Final report

A novel semen extender to accelerate genetic improvement programs

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Prepared by: Erin Klein, Dr Mohammad Bahrami, Dr Zamira Gibb
The University of Newcastle

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Abstract

The use of artificial insemination (AI) for the genetic improvement of northern Australian beef cattle herds has been restricted by logistical constraints of conducting AI programs on geographically isolated farms, the reduced fertility of cryopreserved sperm and the limitations of oestrous synchronisation regimens. The aim of this project is to improve access to AI and the conception rates achieved by developing a semen extender which can maintain fertility over several weeks without the need for cryopreservation. We have conducted studies to optimise the sperm processing techniques and the ingredients of a bull sperm preservation medium which has resulted in a sperm storage medium which is able to preserve sperm motility and viability over 14 days at room temperature. In addition, we have shown that sperm stored in this medium for 7 days have an equal or greater field fertility than cryopreserved sperm. High-quality sperm were isolated from semen samples using density gradient centrifugation (DGC) and stored at a concentration of approximately 50 million sperm/mL. Sperm were stored at room temperature (in an air-conditioned room at approximately 22 °C) without the use of a specific device to control temperature. Ensuring longer survival of sperm stored at room temperature will extend their viability within the female reproductive tract in comparison to cryopreserved sperm, allowing more flexibility in fixed-time AI insemination regimens. This development will markedly reduce the logistical constraints associated with the collection, transport and adoption of AI in northern beef herds.

Executive summary

Background

Poor reproductive performance (McCosker et al., 2010) and variability in reproductive traits (Johnston et al., 2009) that impact productivity have been reported by northern Australian beef cattle producers. These producers have been slow to adopt assisted reproductive technologies (ART), with it estimated that less than 1% are utilising artificial insemination (AI) in their breeding and management programs. This is largely due to the logistical challenges associated with fixed-time AI (FTAI), namely precise timing of insemination in relation to ovulation. Traditionally, AI is done using sperm that have been cryopreserved, however this process causes significant damage to the sperm, reducing the number of viable cells following thawing and limiting their longevity within the female reproductive tract. This poses less of a challenge in dairy herds where AI can be performed on observed heat. By improving the logistics and flexibility around AI regimens and the viability of sperm intended for insemination, AI for selective breeding programs can become a feasible option for producers managing their herds extensively. To do so, we have developed a sperm storage medium capable of maintaining viability without chilling or freezing for up to 14 days following collection.

Objectives

Objective 1: Extender development

To develop and validate a novel, innovative sperm preservation medium that will maintain the viability of fresh-chilled semen for between 2 to 3 weeks after collection.

Objective 2: Extender validation

To confirm through laboratory testing and field trials the fertility of fresh-chilled sperm preserved in the novel medium in terms of iatrogenic damage to the sperm, pregnancy rates and weaning rates.

Methodology

- Semen collection – electroejaculation
- Sperm Processing – density gradient centrifugation (DGC)
- Sperm motility assessment – Computer-assisted sperm analysis (CASA)
- Flow cytometry
- In vitro maturation (IVM) and fertilisation (IVF)
- Oestrus synchronisation
- Fixed-time artificial insemination (FTAI)

Results/key findings

The results of this project show the development of a medium capable of maintaining the fertility of bull sperm during ambient temperature storage. The developed medium 'NEW Bull' is compared to the current commercial sperm storage methods (cryopreservation and chilling) with significant improvements in motility parameters. Sperm stored in 'NEW Bull' for 7 days were used in a small field trial demonstrating field fertility.

Benefits to industry

'NEW Bull' will have many benefits to the industry once commercialised. For bull sperm intended for use within two weeks, 'NEW Bull' overcomes the need for cryopreservation. We hypothesise that by circumventing the freeze-thaw process of cryopreservation, sperm survival in the female reproductive tract after insemination will be enhanced, allowing for more flexibility in AI timings and higher pregnancy rates as the need for precise timing of insemination with ovulation will be reduced. Aside from its use in AI, 'NEW Bull' can also be used for storage of sperm for IVF and though we have not validated its use with sex-sorted sperm, we have every reason to believe that sperm can be supported before and after the sex-sorting process in this medium as well.

Future research and recommendations

Optimisation of medium manufacturing and development of protocols for best use will need to be done for commercialisation including shelf-storage of the medium, best storage vessels for sperm after extension, and minimum sperm dose used for inseminations. Future research could also be done on determining if this medium is appropriate for use with other ruminants, or if similar media could easily be developed.

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1. Background

1.1 Artificial Insemination

1.1.1 Slow uptake of AI by northern Australian beef producers

Beef cattle production is the single largest contributor to the total gross value of Australia's agricultural production (Australian Bureau of Agricultural and Resource Economics and Sciences, 2019) and is the most common agricultural activity, with beef cattle found on more than half of Australian farms (Australian Bureau of Statistics, 2016). The sustainability of this industry is important to the livelihood of Australian farmers and to the Australian economy. Poor reproductive performance (McCosker et al., 2010) and variability in reproductive traits (Johnston et al., 2009) that impact productivity have been reported by northern Australian beef cattle producers. These producers typically raise breeds of cattle belonging to the Bos indicus subspecies for their adaptive advantages to the harsher tropical environs of northern Australia, however, with these traits come characteristics that can be detrimental to reproduction (Burns et al., 2010, Sartori and Barros, 2011), with Bos indicus females typically maturing later than 100% Bos taurus females and extended lactational anoestrus observed in first lactation Brahman cows (Johnston et al., 2013). Significant focus has been directed at improving the genetics of reproductive performance in Bos indicus females over the past two decades. Artificial insemination (AI), using top ranking sires with desired reproductive traits offers the most practical and cost effective means to improve the fertility and reproductive efficiency in these northern herds, as traits such as age at puberty is highly heritable and lactational anoestrus in first and second calf Brahman cows is moderately heritable (Engle et al., 2019).

1.1.2 Traditional sperm storage for AI

When processing semen for AI, sperm must be stored for a period of time outside of the male reproductive tract, particularly if the male is a great distance from the processing unit. Typically, sperm are stored via cryopreservation or chilling to restrict sperm metabolism, reducing the production of toxic metabolites and the depletion of energy in the form of adenosine triphosphate (ATP).

Cryopreservation of sperm typically involves adding a cryodiluent to the full ejaculate, in a one or two-step dilution, and freezing the sample in straws over liquid nitrogen. While it is commonly used, it is also widely recognised that this process inflicts irreversible damage to sperm. This occurs when sperm encounter osmotic stress (Pegg and Diaper, 1989) and mechanical shock (Mazur, 1984, Hammerstedt et al., 1990), resulting in reduced motility, membrane integrity, and mitochondrial membrane potential (Gürler et al., 2016), leading to premature capacitation-like changes (Watson, 1995). An increase in harmful reactive oxygen species has been observed in frozen sperm samples immediately following post-thaw (Ball et al., 2001, Baumber et al., 2003, Kim et al., 2010, Gürler et al., 2016) and these may have a negative effect on DNA integrity (Baumber et al., 2003, Kim et al., 2010).

The damage caused by storing in liquid nitrogen is not due to the actual storage at -196°C, but instead the intermediate zone between -15 and -60°C that the sperm cell must pass through during freezing and again during thawing (Mazur, 1984). The freezing rate during this phase change is crucial, too fast of a freezing curve and the spermatozoon will not be sufficiently dehydrated and intracellular ice crystals will form, damaging to the cell. However, a slow freezing curve exposes the

sperm to the hypertonic conditions for too long and they become excessively dehydrated, resulting in mechanical damage via shrinking (Watson, 2000, Sieme et al., 2015).

The freeze/thaw process also disrupts regulation of calcium homeostasis, causing an intracellular calcium overload. With the mitochondria taking up large amounts of calcium, the sperm undergo the mitochondrial permeability transition, resulting in a decrease in mitochondrial membrane potential and a cessation of mitochondrial membrane potential dependent functions, including ATP synthesis (Treulen et al., 2018).

To complicate the variation in fertility from male to male, there is also a variation in ability to withstand the freeze-thaw process which appears unrelated to fresh sperm quality. Bulls with low post-thaw motility (<50% motile) are deemed “bad freezers”, and whilst possessing desirable genetics, are ill-suited for an AI program. Relying on natural mating from such animals not only reduces the rate of genetic gain within the herd but prevents transferring its genetics across national herds. Proteomic comparisons of bulls with high and low freezability indicate a correlation exists between certain plasma membrane proteins and the ability to withstand cryopreservation (Westfalewicz et al., 2015). Developing a storage medium that allows longer survival of sperm without cryopreservation will allow more bulls to enter AI programs when insemination can occur within one to two weeks of collection.

1.1.3 Can sperm be stored in a better way for AI?

Cryopreservation will remain an important aspect of assisted reproductive technology (ART), as this method enables indefinite storage of sperm or ART (Mazur, 1984). However, for much of its use in production animal industries, indefinite storage may not be required. For more immediate use, storage at ambient temperature is the preferred method to avoid the damage to sperm caused by reduced temperatures (Watson, 1995, Sieme et al., 2015). Previous attempts at ambient temperature storage have involved CO_2 narcosis (VanDemark and Sharma, 1957), lowered pH (Norman et al., 1958), and N_2 gassing (Shannon, 1965) as methods for metabolic inhibition. While a small decline in motility is observed using these methods, the fertility of the sperm decreased dramatically within the first few days of storage (VanDemark and Bartlett, 1962), possibly as a result of oxidative stress. Recent research from our group has led to the development of a stallion sperm storage medium (Gibb et al., 2015, Swegen et al., 2016) that preserves sperm quality and fertility for a minimum of 7 days (Gibb et al., 2018), and is currently available in Australia as SpermSafe-E (BREED Diagnostics, Newcastle, Australia). Sperm metabolism, uninhibited at ambient temperature, is supported in this novel medium. Following the same approach that led to the development of SpermSafe-E, we hypothesised that a medium appropriate for the storage of bull sperm could be formulated, thereby avoiding the need to cryopreserve bull sperm for the dissemination of new and desirable genetics to northern Australian beef cattle herds.

