) showed lower viability, while freezing at 200×10^6 sperm/mL increased morphological abnormalities ($P<0.05$). Thawing pellets with tris-citrate-fructose media resulted in higher motility, acrosome and membrane integrity 0, 3

and 6 h post-thaw (P>0.05). Thawing one-pellet in tris-citrate-fructose improved motility and viability post-thaw (P<0.05) compared to multiple pellets, while the number of pellets thawed had no significant effect when dry-thawed (P>0.05). The results suggest that the optimal post-thaw quality is achieved when semen is frozen between 400-600×10^6 sperm/mL and thawed in tris-citrate-fructose media, one pellet at a time.

5.2. INTRODUCTION

Cryopreservation is one of the most important reproductive technologies available to the artificial breeding industry as it permits long-term storage and efficient dissemination of elite male genetics. The cryopreservation process is known to invoke a plethora of changes in spermatozoa including early capacitation and acrosome reaction (Gillan and Maxwell 1999), reduced membrane integrity (Salamon and Maxwell 1995b), reduced motility (Gillan and Maxwell 1999), along with several sub-lethal modifications (Pini *et al.* 2018). These changes can impair sperm transport through the female (Warr *et al.* 2022), subsequent fertilisation of the oocyte and ultimately reduce pregnancy rates (Salamon and Lightfoot 1967). As such, it is no surprise that the success of AI has been linked to *in vitro* semen quality (Mickelsen *et al.* 1981; Gillan *et al.* 2008; Love 2011; Kumaresan *et al.* 2017; Macías *et al.* 2020). Given the pivotal importance of producing high-quality frozen-thawed ram sperm for AI, considerable efforts have been made to optimise the method used to freeze ram semen and minimise the aforementioned negative effects of the entire process (Jabbour and Evans 1991; Watson 2000b). However, the effect of some practical aspects of the freezing and thawing process remains unclear, particularly for pellet frozen ram semen, where the commercial protocols used to produce and thaw ram semen pellets are very diverse.

In sheep, semen is overwhelmingly processed for frozen storage using a simple one-step dilution with tris-citrate-glucose-egg yolk-glycerol diluent (Salamon 1968). Following dilution, semen is cooled slowly to 4 °C and frozen either in straws in liquid nitrogen vapour

or in 0.2-0.25mL pellets on dry ice. Each pellet is expected to contain enough spermatozoa to inseminate three ewes (>75×10^6 motile spermatozoa) (Evans and Maxwell 1987) and remains the most common method of semen freezing in sheep in Australia, despite no difference in pregnancy rates between straw and pellet-frozen semen (Wilson *et al.* 2023). Several studies in different species have attempted to determine the optimal pre-freezing concentration of spermatozoa packaged in straws by measuring its effect on *in vitro* sperm quality parameters post-thaw. In sheep, freezing straws at lower concentrations of 100 to 500×10^6 sperm/mL appears to improve acrosome integrity, viability and motility of frozen-thawed ram semen when compared to 800 and 50×10^6 sperm/mL concentrations (D'Alessandro *et al.* 2001). Additionally, in stallions, Nascimento et al. (2008) measured greater motility and intact plasma membranes when spermatozoa were frozen at 100×10^6 sperm/mL. Pena and Linde-Forsber (2000) also measured dog sperm motility and viability to be significantly lower in straws frozen at 400×10^6 sperm/mL. Most recently, Alvarez et al. (2012) confirmed that cryopreserved ram semen packaged in straws at 800×10^6 sperm/mL will adversely affect the post-thawing quality. As such, ram semen frozen in straws is expected to be approximately 200×10^6 sperm/mL in most commercial situations (Evans and Maxwell 1987). For pellets, Salamon and Lightfoot (1969b) demonstrated that semen dilution rates before pellet freezing significantly influence the post-thaw survival of ram spermatozoa, with a four to six-fold dilution yielding superior motility and viability compared to two-fold dilutions. Supporting the initial findings by Platov (1965) and Jones and Martin (1965), freezing ram semen at concentrations outside these thresholds was shown to be detrimental to sperm survival (Jones and Martin 1965; Platov 1965). However, an industry standard is less well established as the majority of research on pellet dilution is by fold, rather than a specific number. Therefore, an updated analysis of the effect of optimal freezing concentration for a pellet is required to standardise industry procedures to ensure the best quality semen post-thaw.

Pellet frozen ram semen can be thawed either in a solution ("wet thawing") or a dry glass tube ("dry thawing"). Throughout history, various thawing diluents have been considered (for full review see (Salamon and Maxwell 1995a)) including sugar-citrate-yolk diluent and tris-citrate-fructose thawing media (Evans and Maxwell 1987), Phosphate Buffer Saline (PBS) (Putri *et al.* 2019) with and without dye (used to aid visualisation of semen in the insemination pipette), as well as embryo growth media (eg. *Emcare Holding, ECHM-100, Pacific Vet PTY LTD, Australia*; EMCARE). Initial research suggested post-thaw quality was maximised when pellets were thawed as quickly as possible, namely in solution and at high temperatures 38-40ºC (Platov 1965; Platov 1966; Salamon 1968; Salamon and Lightfoot 1969b). Similarly, the number of pellets in the tube has been thought to affect the thawing rate and, thus, post-thaw semen quality (Salamon 1968; Salamon and Lightfoot 1969b; Srivastava and Kalra 1985). Increasing the rate of dilution at thawing from 3:1 to 1:3 (pellets: thawing solution), demonstrated a linear increase in the revival of spermatozoa (Salamon 1968). Conversely, Srivastava and Kalra (1985) demonstrated improved post-thaw motility when three to six pellets rather than one to two pellets were thawed at 45 °C by dry thawing (Visser and Salamon 1974a; Srivastava and Kalra 1985). In practice, there is a wide variety of thawing methods employed by sheep artificial breeding operators, and much debate over the optimal concentration to freeze semen, the effectiveness of dry vs wet thawing of pellets, the best type of thawing media to use, and the maximum number of pellets that can be thawed at a given time. Therefore, it is timely to investigate the effect of these practices on the quality of pellet-frozen-thawed ram semen.

As such, this series of experiments aimed to determine the effects of 1) sperm concentration during freezing, 2) diluent presence and type during thawing, and 3) the number of pellets present in the tube during thawing on the post-thaw quality of ram sperm. The results of this

study will inform the optimal conditions for *in vitro* processing and thawing of pellet-frozen ram semen.

5.3. MATERIALS AND METHODS

5.3.1. Experimental Design

Three experiments were conducted to assess the effect of *in vitro* semen processing on sperm quality post-thaw. Two ejaculates were collected from Merino rams (n=3), with four replicates per ram (n=12). In Experiment 1) each of the paired ejaculates for each ram was pooled to make a replicate, diluted and frozen to concentrations of 200, 400, 600 or 800×10^6 sperm/mL. In Experiment 2) semen was separated into five treatment groups, consisting of diluent type: Control (DRY), Salamon's Thawing Diluent (29.99 mM tris, 5.55 mM fructose, 9.47 mM citric acid, and pH 7; FTC), Salamon's Chill Diluent (29.99 mM tris, 2.78 mM fructose, 9.47 mM citric acid, 15 % [v/v] egg yolk, and pH 7.3; FTC + EY) PBS + dye (*PBS Blue, 21500/0001, Minitube, Australia*; PBS + Dye), PBS alone (*Phosphate buffed saline, P4417, Sigma-Aldrich, Australia*; PBS) and IVF media (*Emcare Holding, ECHM-100, Pacific Vet PTY LTD, Australia*; EMCARE). In Experiment 3) two ejaculates of each ram were pooled ejaculates to make a replicate and split into six treatment groups consisting of Salamon's thawing diluent (FTC) and control (DRY) with 1, 2 and 3 pellets.

5.3.2. Animals

For all experiments, the studies were carried out in strict accordance with the Australian Research Act 1985 No. 123 and the Australian Code for the Care and Use of Animals for Scientific Purposes Eighth Edition (2013). All experimental procedures were conducted with approval from the University of Sydney Animal Ethics Committee (AEC No: 2023/2330).

5.3.3. Semen Collection and Preparation

Merino rams were housed at the animal house facility at the University of Sydney and fed a live weight maintenance diet. Rams were kept in individual pens with minor visual contact with each other. Semen was collected using an artificial vagina and teaser ewe (Evans and Maxwell 1987). Ejaculates were pooled and diluted 1:0.5 with Salamon's cyrodiluent (300 mM tris, 28 nM glucose, 104 mM citric acid, 15 % [v/v] egg yolk, 5 % [v/v] glycerol, and pH 7.3). Initial concentration was determined by NucleoCounter SP-100 (*ChemoMetec A/S, Allerod, Denmark*).

5.3.4. Semen Freezing

For Experiment 1) samples were diluted to concentrations of 800, 600, 400, and 200×10^6 sperm/mL in Salamon's cyrodiluent. For Experiments 2 and 3), samples were diluted to a concentration of 600×10^6 sperm/mL in Salamon's cyrodiluent. After dilutions, samples were cooled to 4 ºC over 2-3 h. 0.20 mL of diluted semen from each ram was pipetted onto dry ice. Pellets were stored in liquid nitrogen until thawing for analysis.

5.3.5. Thawing Semen

For Experiment 1) 2 pellets were thawed in a glass thawing tube (*13x100 mm Borosilicate; Livingstone International, Rosebery, Australia*). For Experiment 2) 2 pellets were placed in the thawing tube containing either 1.2 mL of each treatment media (1+3) or without media (Control). For Experiment 3) 1.2 mL was placed into each thawing tube as 1, 2 and 3 pellets were added. This was followed by placing 1, 2, and 3 pellets in a dry tube (Control). All treatments were thawed for 2 mins within a water bath at 37 ºC. All dry thaw treatments were diluted 1:0.5 with PBS + 0.3 % BSA and held for 6 h post-thaw.

5.3.6. Morphology Analysis

For Experiment 1, the percentage of abnormal spermatozoa was subjectively assessed using a phase-contrast microscope (×400 magnification) (Gillan *et al.* 2008). Capturing a minimum of 200 cells, the results were converted into a percentage by calculating the proportion of abnormal spermatozoa in each sample.

5.3.7. Sperm Motility Analysis

At each time point (0, 3 and 6 h post-thaw), the motility of spermatozoa of the initial 1:0.5 dilution with PBS + 0.3 % BSA and 50×10^6 sperm/mL was assessed. The percentage of motile spermatozoa was subjectively assessed using a phase-contrast microscope (×100 magnification). Samples (6 µL) were aliquoted onto slides warmed to 37 ºC and enclosed using a 22 × 22 mm coverslip. Values were obtained to the nearest 5 % by examining five fields of each sperm sample (kept on a heated slide and coverslip at 37 ºC), as (Evans and Maxwell 1987).

