New insights into AI semen use and objective semen evaluation in UK Veterinary Practice

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Authors Declaration

Other than the help acknowledged, all the work presented in this thesis was carried out by the author. It has not been submitted, in full or in part, for consideration for another degree or professional qualification.

Mark William Spilman

Some of the work presented in this thesis has been used in presentations at:

BCVA Congress 2016

International Bull Fertility Conference 2018

Abstract

The reproductive performance of breeding cattle is a key driver of sustainable beef and dairy production. Good fertility performance in breeding cattle is a multifactorial phenomenon and the male factors of fertility should not be overlooked. The aim of this study was to investigate the semen handling and storage practices on 47 farms utilising Artificial Insemination (AI) through a questionnaire, and then to use objective multi parametric semen analysis equipment in a veterinary practice to assess the quality of the AI semen in storage on the same beef and dairy farms in North Yorkshire. From these data, the proportion still above breeding company pre-release standards, and differences between conventional and sexed semen, beef and dairy, breed of bull, beef and dairy type of farm were investigated for all assays performed using Students t-test. Fresh semen from bull breeding soundness evaluations was also assessed and in the same laboratory and subjective and objective assessments of motility and morphology assessments compared. Finally semen evaluation results were compared with field fertility data on one farm and correlations reported.

There were inconsistencies in how farmers stored and handled semen in storage on their farms. 12.1% of flasks in use were over 14 years old, over half of the herds surveyed (51.7%) did not respond with the age of flask, but 13.8% were new in the last 4 years. Of the herds that responded, the most common response (45.1%) was that liquid nitrogen levels were not checked at all and 6.1% herds checked their liquid nitrogen levels weekly. The majority (19.5%) of farms used a thaw temperature between 37^0C and 37.9^0C, next most common was 35 – 35.9^0C, 36 -36.9^0C accounted for 2.4% of farms, and only 1.2% of farms used >38^0C. The majority (28%) of farms that responded thawed straws for between 21 and 30 seconds, but some farms (2.4%) were using short thaw times of less than 10 seconds. Only 9.8% of farms thawed straws for longer than 30 seconds. 70.7% of herds in the study were using DIY AI; less than 25% of herds were using a technician service. 40.2% of herds were carrying out less than 50 serves/month. 30.5% of herds were serving more than 99 times per month.

Semen analysis of straws of frozen semen taken from farm storage in North Yorkshire, UK was compared to pre-release standards used in UK and N America. Analysis of conventional and sex sorted semen showed respectively 65% and 70% were above the viability standard; 46% and 0% were above mitochondrial activity standard; 44% and 10% above CASA motility standard; 32% and 10% above CASA progressive motility standard in the UK; 86% and 40% above CASA progressive motility standard in North America (see table 3).

Categories of semen were then compared using students t test, following visual assessment of normality. Comparisons between sex sorted and conventional straws showed significant differences in CASA motility ($p=0.0182$), progressive motility ($p=0.0024$), mitochondrial activity ($p<0.0001$) and morphology ($p=0.0257$).

Straws from dairy sires had significantly greater viability (p=0.0432) and morphology (p<0.0001) than beef sires, whereas straws from dairy farms had significantly greater acrosome integrity (p=0.0043), CASA motility (p=0.0129), progressive motility (p=0.0243) and morphology (p=0.0271) than straws from beef farms. There was no significant difference in motility (p=0.1001) or progressive motility between dairy and beef sires (p=0.0804). There was no significant difference in acrosome integrity between beef and dairy sire (p=0.1959).

Investigation of field fertility outcomes on one dairy farm showed significant positive correlations between the flow cytometry viability assay and conception rate (r^2 = 85.3% and P = 0.025), and also the CASA % motile at 2 hours post thaw and conception rate (r^2 = 90% and P = 0.05).

70 semen samples from bull breeding soundness evaluations were evaluated in the same manner, as well as having manual assessment of morphology performed. Significant differences were present when on farm subjective motility assessments were compared with laboratory-based assessments (CASA motility and subjective), (p<0.0001) but not between CASA motility assessment in the lab and subjective assessment in the lab. There was no difference between morphological evaluations assessed by CASA or manually, despite CASA limitations in picking up sperm head abnormalities.