1.2 Taking ART to the North

1.2.1 Reluctance of northern Australian producers

Northern Australian beef cattle producers have been slow to adopt ARTs, with estimates that less than 1% of these producers are currently utilising AI in their breeding programs. The reluctance to incorporate AI practices into their breeding management programs has largely been due to the logistical challenges associated with introducing AI protocols in large herds, undertaking AI programs during the wet season, and poor conception rates from FTAI. These herds are generally managed extensively on immense properties in remote locations. The success of AI programs using the current

best-practice protocols relies heavily on precise oestrus synchronisation of females, ensuring all females are cycling, the skill of AI technicians, and repeat inseminations. Producers must also be able to receive and store straws of semen cryopreserved in liquid nitrogen. However, adoption of AI programs by northern Australian beef cattle producers would be greatly enhanced if producers are able to collect, process semen and inseminate on site or alternatively collect semen at the property of origin and dispatch to a processing unit for cryopreservation. AI facilitates the dissemination of improved genetics throughout a herd (Edwards et al., 2015), including heritable traits such as reproductive capability (Kosova et al., 2010).

A new method of storage that maintains the fertility of sperm without requiring cryopreservation will enable beef producers in northern Australia to utilise AI for genetic improvement across their herds. The conception rates of FTAI should be significantly improved if the timing of insemination is less critical to a successful fertilisation outcome.

1.2.2 AI with ambient temperature stored sperm

Aside from having the potential to improve pregnancy rates, the protocols associated with using sperm stored at ambient temperature would be easier for producers to adopt. Cryopreserved sperm have a drastically shortened life span post-thaw, which requires precise timing of insemination so that the spermatozoon and oocyte are mature, viable, and present at the site of fertilisation at the same time. Sperm stored at ambient temperature would not suffer from this reduced post-insemination longevity, and their improved vitality within the female tract could allow for the timing of inseminations to be less regimented without negatively affecting success. The damage associated with cryopreservation is considered compensable; this means that any damage can usually be overcome by increasing the number of sperm that is inseminated. Typically for cattle AI this is between 15 and 20 million total sperm when post-thaw total motility is over 50% (Shannon and Vishwanath, 1995, Mohanty et al., 2018). If stored in a manner that is less damaging, the insemination dose could be lowered considerably and an additional advantage to this development would be that a single ejaculate could produce many more AI doses than using current methods.

A provisional patent application has been filed for this novel ambient temperature sperm storage medium. After a large-scale field trial and further experiments on protocols for best use, including minimum AI dose, maximum storage length, and increasing antimicrobials and antifungals, this extender will be brought to market for both Australian, and potentially international producers.

2. Objectives

Objective 1: Extender development - achieved

To develop and validate a novel, innovative sperm preservation medium that will maintain the viability of fresh-chilled semen for between 2 to 3 weeks after collection.

Objective 2: Extender validation – achieved, pending large-scale fertility trial

To confirm through laboratory testing and field trials the fertility of fresh-chilled sperm preserved in the novel medium in terms of iatrogenic damage to the sperm, pregnancy rates and weaning rates.

3. Methodology

3.1 General methodology:

3.1.1 Materials

INRA96, EquiPlus and AndroMed were purchase from Minitube Australia (Ballarat, VIC), and BoviPure, used to isolate cells for *in vivo* use (Experiment 11), was purchased from Tek Event (Round Corner, NSW). Unless otherwise stated, all other chemicals and reagents were purchased from Sigma-Aldrich (Castle Hill, NSW).

A modified Biggers, Whitten and Whittingham (BWW) medium (Biggers et al*.* 1971) containing 95 mM NaCl, 4.7 mM KCl, 1.7 mM CaCl2.2H2O, 1.2 mM KH2PO4, 1.2 mM MgSO4.7H2O, 25 mM NaHCO3, 5.6 mM D-Glucose, 275 µM sodium pyruvate, 3.7 µl/ml 60% sodium lactate syrup, 50 U/ml penicillin, 50 µg/ml streptomycin, 0.25 mg/ml gentamicin to prevent growth of Pseudomonas aeruginosa (Aurich and Spergser 2007), 20 mM HEPES and 0.1% (w/v) polyvinyl alcohol, with an osmolarity of approx. 310 mOsm/kg, was utilised throughout this study.

Selection of high-quality bull sperm for storage was achieved using density gradient centrifugation (DGC) on a 45 and 90% discontinuous Percoll (GE Healthcare, Castle Hill, Australia) gradient. For this procedure, Percoll (90 mL) was supplemented with 10 mL of Ham's F10 solution, 370 µL sodium lactate syrup, 3 mg sodium pyruvate, 210 mg sodium hydrogen carbonate and 100 mg polyvinyl alcohol (PVA). This isotonic Percoll solution was diluted with BWW to create the discontinuous gradient (45% and 90% isotonic Percoll solution: 55% and 10% BWW respectively). 3 mL of 90% Percoll was underlaid below 3 mL of 45% Percoll in 15 mL conical centrifuge tubes (BD Falcon).

3.1.2 Semen collection and processing

Institutional and New South Wales State Government ethical approval was secured for the use of animal material in this study (UON: ACEC A-2018-813; CSU Wagga Wagga: ACEC ARA no. A18-014). This research was based on ejaculates from 17 normozoospermic Angus bulls (between 3 and 7 years of age) of proven fertility, held at Charles Sturt University, Wagga Wagga and 22 American Bucking Bulls of unknown fertility (privately owned). Semen was collected via electro-ejaculation, and the ejaculate was diluted (1:1) with INRA96 (with the exception of Experiment 1, 7 and 8). This initial dilution was deemed to be necessary to reduce the damage to sperm caused by toxic seminal plasma proteins during processing (Bergeron and Manjunath 2006). Equipment and extender were maintained at temperatures between 30 and 37 °C for the duration of semen collection and dilution. The tubes of extended semen were then held at room temperature (approx. 20 to 25°C) for up to 1 h prior to further processing. In Experiment 1, up to 6 mL raw semen was either centrifuged directly (700 x g for 10 min), or was overlaid on 3 mL BoviPure and centrifuged at 700 x g for 20 min, for all other experiments up to 5 mL extended semen was layered on top of 6 mL Percoll gradients and centrifuged at 700 x g for 30 min. Following centrifugation, the extended seminal plasma and BoviPure or Percoll were removed and discarded. High-quality sperm pellets were recovered from the base of the tubes, resuspended in 2 mL BWW and washed via centrifugation (300 x g for 5 min). These pelleted cells were then resuspended at a concentration of 50 million sperm/mL in the experimental media.

3.1.3 Sperm motility assessment

Sperm motility was objectively determined with computer assisted sperm analysis (CASA). CASA analysis was performed using an iSperm device (Aidmics Biotechnology, Taiwan) in Experiment 2, and AndroVision (Minitube, Ballarat, VIC) in Experiment 11. All other experiments were analysed with a HT IVOS or IVOS II (Hamilton Thorne, Danvers, MA) using the following settings; negative phase-contrast optics, recording rate of 60 frames/s, minimum contrast of 70, minimum cell size of 4 pixels, low size gate of 0.17, high size gate of 2.9, low intensity gate of 0.6, high intensity gate of 1.74, non-motile head size of 10 pixels, non-motile head intensity of 135, progressive VAP threshold of 50 µm/s, slow (static) cells VAP threshold of 20 µm/s, slow (static) cells VSL threshold of 0 µm/s and threshold STR of 75%. Cells exhibiting a VAP of ≥ 50 µm/s and a STR of ≥ 75 were considered progressive. Cells with a VAP greater than that of the mean VAP of progressive cells were considered 'rapid'. A minimum of 200 sperm in a minimum of five fields were assessed using 20 µm Leja standard count slides (Gytech, Australia) and a stage temperature of 37 °C.

3.1.4 Flow cytometric analyses

100 µL aliquots of sperm samples were pelleted via centrifugation (300 x g for 3 min), resuspended in 200 µL BWW containing FITC-PNA (0.8 µg/mL) and LIVE/DEAD far red fixable stain (0.5 µL/mL; Molecular Probes, Australia), and incubated at 37° C for 20 min. Following staining, sperm were pelleted via centrifugation and re-suspended in BWW for flow cytometric analysis. All flow cytometry was performed using a FACSCanto flow cytometer (BD Bioscience, NJ, USA) with a 488 nm argon-ion laser. Emission measurements were made using 530/30 band pass (green/FL-1) and > 670 long pass (far red/FL-4) filters. Debris was gated out using a forward scatter/side scatter dot plot, and a minimum of 10000 cells were analysed per sample. All data was analysed using FACSDiva software (BD Bioscience). Sperm were classified as being either; live and acrosome intact (neither green nor red fluorescence), live and acrosome damaged (green fluorescence only), dead and acrosome intact (red fluorescence only), or dead and acrosome damaged (red and green fluorescence).