5.3.8. Flow Cytometric Analysis (Acrosome Integrity and Viability)

Samples were assessed using the CytoFLEX (*CytoFLEX and CytExport 2.0 Software Beckman Coulter; USA*); three lasers were employed: 50 mW 488 nm, 50 nW 638 nm and 80 mW 405 nm. All samples were stained with the DNA probe Hoechst 33342 (final concentration of 1 µg/mL), which has a fluorescence detection filter of 450/45 bandpass (BP), to gate any possible debris in the sample. Sperm cells were isolated from total events based on 488 nm forward and side scatter profiles. For each of the below variables, 10,000 sperm cells were analysed, and a minimum of 1000 Hoechst 33342 positive events were required to obtain valid results. Spermatozoa viability and acrosome integrity were examined using a staining combination of both Propidium Iodine (PI, final concentration 6µM) and Fluorescein isothiocyanate peanut agglutinin (FITC-PNA, final concentration 0.4µg/mL) for 10 minutes at 37 ºC. PI and FITC-PNA fluorescence detection was on 690/50, and 525/40 nm BP filters, respectively. Cells were

viable with intact acrosomes if cells were PI and FITC-PNA negative (recorded at a percentage of the parent).

5.3.9. Statistical Design

All statistical analyses were performed using R Studio (Version 2023.09.1). All Averages with ±SEM were initially calculated as part of data exploration. For Experiment 1) a Linear Regression Model (REML) was used to determine if there was an effect of pellet and pre-freeze concentration on *in vitro* semen quality traits. To explore this effect, pellet pre-freezing concentration was used as a fixed effect, followed by ram, replication and timepoint post-thaw as random effects. For Experiments 2 and 3) treatments including thawing diluent, pellet number and timepoint post-thaw were placed as fixed effects followed by ram and replicate as random effects. If the 3-way effect, including timepoint post-thaw, lacked significance, timepoint post-thaw would be considered as a random effect to control for the variation exhibited between time points. The normality and homogeneity of each variable were confirmed using the Shapiro-Wilk and Barlett's tests, respectively. Emmeans pairwise comparisons were used to compare differences between treatment groups. Relations were deemed significant if $P<0.05$.

5.4. RESULTS

5.4.1. Experiment 1: The Effect of Sperm Concentration on the In Vitro Quality of Frozen-thawed Ram Spermatozoa

Sperm motility

There was a significant effect of freezing concentration and time post-thaw on sperm motility ($P<0.001$). Notably, there was also a significant interaction between freezing concentration and time post-thaw ($P=0.002$, Figure 5.1).

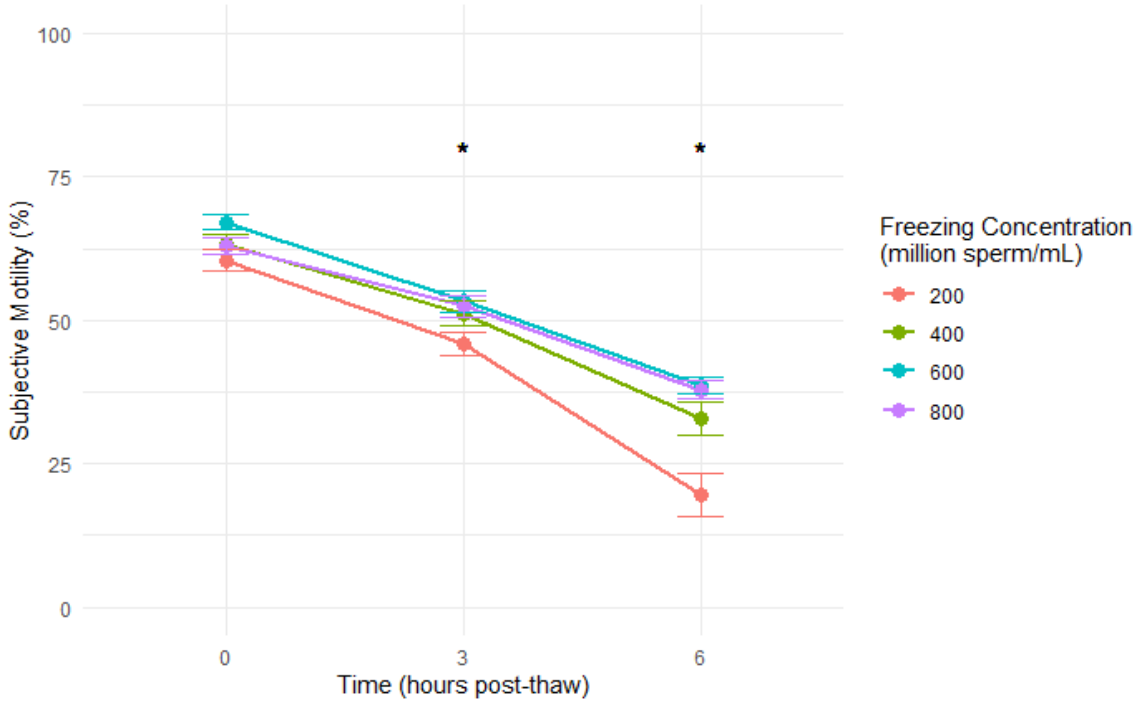


Figure 5.1: The effect of sperm concentration (200, 400, 600 and 800×10^6 sperm/mL) on the mean motility of frozen-thawed ram spermatozoa at 0 h – 6 h post-thaw. Asterisks indicate significant differences in post-thaw motility between sperm concentration treatment groups within each timepoint (*P*<0.05).

At 0 h post-thaw there was no initial difference between pre-freeze concentrations, 200, 400, 600 or 800×10^6 sperm/mL (60.42 ± 1.89 %, 63.33 ± 1.78 %, 67.08 ± 1.30 %, 62.92 ± 1.44 %, respectively, *P*>0.05). At 3 h post-thaw, pellets frozen at a concentration of 200×10^6 sperm/mL recorded a significantly lower motility than 600×10^6 sperm/mL (45.83 ± 2.03 %, 53.33 ± 1.78 %, respectively, *P*<0.05). All other comparisons had no significant differences (*P*>0.05). At 6 h post-thaw, pellets frozen at 200×10^6 sperm/mL recorded significantly lower motility than pellets frozen at 400, 600 and 800×10^6 sperm/mL (19.58 ± 3.72 %, 32.92 ± 2.98 %, 38.75 ± 1.52 %, 37.92 ± 1.68 %, respectively, *P*<0.05). All other comparisons had no significant differences (*P*>0.05).

Percentage of viable and acrosome intact spermatozoa

There was a significant effect of the pellet pre-freeze concentration on the number of viable spermatozoa with intact acrosomes (*P*<0.001, Figure 5.2). There were no interactions (*P*>0.05).

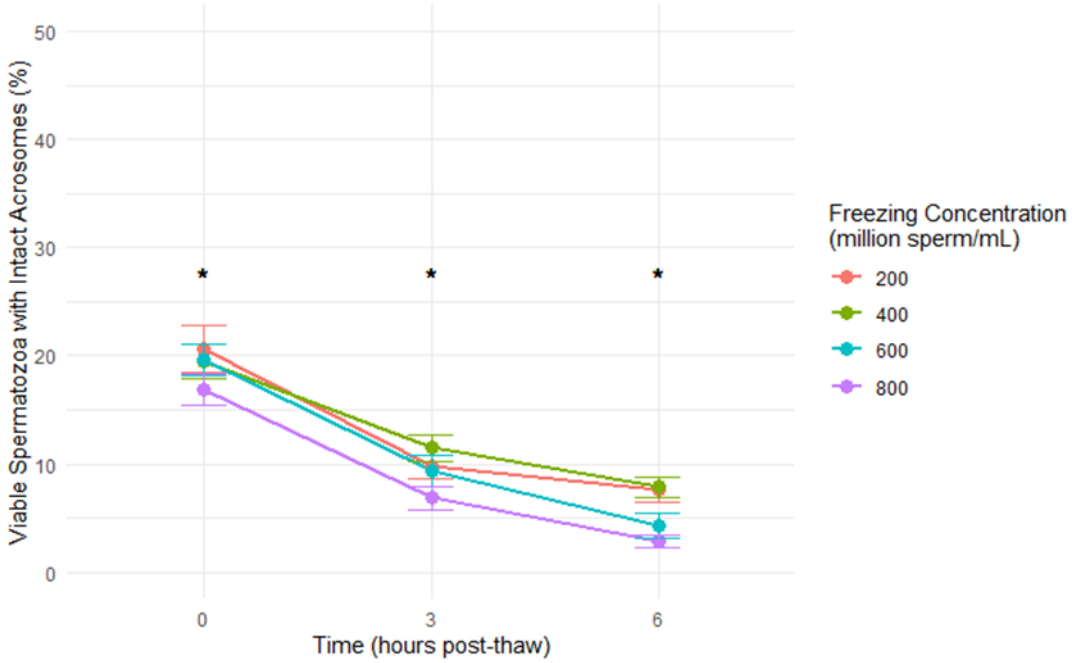


Figure 5.2: The effect of sperm concentration (200, 400, 600 and 800×10^6 sperm/mL) on the mean percentage of viable spermatozoa with intact acrosomes at 0 h – 6 h post-thaw. Asterisks indicate significant differences in post-thaw viability and acrosome integrity between sperm concentration treatment groups within each timepoint (*P*<0.05).

There was a significant decrease in the number of acrosomes intact and viable spermatozoa when frozen at 800×10^6 sperm/mL when compared to pellets frozen at 200 and 400×10^6 sperm/mL (8.85 ± 1.67 %, 12.67 ± 1.30 %, 12.95 ± 1.09 %, respectively, P<0.05). All other comparisons had no significant differences (P>0.05).

Abnormal morphology

There was a significant effect of pellet pre-freeze concentration on the number of abnormal spermatozoa (P<0.001, Figure 5.3). There were no interactions (P>0.05).