Multiparametric objective semen analysis in a Veterinary practice-based laboratory can offer additional information on the semen in use on farm (AI and natural service), to complement traditional subjective methods. This may allow investigation as to what factors impact on semen quality and which parameters may therefore be most important when selecting semen to use on farm.

General introduction

Low fertility in dairy and beef cows

The reproductive performance of breeding cattle is a key driver of sustainable beef and dairy production. In the national dairy herd, it is well established that optimum performance and profitability is achieved by maintaining a calving interval (CI) of 365 days (Esslemont, 2003). Every day CI increases over 365 days is estimated to directly cost the farmer £2.07, or more for lower yielding/ seasonal calving cows (Esslemont & Kossaibati, 2002). It is therefore economically important that dairy cows conceive on average by 85 days post calving. However, CI in the UK herd currently stands at around 418 days (Hudson et al., 2010). For 1,891,000 UK dairy cows (https://dairy.ahdb.org.uk/market-information/farming-data/cow-numbers/uk-cow-numbers/), this represents on average 53 days lost time or £110/cow/year, equating to

over £207,000,000 nationally. Beef herds also rely on good fertility performance. Cows becoming pregnant in their first breeding season and having a calving interval of 365 days are imperative to efficient suckler herd performance (Diskin and Kenny, 2016). The current calving interval in the UK beef herd is 394 days (Gates, 2013). There is therefore much room for improvement in the UK dairy and beef herds using AI and natural service.

Good fertility performance in breeding cattle is a multifactorial phenomenon and although changes in the environment, high production and management of cows have largely been blamed for the decline in reproductive efficiency in dairy cows, male factors of fertility should not be overlooked as the effects of semen quality are well documented but only modestly understood (DeJarnette, 2005).

Artificial Insemination

Artificial insemination (AI) is utilised extensively in the Dairy industry in Europe with 115176785 frozen doses produced annually (Thibier, 2000), but is still only used in 38.7% of Beef suckler herds in the UK, with only 12.6% of suckler cows served by AI (Telford et al., 2003). If AI is to be used more widely and with confidence by beef and dairy farmers they need to be assured of the quality of the product and it is the responsibility of the semen production centres to supply straws containing spermatozoa of good viability that produce acceptable conception rates if all other variables are managed correctly (Vincent et al., 2012). Once it has left the stud it is the responsibility of the courier and the inseminator/ farmer to maintain the quality of the semen through proper transport, storage and handling of the semen as this can have a negative effect on semen quality parameters and fertility following its use (Janett et al., 2007).

Historically in the UK the whole AI process from semen production through to insemination of the cow by the AI technician and analysis of bull performance was controlled by single organisations such as the Milk Marketing board (MMB), Hampshire Cattle Breeders and others. The current situation is very different, whereby semen can be procured by farmers from a number of semen companies, or indeed bought privately from other cattle breeders. The semen is stored on farm in private semen flasks, with varying degrees of monitoring and quality control. The increasing popularity of Do It Yourself Artificial Insemination (DIY AI) means that there is potentially less quality control over the delivery of semen into cows, and less central monitoring of inseminator performance. Thus deciphering the potential causes of variability in fertility performance on farms using DIY AI is difficult.

Advanced breeding techniques such as Embryo transfer (ET) and Ovum Pickup/ *In Vitro* Fertilisation (OPU/IVF), are particularly dependent on utilising semen of optimal quality as it has been shown that sperm from different semen lots of one bull or even straws from the same semen lot can differ in their *in vitro* fertilising capacity (Otoi et al, 1993). Therefore, to maximise *IVF* outcomes, batches and straws from one batch will need to be screened prior to use, allowing the selection of straws with best assessment results.