3.1.5 In vitro fertilisation

Ovaries were collected from the local abattoir and transported to the laboratory in prewarmed (38.5 ^{o}C) saline. Ovaries were washed with sterile, prewarmed saline and kept on a heated stage set to 38.5 ^{o}C. Antral follicles on the surface of the ovaries were aspirated using an 18-gauge needle attached to a 10 mL syringe. The follicular aspirate was transferred to 15 mL tubes and left to sediment in a heated block pre-set to 38.5 ^{o}C. The resulting pellet was added to 5 mL prewarmed HEPES-TALP medium and the search for grade A (Boni et al. 2002) cumulus oocyte complexes (COCs) commenced. COCs were matured in TCM199 supplemented with 7 mg/mL BSA, 0.2 mM sodium pyruvate 1 µg/mL FSH (Folltropin-V, Bioniche Life Sciences) at a density of 20 per 100 µL at 38.5 ^{o}C and 5% CO_2 in humidified air for 22 – 24 hours. All COCs with expanded cumulus cells were washed 2 mL HEPES-TALP and transferred to a fertilisation dish that contained 1700 µL pre-equilibrated FERT-TALP medium supplemented with 0.2 mM sodium pyruvate, 10 µg/mL heparin and 7 mg/mL BSA. The dish was seeded with 1 million sperm/mL and left to incubate at 38.5 ^{0}C and 5% CO2 for 18 hours For cryopreserved semen, 2 – 3 straws were thawed in a water-bath (37.5 ^{o}C) for 30 s. Straws were wiped dry and the semen was dispensed into a 15 mL Falcon tube containing 10 mL pre-warmed HEPES-TALP. The tube was gently inverted and centrifuged at 500 x g for 10 min. Following centrifugation, the supernatant was removed carefully to avoid disruption of the sperm pellet. 200 µL of fresh pre-equilibrated HEPES-TALP was gently overlaid on the sperm pellet. Sperm were left to 'swim up' for 45 min at 38.5 ^{o}C. The top 200 µL medium containing sperm was removed and total cell count and motility was determined using the NucleoCounter SP-100 (Chemometec, Allerod,

Denmark) and CASA (IVOS II, Hamilton Thorne, Danvers, MA), respectively. For sperm stored in the novel medium, a 3 µL aliquot of sperm cells in the novel extender was checked for total cell count and motility and added directly to the IVF dish, COCs and sperm cells in the novel extender were co-incubated as described earlier. After 18 hours, all COCs were transferred into an Eppendorf tube containing 200 µL pre-warmed HEPES-TALP and denuded by repeated pipetting using a p200 Gilson pipette. Presumptive zygotes were transferred into 100 µL 4% PFA and fixed for 30 min at room temperature. Presumptive zygotes were transferred to 100 µL 0.3% Triton-X and permeabilised for 10 min at room temperature. After 4 washes in 300 µL PBS + 1 mg/mL PVA presumptive zygotes were transferred into 3 µL Vectashield mounting medium containing DAPI (Vector Laboratories) on a standard microscope slide. A coverslip was gently applied and kept in the dark for 2 hours. Presumptive zygotes were imaged using an Olympus BX51 fluorescent microscope to determine the presence of two pronuclei (2PN).

3.1.6 Fixed-time Artificial Insemination (FTAI)

Transcervical AI was performed by an AI technician following a 12 day oestrus synchronisation program. At day 0, a CIDR implant (Zoetis Australia, Rhodes, NSW) was inserted and 2 mg oestradiol benozoate injected intramuscularly (IM). 500 mcg prostaglandin was injected IM at day 7, the CIDR removed at day 10, 100 mcg gonadotropin-releasing hormone (GnRH) was injected IM at day 11, and at day 12 the cows were inseminated.

3.2 Experimental design

3.2.1 Experiment 1: Extender development: 'SpermSafe-E' for bull sperm

The aim of Experiment 1 was to conduct a preliminary investigation to ascertain whether bull sperm could be effectively stored in the ambient temperature stallion sperm extender ('SpermSafe-E'), developed by our group, and whether the isolation of high quality sperm from dead cells, seminal plasma and microbial contaminants using a BoviPure centrifugation step would be beneficial to sperm longevity during storage. INRA96, a stallion semen extender which has previously been shown to be optimal for the liquid storage of bull sperm (Murphy et al. 2017), was utilised as the control medium for this study. Sperm from 6 bulls were processed as outlined in section 3.1.2, and CASA motility analyses (section 3.1.3) were performed after 3 days of storage at 17 °C.

3.2.2 Experiment 2: Extender development: formulation experiments

Based upon the results of Experiment 1, in which the motility of bull sperm stored in the 'SpermSafe-E' stallion sperm extender were significantly poorer than those stored in INRA96, an extensive study to investigate the effects of each novel component of the medium was carried out. A total of nine individual experiments were undertaken. These experiments are considered confidential at this time, due to the filing of a provisional patent, and cannot be included in this report as they contain information on the components of the medium. Sperm from a total of 15 bulls (N=3 to 6 per treatment) were processed as outlined in section 3.1.2, and CASA motility analyses (section 3.1.3) were performed after 24 h of storage at room temperature using the iSperm device.

3.2.3 Experiment 3: Extender development: 17 vs. 22 degree storage, + Nystatin

As Experiment 2 was conducted under field conditions, it was only possible to make one objective measure of motility as consumables were limited. While the 24 h time point which was selected was too early to be able to observe the effects of some treatments, the subjective analyses that we were able to conduct using a phase contrast microscope did provide us with sufficient information to

determine the optimal combination of ingredients, termed 'NEW Bull' (Appendix 8.2). For this experiment, 'NEW Bull' was stored at either 17 °C or at room temperature (~22 °C) and supplemented with or without Nystatin (100U/mL) to inhibit fungal growth that was observed sporadically during Experiments 1 and 2. Sperm from a total of 10 bulls were processed as outlined in section 3.1.2, with CASA motility measurements (section 3.1.3) being performed every 24 h over 8 days. Experiment 9 was ran concurrently with sperm from split ejaculates stored chilled (5 °C) in INRA96. Acrosome integrity analyses (section 3.1.4) were performed at 3 days for all samples, and at 7 days for the 'NEW Bull' extender treatments.

3.2.4 Experiment 4: Extender development: amino acid comparison

This experiment contains information on the final formulation of the medium and is considered confidential at this time.

3.2.5 Experiment 5: Extender development: fungicide comparison

This experiment contains information on the final formulation of the medium and is considered confidential at this time.

3.2.6 Experiment 6: Extender development: collection temperature optimisation

Following the first part of Experiment 4 (Part 1, 3.2.4) it became apparent that low ambient temperatures during semen collections was effecting the quality of the sperm, resulting in low motilities across all treatments. For this reason, we investigated the effect of using a pre-warmed collection vessel during the electroejaculation procedure.

We fabricated simple water jackets which would fit over the collection tube using 50 mL Falcon tubes fitted with appropriately-sized rubber grommets (Fig. 1).

Figure 1. Simple water jackets to prevent cold-shocking sperm during semen collection.

Prior to running the test, we plotted the loss of temperature over time at an ambient temperature of 5 °C, which was the temperature inside the shed during collections. A 15 mL falcon tube containing 37 °C water cooled to less than 20 °C in under 4 minutes, while a 15 mL falcon tube containing 37 °C water inside our 37 °C water jacket took over 17 minutes to cool to less than 20 °C.

For Part 2 of Experiment 4, the tubes were filled with warm water and stored in a water bath at 37 °C until use. Semen was collected, processed and stored according to section 3.1.2. The total and progressive motilities of sperm collected without the water jacket (at an ambient temperature of 5 °C) were assessed (section 3.1.3) and compared with the total and progressive motilities of sperm collected using the water jacket following 3 days of storage in 'NEW Bull' extender at room temperature, Part 1 and Part 2 of Experiment 4, respectively.

3.2.7 Experiment 7: Extender development: initial dilution extender

As described in section 3.1.2, immediately upon collection, semen is diluted with an initial extender (typically INRA96) to reduce damage to sperm from toxic seminal plasma proteins. This is also done to dilute the concentration of sperm in the ejaculate as during collection via electroejaculation the pre-ejaculate is skipped, collecting only the most concentrated semen. While INRA96 performed well in this role, the milk-based components and regulations around import make it hard to source in Australia. We questioned whether other media, including our 'NEW Bull' medium, could be used in this initial dilution role in its place. Immediately following collection, semen from each bull (N=5) was split between 5 tubes and diluted 1:1 with INRA96, EquiPlus, 'NEW Bull', BWW, or AndroMed; sitting up to 1 h at room temperature before processing. The rest of the sperm processing steps were done as described in section 3.1.2 with all samples resuspended in 'NEW Bull.' CASA motility measurements (section 3.1.3) were made at 0, 3, 7, and 10 days of storage.

3.2.8 Experiment 8: Extender development: fresh extender vs. freeze-dried

For most experiments, 3x concentrated 'NEW Bull' medium was freeze-dried ahead of transportation for fieldwork and reconstituted with ddH_2O on the day of use. However, when the 'NEW Bull' medium was made fresh on the day of use, it was anecdotally observed that samples maintained a higher motility past 7 days of storage. To assess this, sperm was collected (section 3.1.2) and resuspended in the 'Fresh' medium, 1x concentrated freeze-dried medium, or 3x concentrated freeze-dried medium with CASA motility measurements assessed at 3, 7, 10, and 14 days (section 3.1.3).