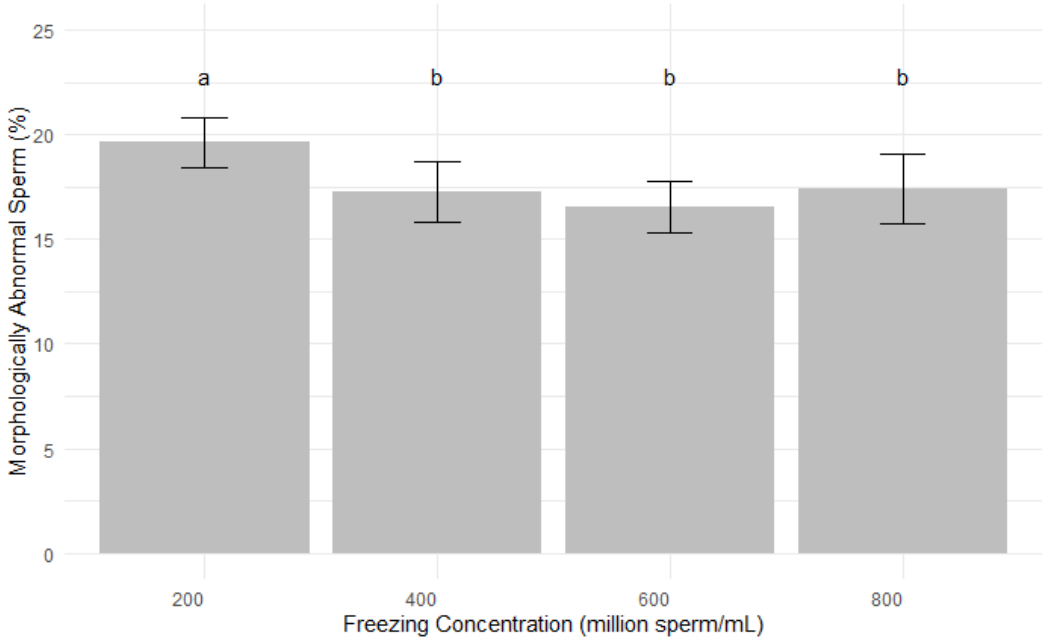


Figure 5.3: The effect of freezing concentration (200, 400, 600 and 800×10^6 sperm/mL) on the number of morphologically abnormal spermatozoa at 0 h post-thaw . Columns that differ in subscript indicate significant differences between treatments (*P*<0.05).

Semen frozen at a pre-freeze concentration of 200×10^6 sperm/mL (19.63 ± 0.67 %) produces a significantly higher amount of abnormal sperm compared to pellets frozen at 400, 600 and 800×10^6 sperm/mL (17.25 ± 0.81 %, 16.54 ± 0.71 %, 17.42 ± 0.92 %, respectively).

5.4.2. Experiment 2: The Effect of Thawing Diluents on the In Vitro Quality of Frozen-thawed Ram Spermatozoa

Sperm Motility

There was a significant effect of diluent type and time post-thaw on sperm motility (*P*<0.001). There was a significant interaction between diluent and time (*P*<0.001, Figure 5.4).

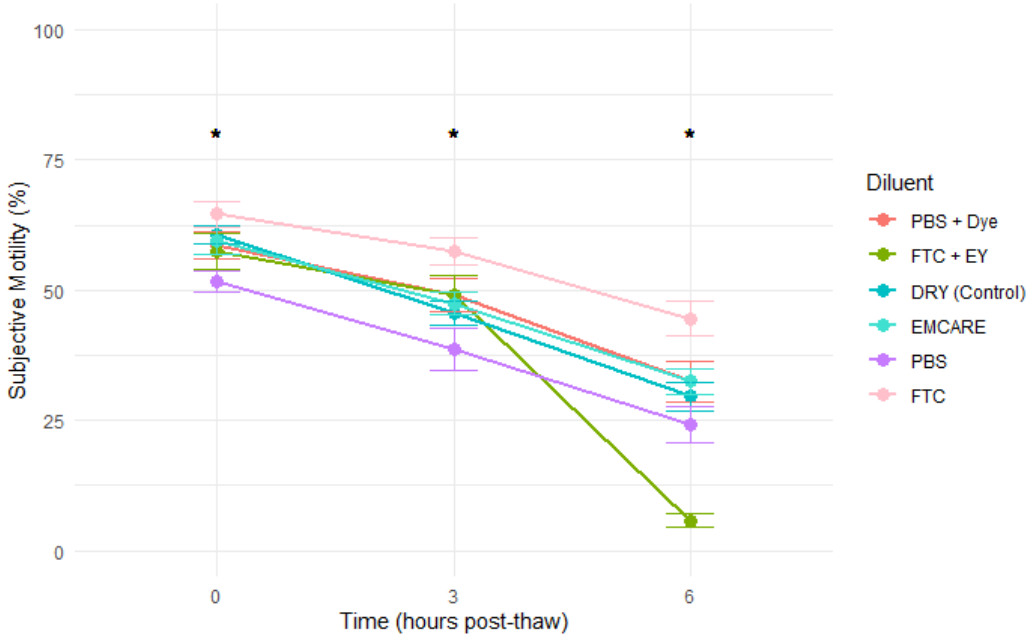


Figure 5.4: The effect of thawing diluents (PBS +Dye, FTC + EY, DRY (Control), EMCARE, PBS and FTC) on the motility of ram sperm at 0 h – 6 h post-thaw. Asterisks indicate significant differences in post-thaw motility between treatment groups within each timepoint (*P*<0.05).

At 0 h, the FTC diluent had the greatest motility, 64.58 ± 2.42 %, significantly different from PBS, which recorded the lowest motility, 51.67 ± 1.98 % (*P*<0.05). All other comparisons had no significant differences (*P*>0.05). At 3 h, FTC diluent had the greatest motility at 57.50 ± 2.65 % compared to PBS, recording the lowest motility at 38.75 ± 4.09 %. FTC had a greater motility than DRY, EMCARE and PBS, while PBS recorded a lower motility to PBS + dye, FTC + EY and FTC (*P*<0.05). All other comparisons had no significant differences (*P*>0.05). At 6 h, FTC diluent had the greatest sperm motility of 44.58 ± 3.23 %, and FTC + EY recorded the lowest motility of 5.83 ± 1.20 %. FTC diluent motilities were significantly higher than all other diluents, and FTC + EY was significantly lower than all other diluents (*P*<0.001). All other comparisons had no significant differences (*P*>0.05).

Percentage of viable and acrosome intact spermatozoa

There was a significant effect of diluent and time on the number of viable and acrosome intact spermatozoa (P<0.001). There was a significant interaction between diluent and time (P<0.001, Figure 5.5).

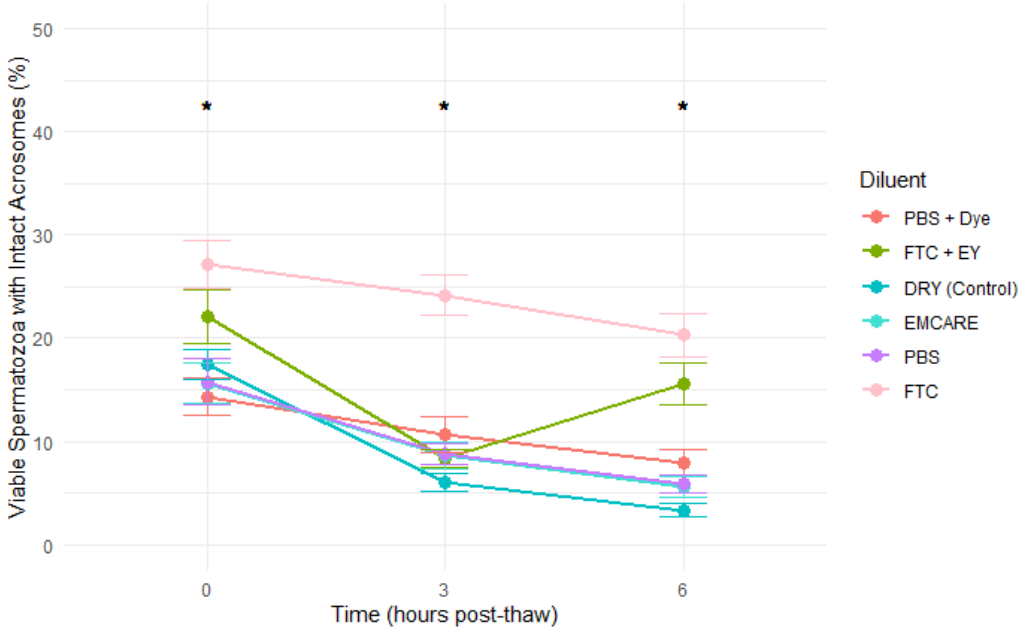


Figure 5.5: The effect of thawing diluents (PBS +Dye, FTC + EY, DRY (Control), EMCARE, PBS and FTC) on the number of spermatozoa with intact viable acrosomes of ram sperm at 0 h – 6 h post-thaw. Asterisks indicate significant differences in post-thaw viability and acrosome integrity between treatment groups within each timepoint (P<0.05).

At 0 h, FTC diluent had the greatest viability of 27.16 ± 2.25 %, whereas PBS + dye recorded the lowest viability of 14.29 ± 1.82 %. There was a significant difference between diluents FTC and all diluents (P<0.001). FTC + EY was significantly different to PBS + dye (P<0.05). All other comparisons had no significant differences (P>0.05). At 3 h, FTC diluent had the greatest viability of 24.17 ± 1.91 % compared to DRY, recording the lowest viability of 5.98 ± 0.85 %. There was a significant difference between the FTC compared to all other diluents (P<0.001). DRY was significantly different to PBS + dye (P<0.05). All other comparisons had no significant differences (P>0.05). At 6 h, FTC diluent has the greatest sperm viability of 20.33 ± 2.09 % compared to DRY with the lowest sperm viability of 3.35 ± 0.60 %. There was a significant difference between the viability of FTC and PBS + dye, DRY, EMCARE and PBS

(P<0.001). FTC + EY was also significantly different to DRY, EMARE, PBS and PBS + dye (P<0.001). DRY was significantly different to PBS + dye (P<0.05). All other comparisons had no significant differences (P>0.05).

5.4.3. Experiment 3: The Effect of Thawing Diluents and Pellet Number on the In Vitro Quality of Frozen-thawed Ram Spermatozoa

Sperm Motility

There was a significant effect of diluent and pellet number on sperm motility (P<0.001). There was a significant interaction between pellet number and diluent (P<0.001, Figure 5.6).