Natural service

With only 65-85% of bulls being classed as satisfactory potential breeders at breeding soundness examinations (Chenoweth and McPherson, 2016) it is essential that bulls to be used in natural service are evaluated as thoroughly as possible prior to use with respect to natural service. Bulls should be capable of depositing high quality semen in the correct place (Penny, 2009). The British Cattle Veterinary Association (BCVA) Bull breeding soundness evaluation includes objective measurements such as scrotal circumference and percentage of morphologically normal sperm, that have been shown to be correlated with fertility (Penny, 2009). The other components of the BCVA semen evaluation include assessment of mass motility (on a scale of 1-5) and individual progressive motility percentage. Both of these measures are subjective and are dependent on operator skill, experience and consistency and it has been shown that manual analysis is prone to within and between technician errors (Vincent et al., 2012). Computer Assisted Semen Analysis (CASA) systems provide precise and accurate information of measures of sperm motion characteristics and allows objective classification of a sperm population (Kathiravan et al., 2011) Research has shown that subjective assessments of semen motility are not comparable to measurements by CASA on the same samples (Broekhuijse et al, 2011). Therefore until veterinarians have built up a high level of experience the level of error is likely to be higher and the use of an objective and repeatable measurement of semen motility such as CASA should be considered as it is a powerful tool in objective measurements of semen motility (Vincent et al., 2012). It may not be economically feasible for veterinary practitioners to acquire CASA systems but being able to submit samples via the post to a central semen laboratory may provide a solution to repeatable and objective semen motility evaluation.

With as low as 65% of bulls being classed as satisfactory potential breeders at breeding soundness examinations (Chenoweth and McPherson, 2016), it is essential that bulls to be used in natural service are evaluated as thoroughly as possible prior to use. Bulls should be capable of depositing high quality semen in the correct place (Penny, 2009). The British Cattle Veterinary Association (BCVA) Bull breeding soundness evaluation (https://www.bcva.eu/system/files/CPD_documents/17_64.pdf) includes objective

measurements such as scrotal circumference and percentage of morphologically normal sperm that have been correlated with fertility (Penny, 2009). The other components of the BCVA semen evaluation include assessment of mass motility (on a scale of 1-5) and individual progressive motility percentage. Both of these measures are subjective and are dependent on operator skill, experience and consistency and it has been shown that manual analysis is prone to within and between technician errors (Vincent et al., 2012). CASA systems provide accurate information of measures of sperm motion characteristics and, therefore, allow objective classification of a sperm population (Kathiravan et al., 2011). Interestingly, subjective assessments of semen motility are not comparable to measurements by CASA on the same samples (Broekhuijse et al., 2011). In summary, until veterinarians have built up a high level of experience the level of error in such motility assessments is likely to be higher than when measurements of semen motility are objectively and repeatedly assessed using CASA (Vincent et al., 2012). It may not be economically feasible for veterinary practitioners to acquire CASA systems but being able to submit samples via the post to a central semen laboratory may provide a solution to repeatable and objective semen motility evaluation.

Chapter 1

Literature Review

Spermatogenesis

Spermatogenesis is the process that involves germ cell multiplication and differentiation resulting in the production of spermatozoa in the seminiferous tubules, released at the apical point of the Sertoli cell. The process takes 61 days to occur in the bovine and is composed of three distinct parts – spermatocytogenesis, meiosis and spermiogenesis (Staub and Johnson, 2018).

During spermacytogenesis, which begins in the basal compartment of the seminiferous tubules, spermatogonia undergo mitotic cell division, resulting in the formation of large primary spermatocytes, stem cells are renewed and more Spermatogonia produced (Staub and Johnson, 2018).

Primary spermatocytes then undergo the first division of meiosis to produce haploid secondary spermatocytes. Secondary spermatocytes divide again (2^{nd} division of meiosis) to produce the two haploid spermatids.

Spermiogenesis is the process whereby the spermatids undergo differentiation. Each sperm undergoes a number of changes, including condensation of the nucleus and organisation of the genetic material, acquisition of the acrosome in the head of the sperm and reorganisation of the mitochondria in the midpiece of the sperm. These changes are key to the sperm functioning normally once ejaculated and achieving its function as a self-propelled, penetrative enzyme containing male genome delivery system (Staub and Johnson, 2018). The tail of the sperm also develops with the excess cytoplasm moving caudally and being lost as a residual body which will get phagocytosed by the Sertoli cell. The fully developed Spermatozoon is then released into the lumen of the seminiferous tubule. At this stage the spermatozoon is non-motile and is transported passively from the seminiferous tubule to the Rete testis and then on into the head of the epididymis.