3.2.9 Experiment 9: Extender validation: 'NEW Bull' vs. chilled

Alongside Experiment 3 (optimal formulation of 'NEW Bull' extender at 17 °C or room temperature + Nystatin), sperm from split ejaculates were resuspended at a concentration of 50 million sperm/mL in INRA96 and stored chilled (5 °C). CASA motility measurements were assessed at 3 and 7 days (section 3.1.3), with acrosome integrity analyses performed at 3 days (section 3.1.4).

3.2.10 Experiment 10: Extender validation: 'NEW Bull' vs. cryopreserved sperm

Following optimisation of the 'NEW Bull' extender formulation (see Appendix 8.5), a study was conducted to compare the functionality and fertility of bull sperm stored in 'NEW Bull' for 7 and 14 days with sperm cryopreserved using a standard commercial protocol in AndroMed. Sperm were collected and processed (as per section 3.1.2) before being split into 2 aliquots and either cryopreserved, or stored in 'NEW Bull' extender. Following storage for 7 and 14 days, sperm were analysed for motility (as per section 3.1.3) and utilised for IVF (section 3.1.5).

3.2.11 Experiment 11: Extender validation: 'NEW Bull' field fertility trial

To confirm the fertility of sperm stored in 'NEW Bull' a field fertility trial was performed in Wagga Wagga, with heifers housed at Charles Sturt University. Following a 12 day oestrus synchronisation protocol (section 3.1.6), 18 2-year-old virgin Angus or Angus cross heifers all with a Body Condition Score (BCS) between 3 and 4 were inseminated with sperm from Angus bulls that had been stored for 7 days at room temperature. Semen was collected and processed (section 3.1.2) and isolated sperm were stored for insemination, with motility analysis (AndroVision; section 3.1.3) after processing on Day 0 and on the day of insemination Day 7. No control was used in this trial, the sample size was too small to create statistical significance so this preliminary trial was used to establish proof of field fertility.

3.3 Statistical analyses

All data used in this study were tested for normal distribution prior to analyses. If data could not be transformed to fit a normal distribution, a non-parametric test was performed. Data for all experiments were analysed by one way ANOVA with JMP, Version 14.0 software (SAS Institute Inc., Cary, NC). Where significant treatment effects were identified by ANOVA ($\alpha = 0.05$), means comparisons were performed. Differences between the parameters of sperm stored in various treatment media in all experiments were identified using Student's t-tests for pair-wise mean comparisons ($\alpha = 0.05$).

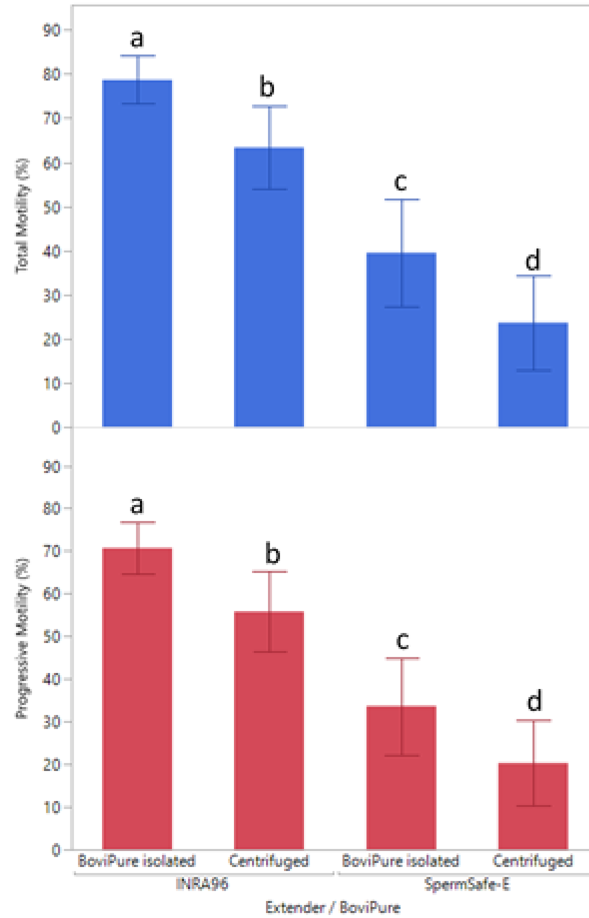
4. Results

4.1 Objective 1: Extender development

4.1.1 Experiment 1 Results: 'SpermSafe-E' for bull sperm

No statistical interaction between centrifugation treatment and sperm storage medium were observed, so data were pooled for further analyses. The progressive and total motilities of sperm stored in INRA96 were significantly higher than those stored in the 'SpermSafe-E' stallion medium (71 vs 31% and 63.3 vs 27% for total and progressive motilities respectively; $P \leq 0.0001$) and the isolation of sperm using BoviPure resulted in significantly improved total and progressive motilities (59.1 vs 43.5% and 52.2 vs 38.1% for total and progressive motilities respectively; $P \leq 0.01$) compared to direct centrifugation alone. Results of individual treatments shown in Fig. 2.

Figure 2. Total and progressive motilities of bull sperm stored following isolation using either a BoviPure gradient or direct centrifugation techniques in either INRA96 or UON's 'SpermSafe-E' stallion sperm extender for 3 days at 17 °C ($P \leq 0.05$).



4.1.2 Experiment 2 Results: formulation experiments

This experiment contains information on the final formulation of the medium and is considered confidential at this time.

4.1.3 Experiment 3 Results: 17 vs. 22 degree storage, + Nystatin

The total and progressive motility of sperm stored in 'NEW Bull' extender at room temperature did not significantly decrease over a week of storage (Fig. 3). While the room temperature storage (without Nystatin) produced consistently higher results than any other treatment, these were only significant at day 3 of storage, where the progressive motilities of both the room temperature and the room temperature + Nystatin treatment were significantly higher than both the 17 °C treatments. Viability and acrosome integrity (Fig. 4) were assessed using flow cytometry, with no significant difference, though room temperature storage (without Nystatin) maintained higher viability than other treatments.

Figure 3. Total (top) and progressive (bottom) motilities of bull sperm (N=10) stored in 'NEW Bull' extender over 8 days at either room temperature (RT) or 17 °C (17). Nystatin was added for control of fungal growth to additional samples at room temperature and 17 °C. (P≤0.05).