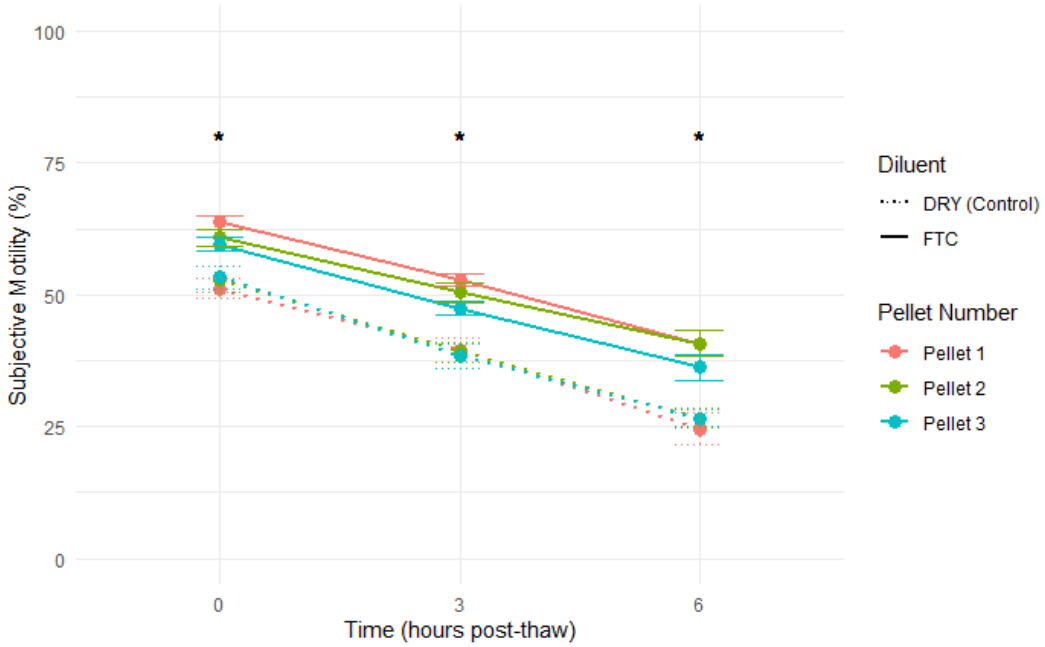


Figure 5.6: The effect of thawing diluents (DRY (Control) and FTC) and the number of pellets (1, 2 and 3) at thaw on sperm motility of ram sperm at 0 h – 6 h post-thaw. Asterisks indicate significant differences in post-thaw motility between treatment groups within each timepoint (P<0.05).

There was no significant difference between pellets when dry-thawed (P>0.05). When thawing in FTC solution, one pellet produced significantly higher motility (52.50 ± 1.87 %) than 2 (50.69 ± 1.77 %) and three pellets (47.78 ± 1.88 %, P<0.05). There was no difference between two and three pellets (P>0.05). Thawing in FTC solution consistently produced better motility results than dry thaw (P>0.05).

Percentage of viable and acrosome intact spermatozoa

There was a significant effect of pellet number and diluent on the number of viable and acrosome intact spermatozoa (P<0.001). There was a significant interaction between pellet number and diluent (P<0.001, Figure 5.7).

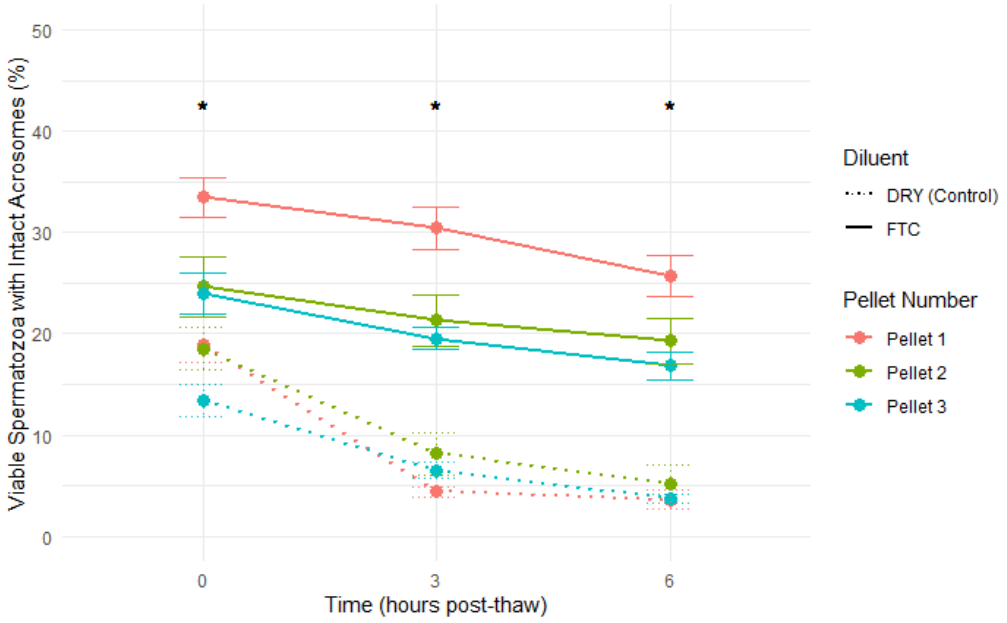


Figure 5.7: The effect of thawing diluents (DRY (Control) and FTC) and the number of pellets at thaw on the number of spermatozoa with intact viable acrosomes of ram sperm at 0 h – 6 h post-thaw. Asterisks indicate significant differences in post-thaw viability and acrosome integrity between treatment groups within each timepoint (P<0.05).

Across all time points, there was no significant difference in the number of spermatozoa with intact and viable acrosomes when DRY-thawed (P>0.05). There was a significantly greater amount of intact and viable acrosomes when one pellet was thawed with FTC diluent compared to two and three pellets (29.88 ± 1.27 %, 21.77 ± 1.50 %, 20.09 ± 1.00 %, respectively, P<0.05). There was a significant difference between DRY and FTC across all pellet numbers (P<0.05).

5.5. DISCUSSION

This study demonstrated the effect of (i) freezing concentration, (ii) thawing diluents, and (iii) the number of pellets thawed at a given time on the *in vitro* quality of frozen-thawed ram spermatozoa. Results indicated that when frozen at a concentration of 400-600×10^6 sperm/mL,

frozen-thawed ram sperm demonstrated the most consistent overall collective performance in terms of post-thaw motility and acrosome viability and membrane integrity, with fewer morphological defects when pooled over time compared to other concentrations. There was an ongoing effect on sperm quality traits when thawed in solution compared to a dry thaw. Thawing pellets in FTC resulted in greater motility and viability of sperm at 0, 3 and 6 h post-thaw than when thawed in a dry test tube or with other thawing diluents. Additionally, there is a clear relationship between pellet number when thawing in solution; however, this is less important in a dry thaw.

A direct comparison of pellet-frozen pre-freeze concentrations for ram spermatozoa has yet to be reported previously. In this study, we had found that motility was significantly reduced, and morphological abnormalities increased when semen was frozen in pellet form at 200×10^6 sperm/mL when compared to pre-freeze concentrations of 400, 600 and 800×10^6 sperm/mL (Figures 5.1-3). Previously studied by Salamon and Lightfoot (1969b), their work measured that a 2-fold dilution (higher sperm concentration) produced less motile spermatozoa when compared to four, six and eight-fold dilutions (lower sperm concentration) at 0 h post-thaw (18.2, 33.4, 36.9 and 34.6 %, respectively). In the context of the "fold", this refers to the factor by which the sperm concentration is diluted. While folding indicates changes to sperm concentration, it does not allow for the precise standardisation of the initial or final concentration. This limitation means that while the folding technique can suggest trends, they do not offer the exact measures of concentration levels through the previous studies. Interestingly, when attempting to make this alignment between fold and concentration, our findings align with the observations of Salamon and Lightfoot (1969b) when examining the effect of dilution rate over an incubation period of 6 h post-thaw. Over this time, the superiority of the four-fold dilution became evident, with higher dilution rates resulting in lower sperm survival rates (9.7, 16.1, 12.8 and 11.1 %, respectively). This suggests that lower freezing

concentrations (higher dilution rates) significantly affect sperm quality over time, whereas extremely high concentrations (lower dilution rates) have an immediate effect on sperm quality traits. Jones and Martin (1965) and Platov (1965) reported similar results, noting that higher dilution rates were harmful when freezing spermatozoa in fructose-yolk-citrate media prior to freezing. Jones and Martin (1965) found that motility was greatest at a tenfold dilution compared to twenty- and forty-fold dilutions (31.9, 24.4, and 6.3 %, respectively). This supports the notion that higher fold dilutions (lower sperm concentration) are not favourable for the preservation of post-thaw sperm motility of ram semen frozen in pellets (Jones and Martin 1965; Platov 1965).

To understand the biological reasoning behind these results, Jones and Martin (1965) initially hypothesised that the effect of dilution rate on sperm quality may be due to the close proximity of spermatozoa, the production of metabolic by-products and diluents and cryoprotectants used. It is hypothesised that lower sperm concentrations (greater dilution rates) allow for greater access to nutrients and metabolites, potentially leading to elevated levels of reactive oxygen species (ROS) and impaired sperm function (Allamaneni *et al.* 2005; Pereira *et al.* 2017; Siristatidis *et al.* 2021). Alternatively, it can be proposed that there is an ongoing interaction between sperm concentration and freezing that explains such changes. Whilst not explored in this chapter, future studies are required to refine the reasoning behind the reduction of motility, viability, acrosome, and morphological integrity at 200×10^6 sperm/mL. Therefore, the ongoing reassessment and adaption of cryopreservation methods are essential to optimise sperm quality for industry applications.

Alternatively, pellets frozen at 800×10^6 sperm/mL displayed greater numbers of abnormal spermatozoa and reduced sperm motility and viability (Figures 5.1-3). Despite focusing their studies on straw freezing, D'Alessandro et al. (2001) and Alvarez et al. (2012) have distinguished a relationship between acrosome and membrane integrity with increasing

freezing concentrations. At higher concentrations, sperm plasma membranes exhibit a lower resistance to cryodamage (Salamon and Visser 1972; Peña and Linde-Forsberg 2000; D'Alessandro *et al.* 2001; Alvarez *et al.* 2012). Notably, the formation of microdomains increases membrane permeability (Neild *et al.* 2003), contributing to premature acrosome reactions (Blottner *et al.* 2001), reduced mitochondrial functions (Vetter *et al.* 1998; Schober *et al.* 2007), and morphological abnormalities (Woolley and Richardson 1978). Our results (Figures 5.1-3) align with these insights, in which at 800×10^6 sperm/mL, morphological abnormalities and the loss of plasma and acrosomal membranes increase. Conversely, the adverse effects are mitigated between the concentrations of 600-400×10^6 sperm/mL. Alongside the work of others, our study adds valuable, current insight and useful recommendations to the existing literature on semen cryopreservation, highlighting the interplay between pellet pre-freezing dilution rates, concentrations, and post-thaw sperm quality. A pre-freeze concentration range of 400-600×10^6 sperm/mL provided the most consistently favourable outcomes across motility, viability, and sperm morphology measures, demonstrating superior performance over time compared to lower and extremely high concentrations.