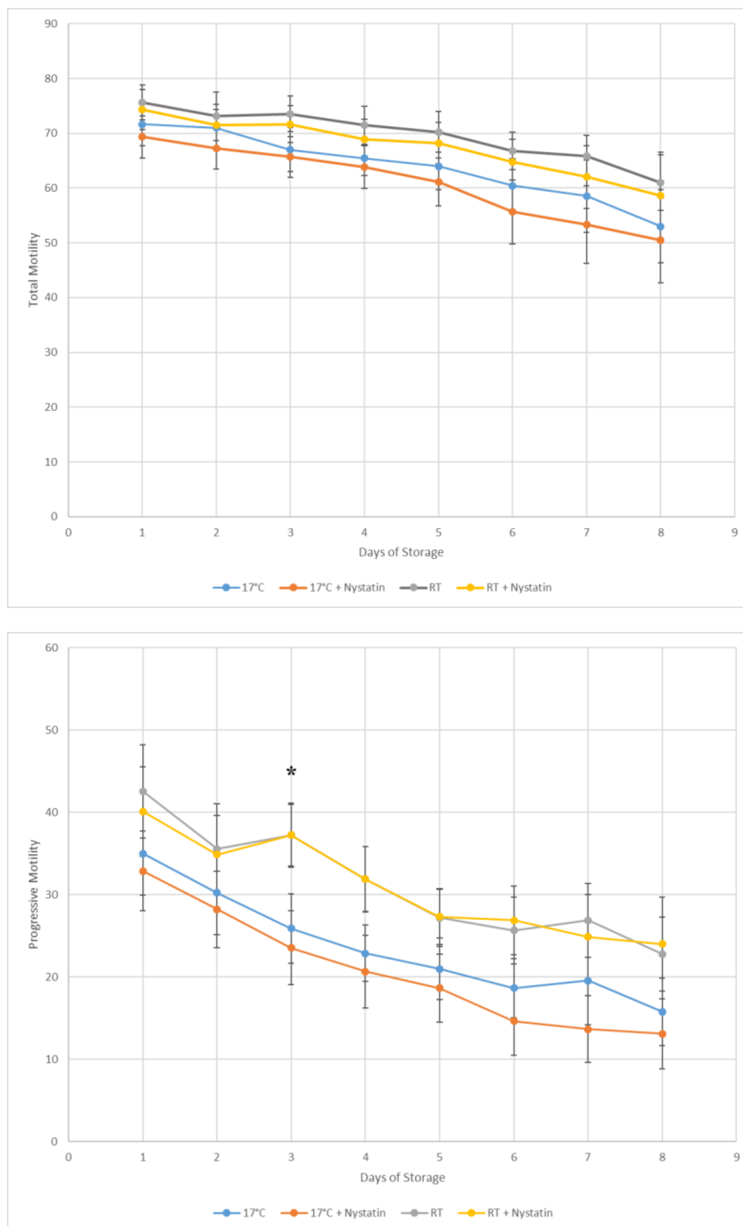
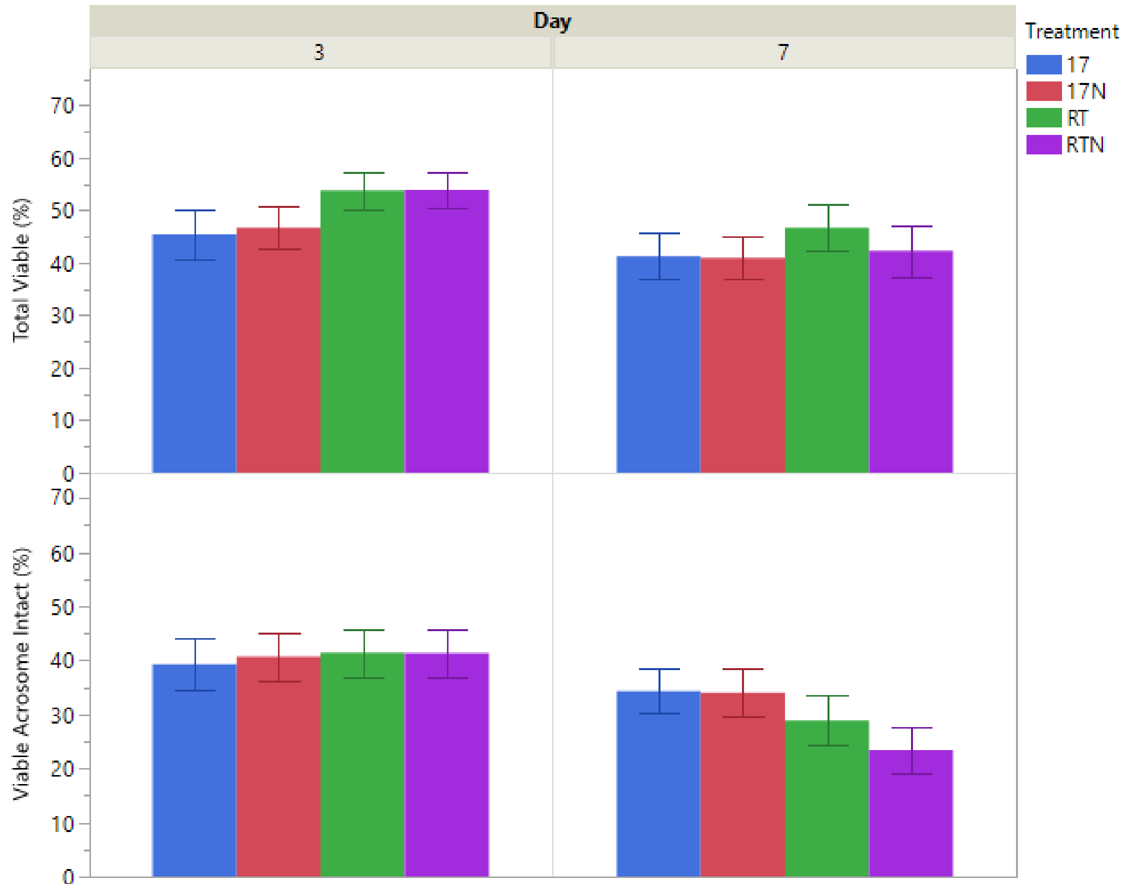


Figure 4. Viability and acrosome integrity were assessed at Day 3 and Day 7 for bull sperm (N=10) stored in 'NEW Bull' extender. Samples were stored at room temperature (RT) or 17 °C (17). Nystatin was added for control of fungal growth to additional samples at room temperature (RTN) and 17°C (17N).



4.1.4 Experiment 4 Results: amino acid comparison

This experiment contains information on the final formulation of the medium and is considered confidential at this time.

4.1.5 Experiment 5 Results: fungicide comparison

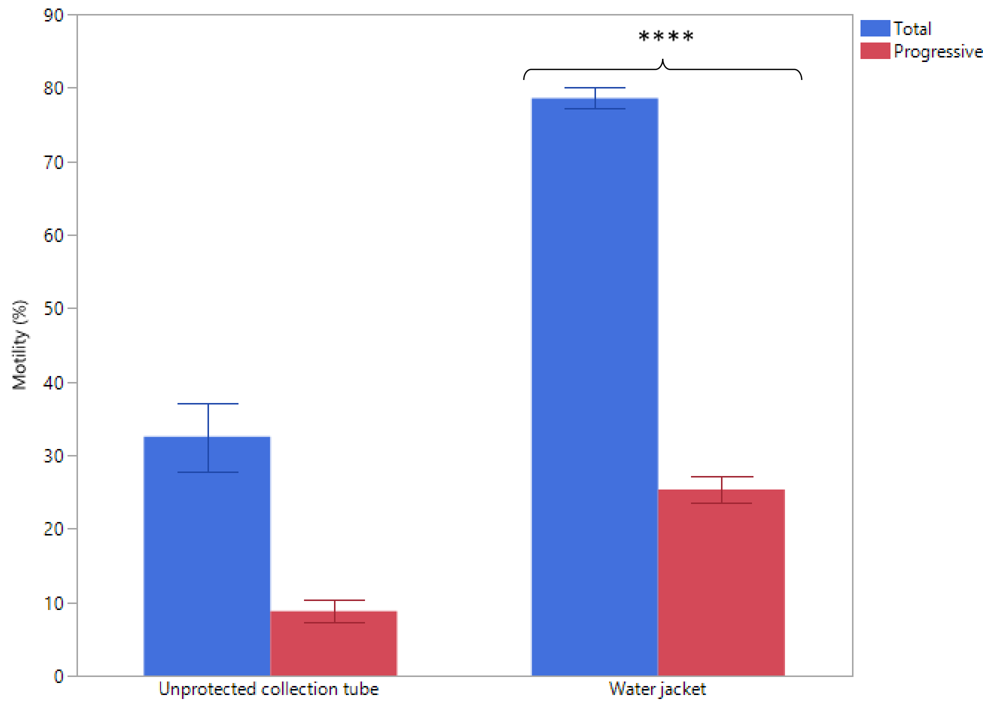
This experiment contains information on the final formulation of the medium and is considered confidential at this time.

4.1.6 Experiment 6 Results: collection temperature optimisation

An initial testing of the water jackets found that the temperature of 7 mL of 37 °C water inside the collection tube remained above 20 °C for 17.5 min following exposure to 5 °C ambient temperatures, whereas the water in the unprotected tube was cooler than 20 °C in less than 4 min.

Following storage, sperm which had been collected using a protective water jacket displayed significantly higher total and progressive motilities than sperm collected into an unprotected tube (Fig. 12).

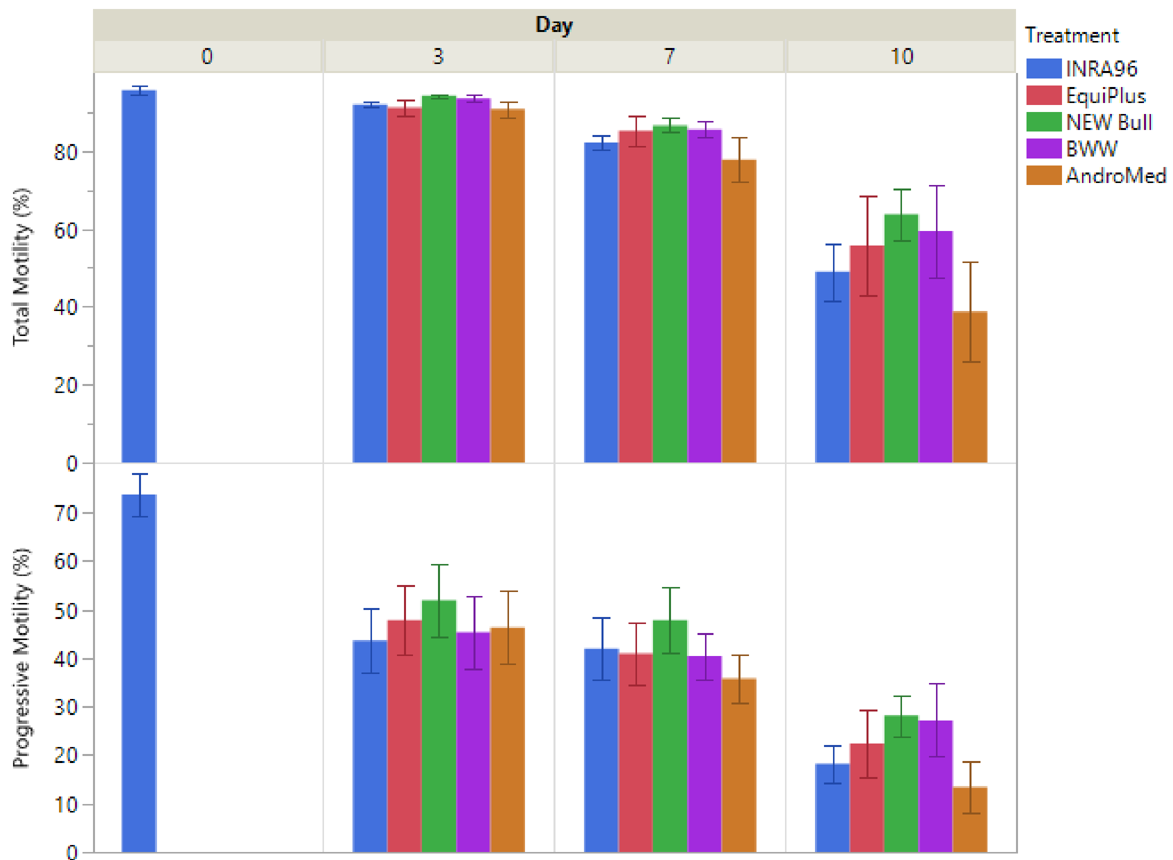
Figure 12. Total and progressive motilities of sperm which had been either collected into an unprotected collection tube, or into a tube within a pre-warmed water jacket. Motility was assessed 3 days post-storage in 'NEW Bull' extender. * P≤0.05, ** P≤0.01* P≤0.001 **** P≤0.0001.**



4.1.7 Experiment 7 Results: initial dilution extender

Though no significance difference was observed between any of the alternative initial extenders and INRA96 as the control (Fig. 13), 'NEW Bull' was selected as the best initial extender as it maintained slightly higher total and progressive motilities and makes for the most simplified protocol, without requiring the purchase of an additional extender.