A clear distinction emerges between wet and dry thawing methods when comparing pellet thawing procedures. FTC consistently outperformed the control dry thaw, demonstrating superior viability, intact acrosome rates and motility (Figures 5.4 and 5.5). The superior performance of wet thawing is attributed to the enhanced thawing rate facilitated by the media (Salamon 1968). Semen frozen in pellet form undergo rapid and significant heat transfer from dry ice, resulting in quick freezing (Maxwell *et al.* 1995). When these pellets are thawed in media, the process is believed to enable a quicker and more efficient diffusion of heat (Salamon and Lightfoot 1969b; Fiser *et al.* 1986). This rapid warming during thawing effectively mirrors reverse freezing, preventing *in vitro* sperm damage. Effectively, this is what we see in our results. To determine if these results are linked to an increase in thawing rate, a subsequent

analysis was performed to assess the time taken for each diluent to reach 37ºC. The results showed that the pellets in the FTC diluent reached 37ºC on average 33.5 seconds timed from the beginning of the thaw. This is compared to a dry thaw, which took, on average, 48.5 seconds to reach 37ºC. These results confirm that thawing pellets in solution increase the warming rate, consequently improving post-thaw semen quality (Salamon and Maxwell 1995a). The use of diluents, in this case, FTC media, can more closely mimic the freezing processes and limit cryodamage by improving *in vitro* semen traits post-thaw.

In addition to the thawing methods, the composition of the diluent plays a crucial role in the post-thaw quality of ram sperm. When comparing pellet thawing procedures across all time points, the FTC diluent treatment showed continuous benefits to sperm motility, viability, and total motility compared to all other treatment groups (PBS, PBS + dye, FTC + EY, DRY, EMCARE, Figures 5.4 and 5.5). The inclusion of sugar, particularly fructose, in FTC and FTC + EY diluents aligns with the previous findings by (Salamon 1968; Salamon and Lightfoot 1969b). Both authors saw sugar-based thawing diluents improve both the revival of pellet-frozen ram spermatozoa and subsequent viability during post-thaw incubation (Salamon 1968; Salamon and Lightfoot 1969b). However, it is complicated because the composition of sugar-based media can affect sperm quality immediately after thawing or during the incubation (Salamon and Lightfoot 1969a; Visser and Salamon 1973). Of particular interest was the FTC + EY thawing media, which is an ideal candidate for this misconception. Spermatozoa thawed in FTC + EY media presented high motility immediately after thaw; however, at 3 and 6 h post-thaw, these results significantly decreased and became the worst-performing diluent alongside the DRY thaw. Similarly, the converse effect was seen in PBS-based solutions, both PBS + dye and PBS reports have a lower immediate effect compared to greater prolonged incubation survivability, indicating it is better for incubation than thawing. These complicated relationships indicate that it is pivotal for artificial breeding programs to be specific about the

thawing media used. This will affect the timing of AI, the program's length and the quality of semen used.

In this investigation, we sought to determine the influence of pellet number on semen quality, comparing wet and dry thawing methods. Our results highlight that the pellet number does not affect semen qualities post-thaw when using a dry thaw (Figures 5.6 and 5.7). Alternatively, when thawed in a diluent, a 1-pellet thaw had a greater number of viable and acrosome intact spermatozoa and greater sperm motility when compared to 2- and 3-pellet thaws (Figures 5.6 and 5.7). The results following a wet thaw are consistent with the work of (Salamon 1968), which suggests that thawing in diluent enhances thawing speed and dilution rates. On the other hand, a dry thaw showed a comparatively lower thawing rate, whilst unexpectedly, pellet number had no effect on post-thaw semen quality. It is believed that as additional pellets are included in the thawing process, this will increase the time required to thaw and, as a result, reduce sperm quality (Srivastava and Kalra 1985). An additional experiment further explored this to determine the effect of pellet time on time to each 37 °C. The results found that thawing with 1, 2, and 3 pellets increased the time to reach 37 °C to 41.5 seconds, 48.5 seconds, and 1.02 minutes, respectively, when dry thawed. Whilst not seen in this study, alternatively, Srivastava and Kalra (1985) reported an increase in motility and higher percentages of live sperm with three to six pellets thawed compared to one to two pellets using dry thawing at 45 °C. While we know there is a clear relationship between pellet number, thaw time and sperm quality post-thaw, especially in wet thawing scenarios. Future investigations are needed to understand this relationship more clearly when dry thawing to provide more valuable insights into optimising thawing protocols.

As the cryopreservation and thawing process has become more streamlined for artificial breeding companies, the current results hold importance to the most updated process to freeze and thaw pellets. The information presented in the current results can help guide artificial

breeding companies on the best thawing protocol to use for each breeding program, to better improve *in vitro* processing for the Australian merino industry.

5.6. CONCLUSION

The results of the present study demonstrate there is an effect of sperm concentration during freezing, type of thawing diluent, method of thawing and the number of pellets when thawed on the *in vitro* quality of frozen-thawed spermatozoa. When pellet freezing, our results indicate that a pre-freeze concentration range of 400-600×10^6 sperm/mL should be used to maximise post-thaw motility, acrosome and membrane post-thaw and normal morphology. Further, it is recommended that one pellet is thawed in solution with tris-citrate-fructose media. Taken together, the experiments within this study have established the optimal freezing and thawing processes for ram spermatozoa frozen in pellet form. Widespread application of these processes across the artificial breeding community will improve the quality of pellet-frozen ram semen destined for artificial insemination.

Chapter 6. Discussion and Conclusion

Artificial insemination is a reproductive technology used to accelerate genetic and production gains across livestock species. In sheep, it is a method by which semen is collected from sires with superior genetics and deposited artificially either within the cervix (vaginal or cervical AI) or uterine horns (intrauterine laparoscopic AI) of the ewe. Unfortunately, frozen-thawed ram semen is unable to achieve acceptable pregnancies following cervical AI (Salamon and Lightfoot 1967), meaning laparoscopic AI must be relied upon to achieve pregnancies and take advantage of the genetic benefits associated with frozen semen. Thus, LAI is the only artificial insemination method commercially available within the Australian sheep industry. While it is expected that 70 % of ewes inseminated via LAI with frozen semen are expected to fall pregnant (Geenty *et al.* 2014), this is not always the case, and results have been measured to vary between season, site, and sire (Eppleston and Maxwell 1995; Hill *et al.* 1998; Del Olmo *et al.* 2013; Gibbons *et al.* 2019). It is believed that this variability has contributed to a low uptake of AI and other reproductive technologies across the industry and, subsequently, a reduced rate of genetic improvement for the industry (Kleemann and Walker 2005). While extensive research over the past decade has focused on a myriad of factors that influence fertility, limited studies have successfully pinpointed the cause of these 'failed' programs, examining the interplay between male and female factors, specifically during LAI, examining fertility of the individual ewe. The industry also lacks consensus on a set of scientifically supported reference thresholds to help standardise the *in vitro* processing, cryopreservation, and artificial insemination of ram semen in the field.

Studies contained within this thesis have addressed these industry limitations and gaps identified within the literature. This has led to the creation and validation of a fertility model that has facilitated the standardisation of fertility traits to predict the outcome of an LAI

breeding program in sheep. Additionally, studies explored the impact of *in vitro* processing techniques on these semen traits necessary for fertility. Most importantly, from a commercial point of view, high fertility levels can now be achieved when using the model as a selection tool, identifying ewes and semen batches from rams with high fertility potential, achieving pregnancy success and accelerating genetic and production gains throughout the industry.

During an industry-wide collation of fertility traits at the time of AI and post-thaw following advanced *in vitro* semen assessment, this thesis successfully identified that the pre-freeze concentration of the semen sample, the motility of ram spermatozoa at 0 h post-thaw, the percentage of viable and acrosome intact spermatozoa at 6 h post-thaw, the percent of sperm within the sample with abnormal morphology, the uterine tone and intra-abdominal fat score of ewes, influenced the likelihood of pregnancy occurring post-LAI (Chapters 2 and 3). When validated under field conditions with new 'unseen' data, this model demonstrated high accuracy (74 %), excellent precision (96 %), lower specificity (33 %), strong recall (76 %), and a final area under the curve (AUC) score of 0.62 (Chapter 4). There was no difference in the number of pregnancies predicted by the fertility model versus that diagnosed via ultrasound approximately 55 days post-LAI. Finally, Chapter 4 used the model to identify fertility thresholds for each predictor under field conditions. The model identified the likelihood of pregnancy to be highest when semen samples were frozen at a concentration of 680.8×10^6 sperm/mL, viability and acrosomes integrity at 6 h post-thaw was 6.31 %, and the percentage of abnormal sperm within a sample was equal to or less than 15.5 %. Ewes with a uterine tone and intra-abdominal fat score of 4 or 5 were also found to have a higher likelihood of pregnancy. Together, these standards recorded a cumulative chance of pregnancy of 64.3 %. The remaining 35.7 % is likely attributed to the influence of the environment during AI, which was unable to be accurately investigated within this thesis. To improve the survival of ram spermatozoa following cryopreservation for AI programs, it was determined that semen frozen

between 400–600×10^6 sperm/mL and thawed one pellet at a time in a tris-citrate-fructose media, would result in superior motility, viability and acrosome integrity (Chapter 5). Combined, these results provide conclusive evidence of a direct link between *in vitro* semen assessment and pregnancy, standardising procedures for the optimal preparation of ewes and the preservation, assessment and insemination of ram spermatozoa for successful LAI within the Australian sheep industry.