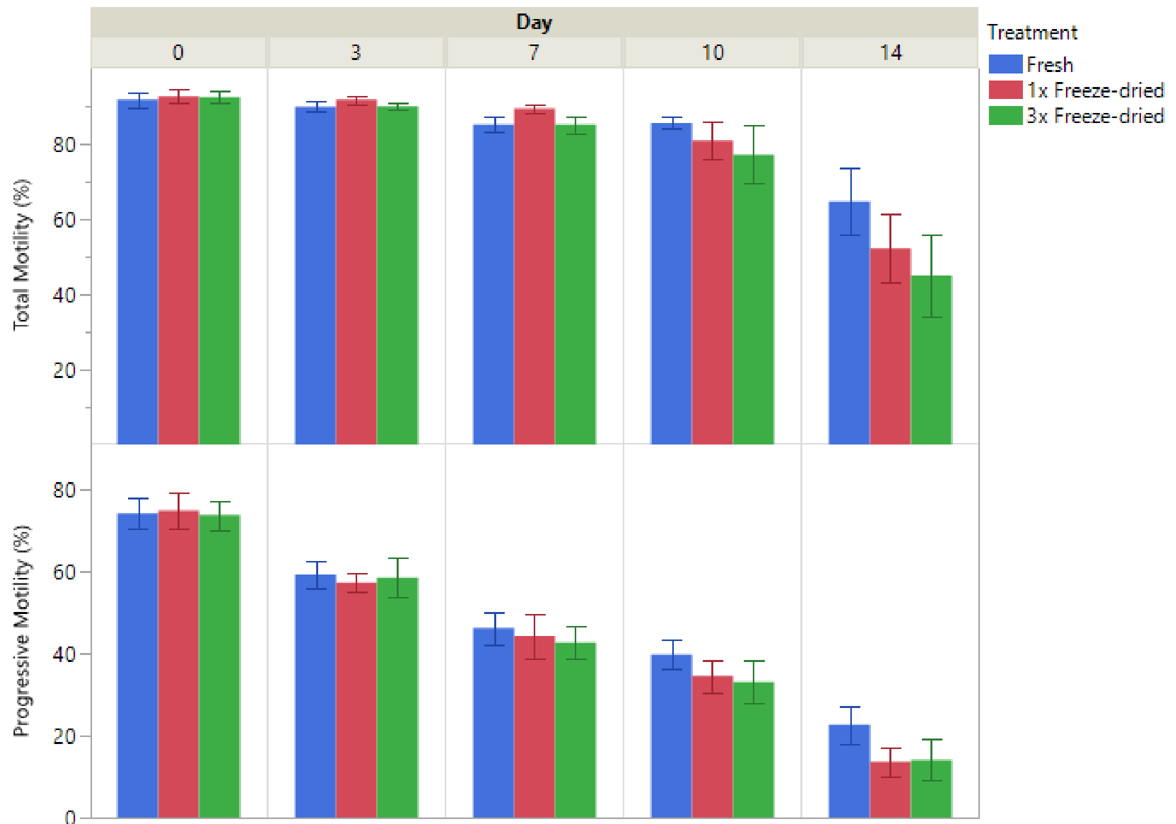
Figure 13. Total and progressive motilities of bull sperm (N=5) diluted with INRA96, EquiPlus, 'NEW Bull,' BWW, or AndroMed prior to processing, all stored in 'NEW Bull' extender for 3, 7, and 10 days at room temperature.



4.1.8 Experiment 8 Results: fresh extender vs. freeze-dried

No significant difference was seen between fresh-made 'NEW Bull' medium and freeze-dried 'NEW Bull' medium (Fig. 14), however, after 7 days of storage motility in either freeze-dried extender appear to decline faster than sperm stored in fresh-made 'NEW Bull.'

Figure 14. Total and progressive motilities of bull sperm (N=7) stored in 'NEW Bull' extender that was fresh-made, freeze-dried at 1x concentrated, or freeze-dried at 3x concentrated at 3, 7, 10 and 14 days of room temperature storage.

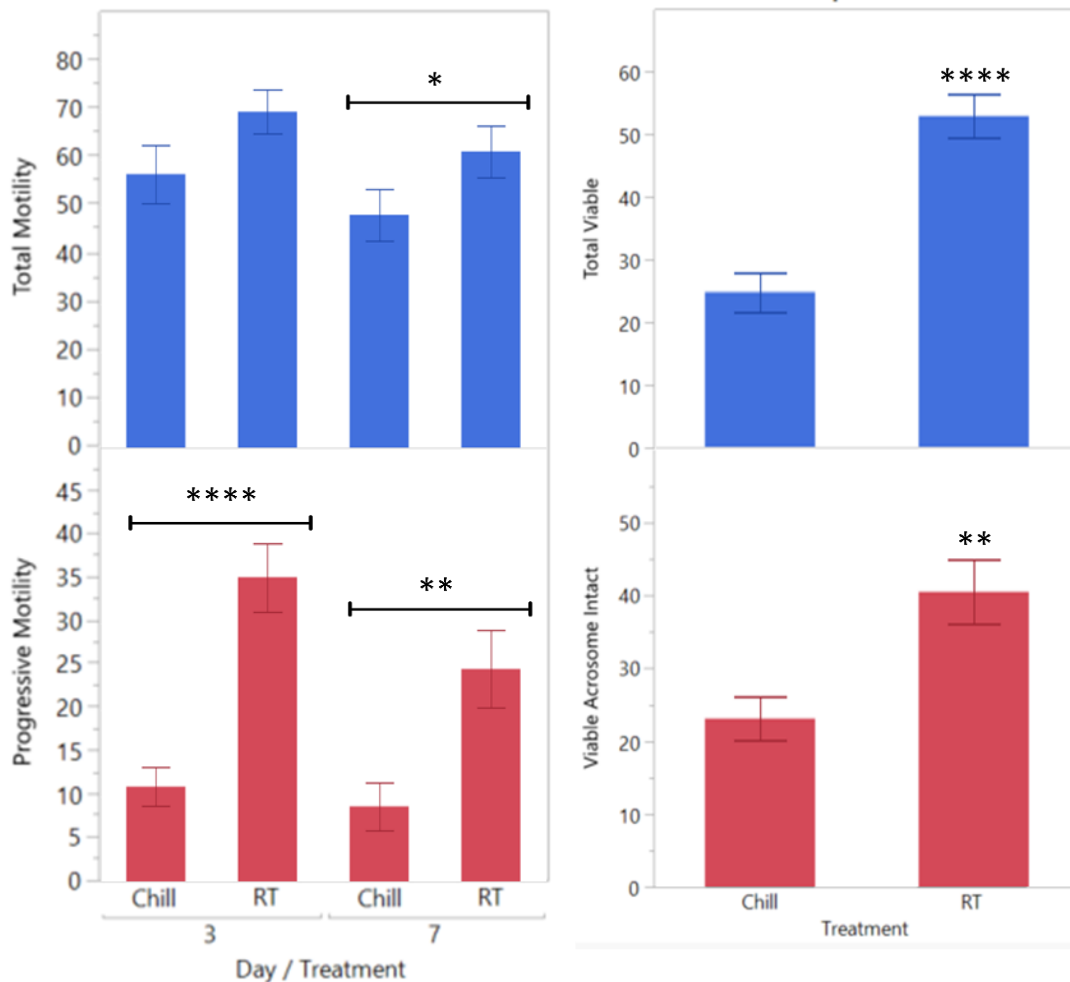


4.2 Objective 2: Extender Validation

4.2.1 Experiment 9 Results: 'NEW Bull' vs. chilled

When compared with chilled sperm stored in INRA96 (split ejaculates; N=10), the progressive motility was significantly higher in the 'NEW Bull' extender after 3 days of storage; after 7 days of storage both total and progressive motilities were significantly higher. A similar pattern was observed for acrosome integrity and viability; after 3 days of storage (considered to be the maximum for sperm fertility following chilling), sperm stored in the 'NEW Bull' extender had significantly higher viability and acrosome integrity that chilled sperm stored in INRA96 (Fig. 15).