Prior to an artificial breeding program, it is paramount that ewes are in top condition to not only support pregnancy but also aid in the synchronisation of oestrous and fertilisation. The uterine tone of ewes assessed during insemination in Chapter 2 was found to significantly influence the likelihood of pregnancy. The importance of uterine tone was retained within the model in combination with intra-abdominal fat score when male semen factors were considered in the same model as outlined in Chapter 3. Assessing uterine tone enables investigation into the interplay between hormone concentration, oestrus synchronisation, and the timing of semen deposition (Hawk and Conley 1974; Hawk and Cooper 1977). Specifically, when ooestrogen levels increase during the follicular phase, prior to oestrus (Maxwell and Barnes 1986), the thickness of uterine epithelium increases (Ford 1982), contributing to a higher uterine tone score, hypothesised to indicate imminent ovulation (Bonafos *et al.* 1995). Whilst it is tempting to correlate uterine tone levels directly with hormone concentration and eventual ovulation, this was not measured within studies in this thesis. Indeed the time of AI was also not retained as significant within the model. In retrospect, recording hormone concentrations before, during and after AI, in addition to examining the ovaries of ewes via ultrasound to examine the timing of ovulation, could solidify our understanding of the relationship between uterine tone, oestrogen levels and eventual time of ovulation to interpret findings practically in relation to semen deposition. The links between intra-abdominal fat and fertility offer a novel perspective, as intra-abdominal fat provides a more accurate representation of a ewe's internal energy

reserves than body condition scoring (BCS) alone. While BCS is non-invasive and easily assessed on-farm, intra-abdominal fat measurements are typically conducted during AI by a veterinarian or AI technician. Unlike BCS, which evaluates external fat and muscle cover and may not fully reflect internal nutritional status, intra-abdominal fat directly assesses fat reserves within the abdominal cavity, which are closely linked to reproductive function. Although studies such as (Bravo *et al.* 1997) have demonstrated a linear relationship between fat deposits, body weight, and BCS, this correlation does not imply that BCS can always serve as a reliable proxy for intra-abdominal fat conditions. Variability in this relationship could arise in cases where BCS is influenced by external factors such as wool or muscling, which fail to capture the internal state of the ewe. Chapter 3 identified intra-abdominal fat as a key reproductive trait and a more accurate predictor of fertility. While BCS was not included in this study, its omission highlights the need to evaluate how these two metrics compare in predicting reproductive outcomes. For instance, if highly correlated, how can BCS be practically incorporated into a breeding evaluation to help cement the link between intra-abdominal fat and fertility? Conversely, had the BCS score been included in this model, would intra-abdominal fat have remained a significant predictor? Further research is required to clarify the relationship between BCS and intra-abdominal fat, particularly in scenarios where they may diverge. This understanding will help refine fertility prediction models and improve breeding outcomes. While optimal uterine tone and intra-abdominal fat in the ewe is critical for successful AI, it is equally important that the ewe is inseminated with high-quality semen.

Before AI is performed, semen from a sire needs to be collected, prepared for immediate insemination, or frozen and thawed according to industry standards. The model created in Chapter 3 identified that the concentration at which ram sperm is frozen influences pregnancy rates, likely owing to its direct relationship to insemination dose rather than any benefit associated with the ratio of cryomedia to sperm (Visser and Salamon 1974b; Alvarez

et al. 2012). Chapter 4 identified an optimal freezing concentration of 680×10^6 sperm/mL, which ensures that post-thaw (regardless of whether samples are frozen as a pellet or straw) samples contain enough motile spermatozoa to inseminate a ewe with approximately 25 million motile spermatozoa (Evans and Maxwell 1987). Interestingly, Chapter 5 demonstrated that ram spermatozoa frozen at concentrations between 400 to 600×10^6 sperm/mL exhibited the best overall post-thaw performance across various *in vitro* semen traits. While 680×10^6 sperm/mL technically falls outside the optimal range recorded in Chapter 5, the slight discrepancy highlights a potential trade-off between maximising insemination dose and maintaining post-thaw sperm quality. Should the concentration of sperm drop below the optimal range, this could result in insufficient motile spermatozoa per insemination dose (Maxwell 1986a; Findlater *et al.* 1991; Eppleston *et al.* 1994), while concentrations above the range could negatively impact sperm quality during freezing and thawing as previously mentioned in the literature (Salamon and Visser 1972; Peña and Linde-Forsberg 2000; D'Alessandro *et al.* 2001; Allamaneni *et al.* 2005; Alvarez *et al.* 2012; Pereira *et al.* 2017; Siristatidis *et al.* 2021). In any case, combined, these findings underscore the importance of determining precise concentrations when preparing semen for freezing or insemination, ensuring appropriate quality assurance is maintained and accurate doses are prepared for successful insemination.

On the day of AI, it is essential to thaw pellets correctly to reduce any processing damage on sperm quality traits post-thaw. Chapter 5 of this thesis explored the relationship between wet and dry thawing when using pellets as an example. The results showed that motility, viability, and acrosome integrity were consistently higher when thawed with media, "wet thaw", likely due to the media facilitating rapid diffusion of heat throughout the pellet and return to 37ºC (Salamon and Maxwell 1995a), protecting spermatozoa from cryodamage. Additionally, when comparing diluents, the inclusion of fructose in both 1+3 tris-citrate-fructose and tris-citrate-fructose + egg yolk diluents aligned with previous research by

(Salamon 1968; Salamon and Lightfoot 1969b), which suggested that sugar-based thawing media could improve the revival of pellet-frozen ram spermatozoa and enhance their viability during post-thaw incubation. These findings are consistent with the notion that sugars like fructose are an immediate energy source for spermatozoa, aiding their recovery after thawing (Tsujii *et al.* 2006). However, while the presence of sugar appears beneficial in the short term, the overall impact on sperm quality post-thaw is less well-defined (Visser and Salamon 1973). Some studies have indicated that the composition of sugar-based thawing media can have complex effects on sperm viability, with some media performing better than others depending on the incubation duration. The FTC+EY media showed high sperm motility immediately post-thaw, suggesting rapid energy uptake from fructose and egg yolk. However, motility declined significantly after 3 and 6 h, indicating limitations as a long-term preservative. In contrast, FTC media maintained good motility and viability up to 6 h post-thaw, demonstrating its effectiveness as a more stable long-term preservative medium for industry, especially as we consider its potential correlation to the survival of sperm inside the female reproductive tract post-insemination. Results also showed that motility, viability, and acrosome integrity decreased when more than one pellet was thawed at one time during 'wet thaw'. This is thought to be linked to the uneven distribution of heat during warming, causing cellular stress (Zenteno *et al.* 2023). In contrast, the number of pellets thawed did not affect dry-thawing, likely due to the uniform heat exposure, minimising the risk of the uneven distribution of heat during thawing. This is evidently important because it highlights the need for quality control protocols to ensure optimal sperm recovery during thawing prior to insemination.

The *in vitro* semen assessment is common in the industry, yet it is not standardised across artificial breeding centres and technicians. Its use as a predictor of sperm fertility has long been sought owing to its practical applications. The model created in Chapter 3 highlighted three key post-thaw *in vitro* semen traits found to determine the likelihood of

pregnancy occurring following LAI: abnormal morphology, motility (recorded at 0 h post-thaw) and sperm viability and acrosome integrity (recorded at 6 h post-thaw). When validated in Chapter 4 under field conditions, each trait was used collectively within the model to predict whether a ewe would fall pregnant and differentiate the threshold of optimal pregnancy results. As highlighted in the literature (Chapter 1), there is a wealth of knowledge linking individual *in vitro* semen traits, including motility (Del Olmo *et al.* 2013), concentration (Visser and Salamon 1974b; Alvarez *et al.* 2012; Macías *et al.* 2020), viability (Santolaria *et al.* 2015), acrosome integrity (O' Meara *et al.* 2008), and morphology (Almadaly *et al.* 2016; Almadaly *et al.* 2019), to ram fertility. However, with these above results, consensus on a direct link or value predicting pregnancy had not been reached for LAI in sheep in Australia.

Within the experiments reported in this thesis, morphology was assessed immediately post-thaw, fixed in a saline solution, and calculated as the percentage of abnormal cells in a given sample. Initial results from Chapter 3 showed that an increase in abnormal sperm was associated with a reduced chance of pregnancy. Chapter 4 identified a threshold of 15.5 % abnormal sperm, where samples with fewer abnormalities (below the threshold) were linked to higher pregnancy rates. Notably, using a larger dataset, this study replicated previous reports which have recommended a threshold of 15 % and supported previous findings of existing literature in rams (Almadaly *et al.* 2016; Almadaly *et al.* 2019). A predictive value of 0.54 was calculated based on the performance of using the morphological assessment alone to classify pregnant and non-pregnant ewes. This value indicates a moderate ability to predict pregnancy outcomes. The lower-than-expected predictive value could be related to the binary classification system used in the study, where sperm morphology was classified as either abnormal or normal. Previous studies have attempted to correlate specific head, body, and tail morphological abnormalities with fertility outputs (Kenney *et al.* 1983; Love 2011), as summarised in Chapter 1. While such classifications provide useful insights, the lack of

standardisation in assessing sperm morphology, at least in ram sperm, caused high variability amongst assessors and labs. Standardising these assessments could improve accuracy and model predictions of pregnancy. Further research is required to refine morphology classification systems in rams and create objective, standardised methods better to understand the impact of sperm morphology on fertility.

At 0 h post-thaw, sperm motility and kinematics measured by CASA influenced the likelihood of pregnancy using a principal component analysis (PCA). Due to the high collinearity and correlation between CASA variables, a PCA combined each variable into a standardised value, allowing for more variables to be included within the fertility model (Chapter 3). As summarised in the literature, higher sperm motility is consistently linked to greater fertility across many livestock species (Farrell *et al.* 1998; Januskauskas *et al.* 2000; Malo *et al.* 2005; Gillan *et al.* 2008; Love 2011; Del Olmo *et al.* 2013). Whilst this trend was confirmed in the current fertility model, using a PCA presents challenges when attempting to interpret and standardise sperm motility traits for the industry. Applying direct standards to the arbitrary values obtained from the CASA PCA would be impractical, as the model generates a wide range of motility and velocity metrics that are difficult to categorise into predefined standards. This challenge stems from the complex relationship between the CASA parameters and the PCA, where multiple factors, such as loading weights, distribution and variability across sperm populations, influence these metrics. This interdependence makes it hard to establish a threshold for each parameter within the PCA model. However, based on the insights provided by the model and supported by previous literature (Del Olmo *et al.* 2013), it can be anticipated that an increase in motility and velocity traits will be correlated to higher pregnancy rates. Further investigation is required to try to extrapolate practical standards for motility and velocity from complex PCA values using sperm motility values linked to fertility following AI.