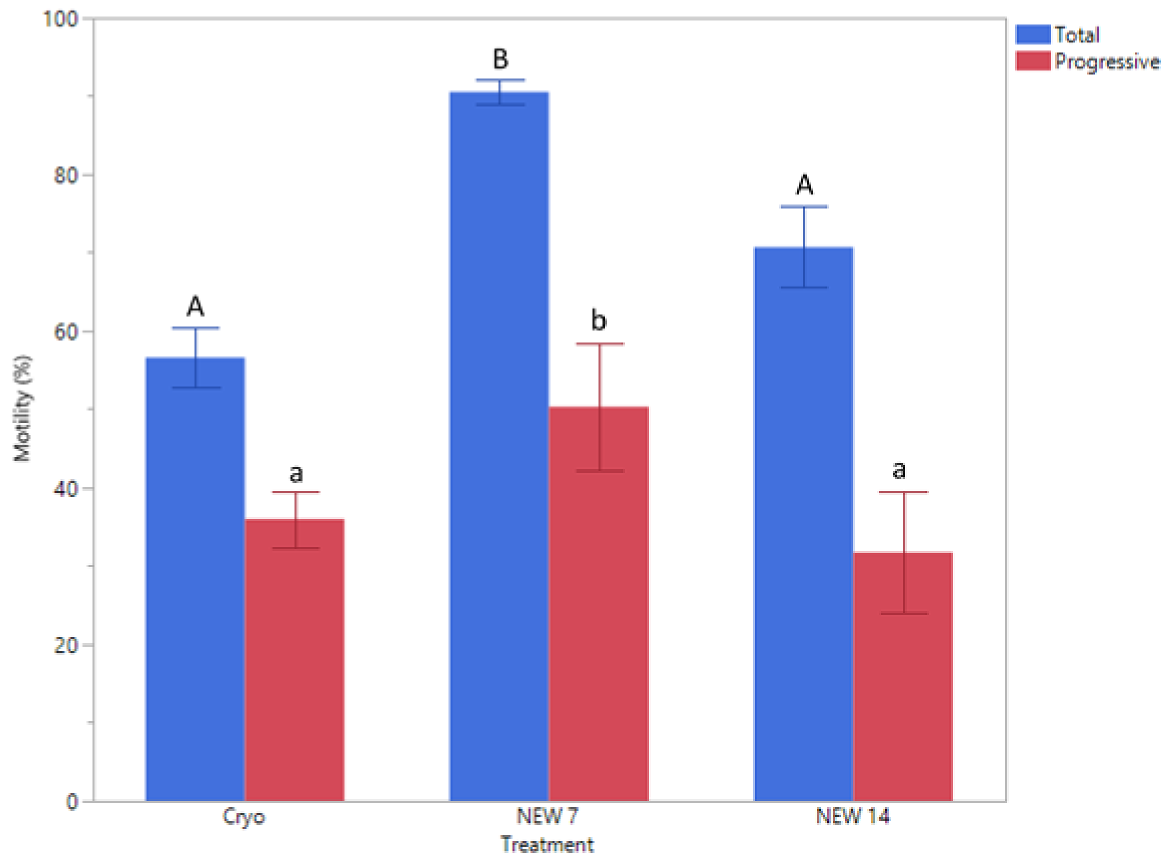
Figure 15. Total and progressive motilities (left) and viability and acrosome integrity (right) of bull sperm stored for 3 and 7 days in either INRA96 at 5°C and 'NEW Bull' extender at room temperature; *P≤0.05, ** P≤0.01, *** P≤0.001, **** P≤0.0001.



4.2.2 Experiment 10 Results: 'NEW Bull' vs. cryopreserved sperm

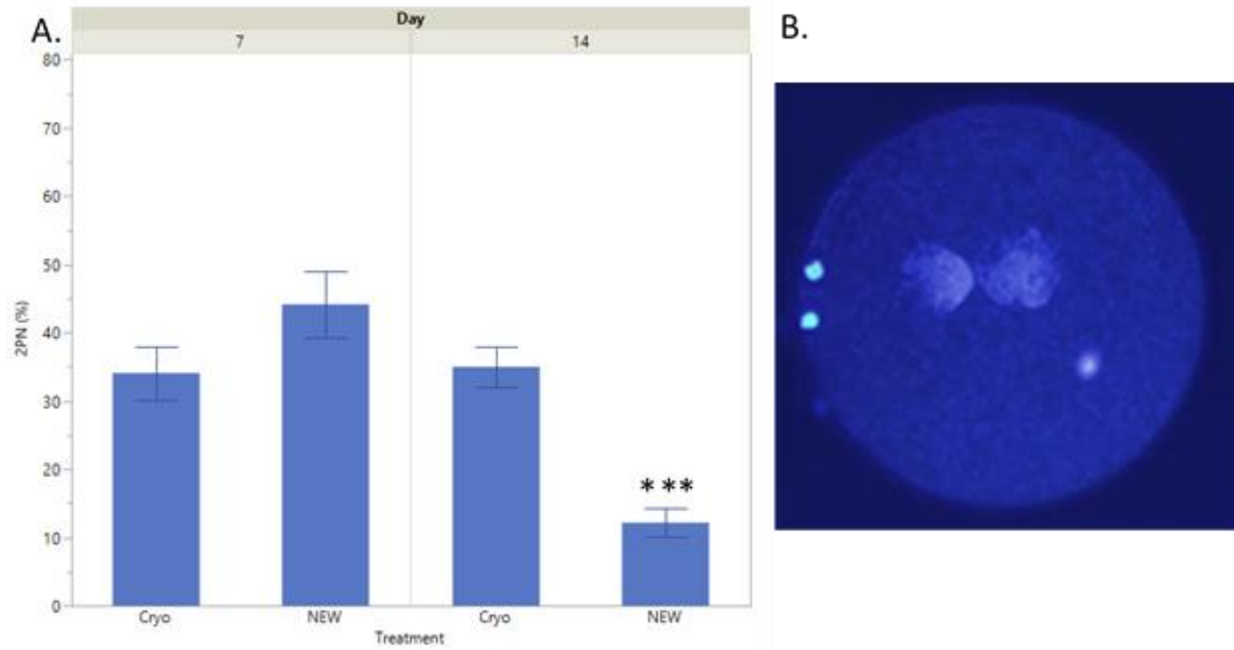
After 7 days of storage, the total and progressive motilities of sperm stored in 'NEW Bull' extender were significantly higher than those of cryopreserved sperm (P ≤ 0.0001 and 0.05 for total and progressive motilities respectively). By 14 days of storage, the motilities of sperm stored in 'NEW Bull' extender had significantly declined, though they were still akin to those of cryopreserved sperm (Fig. 16).

Figure 16. Total and progressive motilities of sperm stored in 'NEW Bull' extender for 7 (NEW 7) and 14 days (NEW 14) compared to sperm cryopreserved in AndroMed (Cryo). Different superscripts denote a significant difference. Individual differences: 'NEW 7' over Cryo total motility P≤0.0001, progressive motility P≤0.05; 'NEW 7' over 'NEW 14' total motility P≤0.001, progressive motility P≤0.05. No difference between Cryo and 'NEW 14' for either parameter.



In a similar pattern, the *in vitro* fertilisation rates were higher (though not significantly so, possibly given the sample size) for sperm stored for 7 days in the 'NEW Bull' extender, though by day 14, the fertilisation rates were significantly lower than those obtained using cryopreserved sperm (Fig. 17).

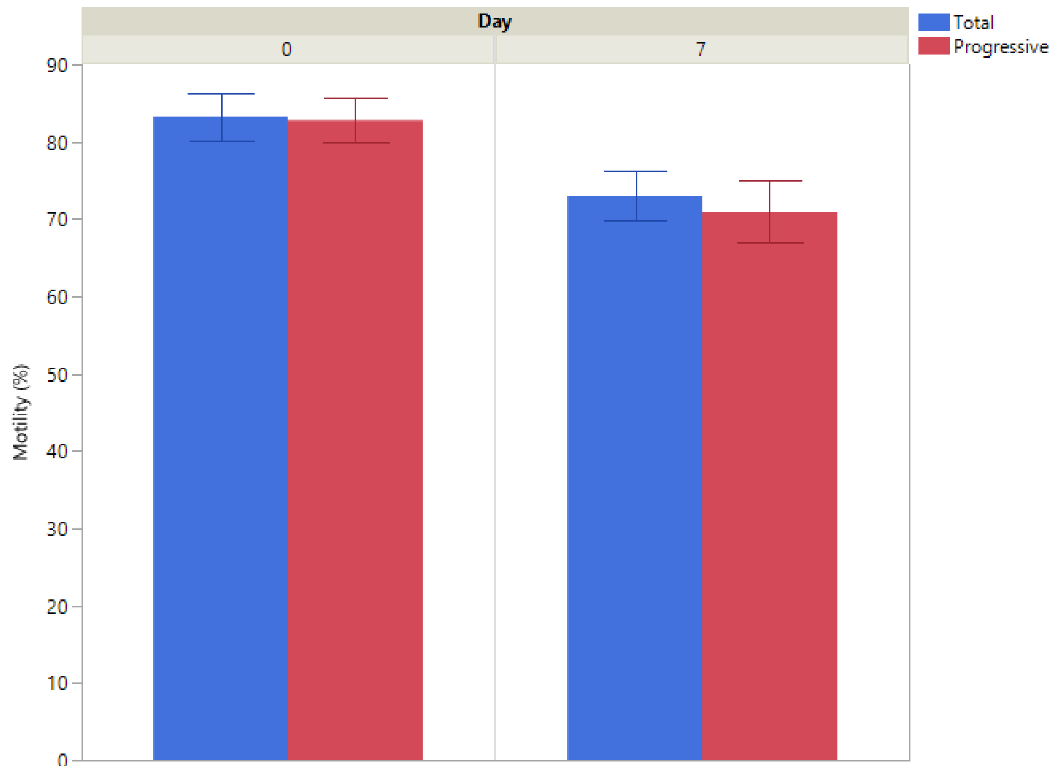
Figure 17. (A) Fertilisation rate (expressed as percentage of presumptive zygotes with two pronuclei; 2PN) following IVF using sperm stored in 'NEW Bull' extender for 7 or 14 days compared to sperm cryopreserved in AndroMed. * denotes P≤0.001. (B) A presumptive zygote with two pronuclei in the centre and two polar bodies on the left side.**



4.2.3 Experiment 11 Results: field fertility trial

Sperm were collected from 6 bulls, with the 3 best samples (based on morphology and motility) stored for inseminations. Only a slight decline was seen from the day of collection to the day of insemination 7 days later (Fig. 18), mean total motility from 83.3% to 73% and mean progressive motility from 82.8% to 70.9%, respectively.

Figure 18. Total and progressive motilities of sperm stored in 'NEW Bull' extender the day of collection and 7 days later, prior to FTAI (N=3).



Eighteen 2-year-old virgin heifers were inseminated following oestrus synchronisation with sperm stored in 'NEW Bull' for 7 days at room temperature, the 3 bulls were split evenly across the heifers with 6 inseminated with each, in case of bull fertility issues. At a 33 day ultrasonographic pregnancy diagnosis, 14 of 18 cows were in calf (~78%). While a chi-square goodness of fit analysis did not reveal significant difference from the industry standard pregnancy rate of FTAI with cryopreserved semen (60%; P=0.1237), we believe this is due to the low sample size of this trial (N=18). We are repeating this trial to increase our sample size and to further validate the use of 'NEW Bull' with Bos indicus cattle.

5. Conclusion

This is the first study in which bull sperm has been stored in liquid form (without cryopreservation) for longer than 3 days (Murphy et al., 2017) while maintaining functionality and fertilising potential. In addition, in the present study, sperm were stored at room temperature (in air-conditioning) without the use of a specific device to control temperature. This development will markedly reduce the logistical constraints associated with the transport of chilled and cryopreserved sperm for field use.

Additionally, we observed that the isolation of bull sperm using DGC significantly improved sperm quality during storage. While others have reported that processing bull sperm through BoviPure and Percoll gradients does in fact improve sperm quality (Arias et al., 2017, Samardzija et al., 2006b, Samardzija et al., 2006a), there are no studies showing the long-term effects of DGC on sperm intended for liquid storage. This step not only removes the dead and dying cells which contribute to oxidative stress in the remaining viable cells (Aitken et al., 2015, Upreti et al., 1998), but it also significantly reduces the microbial and viral contaminants that may be present in the raw ejaculate (Galuppo et al., 2013, Varela et al., 2018) – a step which is vital, particularly when sperm are to be stored at room temperature.

5.1 Key findings

We have demonstrated the field fertility of bull sperm stored at room temperature for 7 days, with laboratory assessments suggesting that 14+ days of storage in this medium is achievable under optimal circumstances. By increasing or changing the antifungals used in this medium we hope to make 14 days the minimum possible storage length for all samples.

5.2 Benefits to industry

Historically, cryopreservation has been the only viable method for sperm storage beyond 72 h, and therefore a necessary part of transporting genetic material destined for use in AI programs. However, in some instances, sperm from bulls with desired fertility and production traits cannot tolerate cryopreservation, and these limitations coupled with the logistical constraints with synchronising oestrus in large cattle herds has prevented AI technology being widely utilised in the northern Australian regions. The success of this project has significant implication for the Australian red meat industry. For this first time, bull sperm viability can be maintained for 14 days without requiring cryopreservation. This allows northern Australian beef producers to better utilise AI technology for selective breeding programs, thereby increasing the rate of genetic gain as it relates to both fertility and carcass traits.

Utilisation of sexing technology has critical implications for the livestock industry. The importance of replacing breeding cows is essential for the longevity of the beef enterprises, and the use of sexed semen can increase the rate of genetic gain by up to 15% (Van Vleck and Everett, 1976). However, sex-sorting has significant effects on sperm viability. Following flow cytometric separation of X- and Y-chromosome bearing sperm, samples are generally cryopreserved before they are distributed to producers (Garner and Seidel, 2008). The cumulative effect of damage from the sex-sorting process and damage from cryopreservation can result in reduced pregnancy rates (Holden and Butler, 2018), and it has been shown that by removing the cryopreservation step, fertility of sex-sorted sperm is significantly enhanced (Xu, 2014). As such, the use of our 'NEW Bull' sperm storage medium for the storage of sperm both before and after sex-sorting will remove the need for cryopreservation if intended for more immediate use, making this technology more accessible for northern Australian producers.

Our 'NEW Bull' medium will also play an integral role in other ARTs used in beef production, specifically IVF. Bos indicus species lend themselves particularly well to IVF programs as their ovaries have more follicles than those of Bos taurus. Beef producers in Canada, Brazil and the US have successfully incorporated IVF into their breeding programs to benefit from both maternal and paternal bloodlines. Whilst oocyte quality is an important factor, sperm viability is critical for IVF success. In IVF programs, frozen-thawed sperm must undergo further processing, either density gradient separation or "swim-up" to isolate live cells. However, sperm stored in our 'NEW Bull' medium is suitable for direct use in IVF, and therefore, there is no need for further laboratory processing as it sperm can be delivered directly to fertilisation dishes as outlined in Experiment 10.

6. Future research and recommendations

A provisional patent on the current formulation of 'NEW Bull' has been filed with discussions around commercialising the product under the name SpermSafe-B. However, before bringing the product to market a few key points need addressed. The current formulation of the medium uses very low concentrations of antimicrobial and antifungal agents when compared to other commercial products

for sperm storage. As such, fungal growth is sometimes seen between 7 and 10 days. Further experiments on increasing the dosages of the current antimicrobials and antifungals, or using alternative antimicrobials and antifungals, without compromising the effectiveness of the extender, would be advantageous prior commercialisation. A large-scale field trial comparing pregnancy rates achieved following FTAI using ejaculates split between cryopreservation and room temperature storage in 'NEW Bull,' will be needed to show statistically significant improvement of this method over industry standard. Smaller field trials were necessary to demonstrate field fertility of sperm in this medium prior to large-scale testing.

6.1 Storage of 'NEW Bull' medium

We developed a protocol to freeze dry the medium in Newcastle so that it could be reconstituted using ddH_2O at CSU, greatly simplifying the logistics of field work in Wagga Wagga. The successful field trial outlined in Experiment 11 used this method of reconstituting freeze-dried extender for the sperm that was stored for 7 days prior to insemination. At that point in the extender's development, a decline was typically seen between 10 and 14 days of storage. When semen collections moved to a privately owned farm closer to Newcastle, fresh-made extender was used for storage of sperm and we anecdotally observed that this media was better at maintaining motility to 14 days and beyond. The theory was tested and as seen in Experiment 8, when freeze-dried the extender is able to perform the same as fresh-made media through 7 days of storage but beyond 7 days the motility of sperm stored in the freeze-dried extender drops off faster than sperm stored in the fresh-made extender. Other ways to store the media for commercial application may be in liquid form following pasteurisation, or by storing and shipping at -20 °C. These experiments are yet to be done, but would substantially enhance the commercial viability of the final product.

6.2 Development of protocols for best use

Further research must also be done on the optimisation of AI protocols for use with this extender. While we stored sperm at 50 million sperm/mL and achieved a high pregnancy rate using a 0.5 mL dose (~25 million total sperm), a high percentage of these were motile compared to the percent required to pass a commercial freeze-thaw test (77-87% vs. 60%, respectively), meaning that the insemination dose required for pregnancies may be much lower than for AI with cryopreserved semen. If this is found to be true it would mean more AI doses per collection for sperm stored in this way. Furthermore, from an industry perspective it would be beneficial to chart the loss of fertilising potential over time by performing inseminations daily over a two-week period, giving an accurate indication of optimal storage durations.

Optimal temperature ranges for maintaining extended sperm and the best vessels for storage and transportation need to be explored. Anything that can smooth the path for industry adoption will be beneficial prior to commercialisation.

A major benefit of this extender would be if AI could be performed without oestrus synchronisation. Oestrus synchronisation is a time consuming process, requiring more intensive management, and can cost A$25-35 per cow. Additionally, the use of progesterone-infused sponges (such as CIDRs) poses an environmental risk (Orlando and Ellestad, 2014), with these hormones entering the ecosystem via water runoff, causing endocrine disruption and reproductive problems in a variety of aquatic species. We predict that sperm stored in our 'NEW Bull' medium will live longer in the female tract than sperm that have gone through the freeze-thaw process. If these sperm can then be kept on-site for multiple days, with AI timing based upon heat detection, this has the potential to reduce the economic and environmental costs of oestrous synchronisation regimens. Facilities that

typically perform AI using fresh semen could also benefit from the extended longevity of this sperm as it would allow greater flexibility in scheduling between semen collection and AIs.

6.3 Testing 'NEW Bull' medium for use in other ruminant species

Given that many commercial sperm storage media can be applied interchangeably for the storage of bull, ram and goat sperm, it would be beneficial to assess whether the formulation described in this document could be utilised for the storage of sperm from these other economically important ruminant species. As we have now optimised formulations for horses and cattle, we are confident that we could also optimise a formulation for rams and bucks which would provide similar advantages to those outlined above, with the additional welfare and economic advantage of avoiding the need for invasive laparoscopic AI methods that are necessary when cryopreserved sperm is utilised.

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