The final *in vitro* sperm parameter within the model was the percentage of viable acrosome intact sperm at 6 h post-thaw. The predictive ability of sperm viability and acrosome integrity to distinguish pregnant and non-pregnant ewes was the highest across all sperm traits at 0.84. Additionally, in Chapter 4, sperm recording a viability and acrosome integrity score greater than or equal to 6.32 % at 6 h post-thaw was identified to maximise pregnancy rates. Assessing spermatozoa over 6 h at 37 ºC 6 h post-thaw is crucial to act as a proxy for the conditions within the female following insemination. The 6 h time point helps provide more information on the status of sperm survival when it is likely to encounter the descending oocyte (Evans and Maxwell 1987; Mahé *et al.* 2021). During this time, spermatozoa undergo capacitation and hyperactivation, critical processes for preparing viable sperm for fertilisation (Januskauskas *et al.* 2000). Acrosome integrity is also essential, as it supports the biochemical changes required for successful oocyte penetration (Abou-Haila and Tulsiani 2000), if these physiological responses occur prematurely, fertilisation is impossible, reducing pregnancy success post-insemination. The 6 h post-thaw assessment period offers a critical window into understanding the functional readiness of sperm for successful fertilisation and helps predict the success of LAI. Assessment of sperm viability and acrosome integrity is not considered routine semen assessment within the industry, owing to the necessity of using advanced equipment such as fluorescent microscopy or flow cytometry for accurate examination. Consultation is now needed with the industry to determine whether this parameter could be feasibly incorporated within the semen assessment toolbox, allowing the industry to benefit from its predictive power.

This thesis has successfully created a comprehensive, validated fertility model that offers the industry a standardised approach to semen cryopreservation, *in vitro* assessment and ewe preparation to improve fertility predictions and AI success rates (Chapters 2, 3 and 4). Using this model would enable more precise identification of suitable samples from rams and

fertile ewes, ensuring more reliable pregnancy rates from LAI. Using the above-recommended standards, the model can account for a cumulative pregnancy score of 64.3 % when all the above is considered. Whilst this is the first time pregnancy following LAI has been predicted accurately, the study recognises that an uncontrollable amount of variation persists within the industry due to changing environments. By placing location, site and breeding seasons within the model as random effects, the study was able to account for their variability and isolate their impact on AI outcomes. This method helped reduce the influence of fluctuating environmental conditions on the results, ensuring the thresholds are predictors across a range of diverse field conditions, which ultimately could still be influenced by environmental conditions.

By incorporating multiple sites across various locations around Australia, the thesis aimed initially to capture and understand the environmental causes of variation in AI success. However, the unbalanced number of breeding seasons and AI sites under varied environmental and climatic conditions meant a meaningful comparison was not possible. To truly quantify the impact of environmental factors, further research is required to design an experiment where the fertility model was examined under controlled conditions using environmentally regulated rooms, where temperature, humidity, and other factors could be carefully managed. This approach would allow researchers to isolate the direct effects of environmental or climatic factors on fertility, providing the industry with actionable insights to mitigate the influence of the environment on AI outcomes. Despite further investigation, it remains clear that predicting the success of an artificial breeding program will always be challenging due to the myriad of external factors involved. At least the work presented in the above thesis has addressed the male and female aspects that contribute to the success of an LAI program within Australia. The model can now be used to study the final component, the environment, and the causes of variability in LAI programs.

In conclusion, this thesis has developed and validated a fertility model that allows for the pre-screening of rams and ewes to predict the likelihood of pregnancy prior to an LAI breeding program. The application of this fertility model and suggested standards throughout the industry, should increase the success and reduce the variation seen in LAI programs. These results will significantly benefit the industry by restoring confidence in the use of reproductive technologies and increasing the adoption and utilisation of superior genetics. Overall, this will drive an acceleration of genetic and production gains for the industry, securing the longevity and sustainability of the Australian sheep industry.

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Appendix 1: Supplementary Files

Chapter 2: Uterine tone influences fertility of Merino ewes following laparoscopic artificial insemination.

Supplementary File 2.1: Influence of individual factors on pregnancy rate following AI. The percentage of ewes pregnant following AI per grouping within each ewe fertility variable. Note semen types include; Frozen (FZ) and Fresh (FR).

Variable	Factors	Ewes Inseminated	Ewes Pregnant by AI (%)
Age of ewe at AI (years)	<2	6 562	4 136 (63.03)
	3	7 000	4 658 (66.54)
	4	3 921	2 659 (67.81)
	5+	3 614	2 423 (67.04)
Uterine tone score	1+2	2 792	1 616 (57.88)
	3	12 417	8 097 (65.21)
	4+5	7 162	5 034 (70.29)
Visual Intra-abdominal fat score	1+2	3 922	2 328 (59.36)
	3	13 427	8 795 (65.50)
	4+5	5 034	3 628 (72.07)
Time of AI post-CIDR removal (h)	<48	460	259 (56.30)
	48-50	3 002	2 016 (67.16)
	50-52	9 108	6 009 (65.97)
	52-54	8 319	5 448 (65.49)
	54-56	2 160	1 396 (64.63)
	>56	240	163 (67.92)
Sperm Type	FR	3 475	2 231 (64.20)

Variable	Factors	Ewes Inseminated	Ewes Pregnant by AI (%)
	FZ	21 225	13 947 (65.71)

Supplementary File 2.2: listed are the results of the univariate analysis. Factora that scores a *P-value* less than 0.2 were included in the multivariable analysis, indicated by the asterisk. Note semen types include; Frozen (FZ) and Fresh (FR).

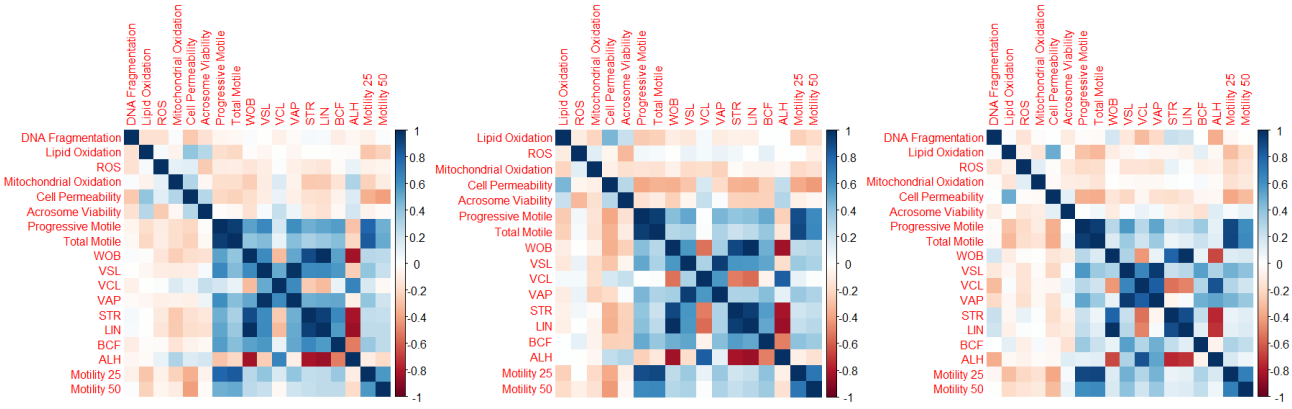
Factor	Individual *P-value*
Age grouped	0.09803*
Tone grouped	<0.0001*
Fat grouped	0.03917*
AI time post-CIDR pull grouped	0.3483
FR or FZ	0.4119

Chapter 3: A multivariate model for the prediction of pregnancy following laparoscopic artificial insemination of sheep.

Supplementary File 3.1: Describes the percentage of ewes pregnancy for each level within the categorical factors recorded in the data set.

Variable	Category	Number of ewes	Number of Pregnant ewes	Percentage of Ewes Pregnant (%)
Intra-abdominal Fat (Grouped)	1+2	3922	2328	59.36
	3	13426	8794	65.50
	4+5	4789	3445	71.94
Ewe Age (Grouped)	<2	6562	4136	63.03
	3	7000	4658	66.54
	4	3920	2658	67.81
	5+	3614	2423	67.04
Uterine Tone (Grouped)	1+2	2792	1616	57.88
	3	12407	8089	65.20
	4+5	7162	5034	70.29
Semen Type	Frozen	27584	18379	66.63
	Fresh	2670	1744	65.32
Package Type	Straw	8260	5395	65.31
	Pellet	16129	10854	67.29
AI Time Post-CIDR Removal	<48	460	259	56.30
	48-50	3002	2016	67.16
	50-52	9108	6009	65.97
	52-54	8319	5448	65.49
	54-56	2160	1396	64.63
	>56	240	163	67.92

a) b) c)



Supplementary File 3.2: Correlation plots for *in vitro* semen traits at (a) 0 h post-thaw, (b) 3 h post-thaw, and (c) 6 h post-thaw

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Chapter 4: The validation and application of an ovine fertility model using standardisation *in vitro* thresholds to predict the likelihood of pregnancy

Supplementary File 4.1: Intercept values given within the fertility model

Intercept term	**Value**
$\hat{\beta}0$	0.206
$\hat{\beta}1$	1.092
$\hat{\beta}2$	-0.0107
$\hat{\beta}3$	0.000496
$\hat{\beta}4$	-0.00368
$\hat{\beta}5$ (3)	0.293
$\hat{\beta}5$ (4 + 5)	0.453

Appendix 2: Conference Proceedings

I. Wilson, H.R., **Spanner, E.A**., McMahon, J., Rickard, J.P., de Graaf, S.P. (2023). Fertility of ram semen frozen in pellets or straws. 11th International Ruminant Reproduction Symposium (IRRS), 28th May – 1st June 2023. Galway, Ireland.

II. **Spanner, E.A**., Rickard, J.P., de Graaf, S.P. (2023). DNA fragmentation is correlated with abnormal morphology of frozen-thawed ram spermatozoa. Proceedings of the Annual Conference of the Society of Reproductive Biology (SRB) 26th – 29th November 2023. Brisbane, Australia.

III. **Spanner, E.A**., Rickard, J.P., de Graaf, S.P. (2024). Predicting the success of laparoscopic artificial insemination in sheep. Proceedings of the 57th Annual Conference of the Society for the Study of Reproduction (SSR) 15th – 19th July 2024. Dublin, Ireland.

IV. **Spanner, E.A**., Rickard, J.P., de Graaf, S.P. (2023). The probability of pregnancy occurring following laparoscopic artificial insemination of sheep. Proceedings of the Annual Conference of the Society of Reproductive Biology (SRB) 10th – 13th November 2023. Adelaide, Australia.

I. FERTILITY OF RAM SEMEN FROZEN IN PELLETS OR STRAWS

H. R. Wilson[1], E. A. Spanner[2], J. McMahon[1], J. P. Rickard[2], S. P. de Graaf[2]
[1]*Westbreed Pty Ltd, Northam WA 6401, Australia*
[2]*The University of Sydney, Faculty of Science, NSW 2006, Australia*

Increase the quality and fertility of frozen ram semen used for artificial insemination.

A potential source of variation in the success of sheep AI programs is the quality of frozen semen used. Many factors contribute to the post-thaw quality of frozen ram semen, including whether semen is frozen as pellets on dry ice or in PVC straws over liquid nitrogen vapour. Contemporary methods of freezing ram semen in straws and pellets have not been directly compared. As such, this study was undertaken to assess the difference in post-thaw motility, morphology and fertility of ram semen frozen in 0.25mL straws or as 0.2mL pellets.

Six to eight ejaculates were collected from four merino rams (N=30). Each ejaculate was diluted with tris-citrate-egg yolk-glycerol extender and split-processed for straw and pellet freezing using industry standard methods (Evans and Maxwell, 1987). Pellets and straws were thawed, incubated (37°C, 6h) and assessed for motility (0, 2, 4, 6h) and morphology (0 h) using light microscopy. A subset of pellet and straw frozen semen from each ram was used for laparoscopic intrauterine insemination of oestrus synchronised ewes (N=536). Pregnancy of inseminated ewes was determined by ultrasound ~50 days post-AI. Statistical analysis (R studio) of the effect of semen freezing method on *in vitro* semen quality and in vivo fertility was undertaken using independent t-tests and binomial logistical regression, respectively.

Post-thaw motility (±SEM) of ram semen at 0h (69.2 ± 0.53% vs 66.8 ± 0.50%, P=0.61), 2h (65.8 ± 0.36% vs 66.8 ± 0.34%, P=0.70), 4h (64.3 ± 0.39% vs 63.8 ± 0.27%, P=0.85) and 6h (64.4% ± 0.49% vs 63.2 ± 0.28%, P=0.74) incubation was not significantly different between samples from straws or pellets, respectively. Sperm morphological abnormalities (± sem) did not differ (P=0.96) between semen frozen in straws or pellets (13.7 ± 0.23% and 13.8 ± 0.30%, respectively). Pregnancy and reproduction rates of ewes inseminated with semen frozen in straws (68.8 ± 0.03%; 103.6 ± 0.03%) did not differ (P=0.32) to that of ewes inseminated with semen frozen in pellets (66.5 ± 0.03%; 101.2% ± 0.03%), respectively.

These findings demonstrate that the post-thaw motility, morphology and fertility of ram semen is similar whether frozen as 0.2mL pellets or in 0.25mL straws using current industry practices. Variation in the success of sheep AI programs is likely caused by other factors, the identification of which requires further research.

II. DNA FRAGMENTATION IS CORRELATED WITH ABNORMAL MORPHOLOGY OF FROZEN-THAWED RAM SPERMATOZOA.

E. A. Spanner[1], M. J. Robertson[1], S.P. de Graaf[1], J. P. Rickard[1]
[1]*The University of Sydney, Faculty of Science, NSW, 2006, Australia*

The morphological characteristics and DNA integrity of spermatozoa have been linked to fertility in a range of species (Morrell *et al.* 2008; Sellem *et al.* 2015). Spermatozoa which record high morphological abnormalities are thought to be less fertile due to their inability to navigate the tract, fertilise the oocyte and often result in pregnancy loss (Mickelsen *et al.* 1981). Compared to humans and other livestock species, where these two parameters have been shown to be correlated, little focus has been given to the potential interplay between the DNA integrity and morphological abnormalities of ram spermatozoa post-thaw (Evenson and Jost 2000). As such, this study directly compares the DNA integrity and morphology of ram spermatozoa following cryopreservation to understand the relationship between semen quality traits.

Semen was collected from Merino rams (N=250) and frozen using industry-standard techniques. Each sample was assessed for the percentage of abnormal sperm using phase-contrast microscopy (x400 magnification, 200 cells) and DNA fragmentation using flow cytometry (Acridine Orange; Thermo Fisher Scientific, NSW) immediately after thawing (3) and following 6h of incubation at 37ºC to establish a DNA fragmentation index (DFI%).

Statistical modelling (R Studio) revealed DFI% at 0 and 6h post-thaw returned a positive correlation with morphological abnormalities (R^2 = 0.53, 0.33, respectively). There was also a significant increase in DFI± SEM% from 0h (3.97±0.24%) to 6h (6.91±0.51%, P<0.001) post-thaw.

These findings demonstrate a positive correlation between DFI% and morphological abnormalities of frozen-thawed ram spermatozoa and an effect of incubation time on DFI% in ram spermatozoa. The results imply that the processes that cause abnormal sperm morphology negatively affect the DNA integrity of sperm. Further research is required to explore the collective impact of such traits on the fertility of semen following artificial insemination and the acceptable levels for processed semen.

III. PREDICTING THE SUCCESS OF LAPAROSCOPIC ARTIFICIAL INSEMINATION IN SHEEP

E. A. Spanner[1], S. P. de Graaf[1], J. P. Rickard[1]
[1]*The University of Sydney, Faculty of Science, NSW, 2006, Australia*

Artificial insemination plays a critical role in facilitating rapid genetic and production gains for the sheep industry, yet variable success rates and diminished confidence have reduced the use of AI and high-merit semen. While several factors are thought to influence sheep fertility, their relevance during laparoscopic AI and the interplay between factors is unknown. As such, this study examined how various female factors collected at the time of AI and male semen factors assessed *in vitro* influence the success of intrauterine laparoscopic AI in sheep.

Over 3 breeding seasons, data was collected during 30 different AI programs in Australia using Merino rams (N=387) and ewes (N= 30,254). Ewes were synchronised and laparoscopically inseminated as per industry standards. During AI, the ID tag, age, uterine tone score (1; pale/flaccid-5; turgid/pink), intra-abdominal fat score (1; little to no fat present-5; high fat), time of insemination post-CIDR removal and sire used, was recorded for each ewe. A subset of the semen used for insemination underwent advanced *in vitro* semen assessment. Thawed samples were diluted (1:0.5) in PBS + 0.3% BSA and immediately assessed for volume (mL), subjective motility (phase contrast microscopy), sperm concentration ($\times 10^6$ sperm/mL; NucleoCounter SP-100), and morphology (% abnormal; x400 objective). Samples were then incubated and aliquots were taken at 0, 3 and 6h for motility and kinematics assessment (Computer-assisted semen analysis; HT CASA IVOS II) as well as sperm viability and acrosome integrity (FITC-PNA/PI), membrane fluidity (M540/Yo-Pro), mitochondrial superoxide production (Mitosox Red/Sytox Green), lipid peroxidation (Bodipy C11) levels of intracellular Reactive Oxygen Species (H_2DCFDA) and DNA fragmentation (Acridine Orange) via flow cytometry (CytoFLEX and Aroura 3L).

Transcutaneous ultrasound was performed approx. 55 days post-AI. Statistical analyses (RStudio) included correlations, CASA Principal Component Analysis (PCA) and a binomial multivariable regression analysis.

The concentration at which sperm were frozen (P<0.001), CASA PCA at 0h (PCA; P=0.03), the percentage of viable, acrosome intact sperm at 6h (P=0.02), percentage of abnormal sperm (P<0.001), as well as uterine tone (P<0.001) and intra-abdominal fat (P=0.03) of ewes significantly influenced the likelihood of pregnancy following AI. A 1% increase in the percentage of acrosome intact, viable sperm, a 1% increase in morphology abnormalities and an additional 100 $\times 10^6$ sperm/mL frozen corresponded to a 1.01% increase (OR = 1.10, 95% CL: 0.34, 2.56), 1.07% decrease (OR = 0.99, 95% CL: 0.98, 1.00) and 5.09% increase (OR = 1.05, 95CL: 1.05, 1.05) in the probability of a ewe falling pregnant, respectively. Furthermore, each standard deviation away from the CASA PCA average led to a 0.37% decrease in the probability of pregnancy (OR = 1.00, 95% CL: 1.00, 0.99). The odds of pregnancy increased by 7.21% (OR = 1.07, 95% CL: 0.44, 2.60) and 6.05% (OR = 1.07, 95% CL: 0.78, 1.47) when semen was inseminated into an ewe with a uterine tone and intra-abdominal fat score of 4+5 compared to ewes which scored 1+2 (P<0.001 and P=0.047, respectively).

The results demonstrate the benefit of analysing multiple male *and* female factors recorded during AI on individual ewes. The identified factors could now be used to standardise and

further enhance AI protocols. Screening semen before AI can help minimise variability in success rates, improve the adoption of reproductive technologies, and ultimately advance the rate of genetic gain for the sheep industry.

IV. THE PROBABILITY OF PREGNANCY OCCURRING FOLLOWING LAPROSCOPIC ARTIFICIAL INSEMINATION OF SHEEP.

E. A. Spanner[1], S.P. de Graaf[1], J. P. Rickard[1]
[1]*The University of Sydney, Faculty of Science, NSW, 2006, Australia*

Variation in pregnancy rates following laparoscopic artificial insemination (LAI) in sheep has resulted in reduced adoption of this reproductive technology, limiting genetic gain for the industry. This study aimed to determine the contribution of various male and female factors on the probability of pregnancy following LAI.

Data from Merino ewes (N=30,254) including age, uterine tone (1; pale/flaccid-5; turgid/pink), intra-abdominal fat (1; no/little fat present-5; high fat), time of insemination and semen sire (N=388), were recorded during AI. Semen used for AI was assessed for volume, concentration, subjective motility and morphology immediately post-thaw, while motility (CASA), viability, acrosome integrity (FITC-PNA/PI), membrane fluidity (M540/Yo-Pro), mitochondrial superoxide production (Mitosox Red/Sytox Green), lipid peroxidation (Bodipy C11), level of intracellular reactive oxygen species (H2DCFDA) and DNA fragmentation (Acridine Orange) were assessed at 0, 3 and 6h post-thaw. A binomial logistic regression analysis and odds ratios evaluated the impact and interplay of factors on pregnancy. New validation data was collected (as described above) and run through the model to predict pregnancy probability. Predicted outcomes were evaluated against ultrasound results using discrimination and calibration statistics.

The concentration at which sperm was frozen ($P<0.001$), a CASA PCA (0h; $P=0.03$), percent of viable, acrosome-intact sperm (6h; $P=0.02$), percent of abnormal sperm ($P<0.001$), uterine tone ($P<0.001$), and intra-abdominal fat ($P=0.03$) of ewes influenced the likelihood of pregnancy post-LAI. The model demonstrated high accuracy (74%), excellent precision (96%), lower specificity (33%), strong recall (76%), and AUC (0.62). There was no difference in the number of pregnancies predicted versus ultrasound detected ($P=0.184$). Thresholds for each predictor (freezing concentration; 680.85×10^6 sperm/mL, CASA PCA; 18.34, %viable; 6.31 and %abnormal; 15.5) were calculated, returning a cumulative 64.3% chance of pregnancy.

These findings enable a practical means of screening semen and ewes before AI to maximise the success of artificial insemination for the sheep industry.