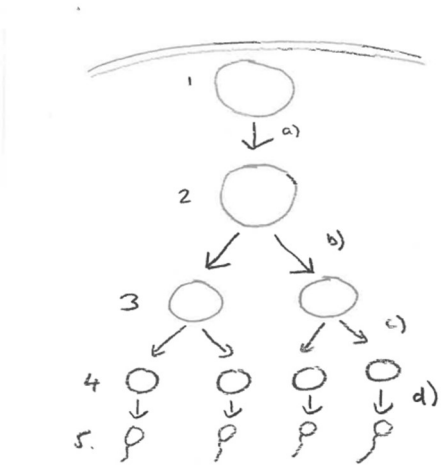


Fig 1 - Schematic representing the stages of spermatogenesis. Cell type are depicted by numbers 1-5, and the stages of the process by letters a -d. Spermatogonium (1), undergoes mitotic cell division (a), to become a primary spermatocyte (2). This undergoes first division of meiosis (b) to become a secondary spermatocyte (3), followed by the second division of meiosis (c) to form Spermatid (4). Spermiogenesis then occurs (d) resulting in the production of spermatozoa.

During its passage through the epididymis there are further changes in the sperm, which enable it to be progressively motile and be capable of fertilising an oocyte. There are changes to the plasma membrane and acquisition of species-specific proteins required for binding with the zona pellucida of the oocyte. Transport through the Caput and Corpus of the epididymis is under control of peristaltic contractions of smooth muscle, and therefore passage is relatively constant and independent of frequency of ejaculation. Sperm are stored in the Cauda epididymis prior to ejaculation and the contraction of these muscles only occurs at point of ejaculation. Therefore the length of time in the cauda epididymis can be dependent on frequency of ejaculation (Peters, 1995). The final maturation process – Capacitation - occurs post ejaculation and this is necessary prior to the Acrosome reaction occurring.

Post ejaculatory maturation

Capacitation occurs in the female reproductive tract and is the process whereby spermatozoa prepare to undergo the acrosome reaction, a pre-requisite for fertilisation. Capacitation results in increased membrane fluidity and alteration in Calcium channels in response to interaction with seminal plasma or exposure to the various parts of the female reproductive tract such as oviductal secretions (Burac and Birtoiu, 2014). The alterations to the membrane allow the influx of calcium ions required for changes in motility and initiation of the acrosome reaction. The motility of the sperm becomes hyperactivated at capacitation and results in 'whiplash' movements, necessary to achieve fertilisation. The most important change in spermatozoa after capacitation is gaining the ability to undergo the acrosome reaction in response to Zona pellucida sperm-binding protein 3 (ZP3) (Burac and Birtoiu, 2014).

The Acrosome Reaction is the process whereby the plasma membrane and outer acrosomal membrane fuse at multiple points over the head of the sperm. The resultant vesicles allow the release of acrosomal enzymes (Hyaluronidase and Acrosin), which enable the sperm to penetrate the cumulus cell layer surrounding the oocyte and then bind to the Zona Pellucida. On reaching the perivitelline space the sperm will fuse with the oocyte plasma membrane (sperm-oolemma fusion) followed by sperm incorporation, oocyte activation, pronuclear development and embryo cleavage (Sutovsky, 2018). The Zona Pellucida becomes impenetrable in response to the fertilising sperm, which causes calcium oscillations in the oocyte, resulting in exocytosis of the cortical granules, and a hardening of the zona pellucida,therefore making polyspermy unlikely, although in vitro situations can result in polyspermy in up to 45% cases (Coy and Aviles, 2010).

Transport of sperm within the female tract

Whether introduced via natural mating into the vagina (outer os of the cervix) or AI into the body of the uterus, the journey of the sperm in the female tract is long and tortuous. The female reproductive tract is filled with viscous fluid, dead ends and potentially hostile immune cells, such as neutrophils as well as Immunoglobulins (Miller, 2018). Movement is achieved through a combination of the midpiece, which contains spirally arranged mitochondria that produce the energy required for sperm movement, and the flagellum that propagates the propulsive waves initiated at the midpiece (Mortimer, 2000). The direction of travel of the sperm is controlled by a combination of chemotaxis (sperm travel in the direction of secretions from the oocyte e.g amino acids, peptides, lipids), thermotaxis (sperm travel towards a warmer environment as there is a temperature gradient between the isthmus and the ampulla) and rheotaxis (sperm travel against the flow of mucous in the uterus) (Taymour et al, 2014). The sperm provide most of the motility required to reach the site of fertilisation, but are also helped by the active motions of organs within the female

reproductive tract, and actually inanimate objects are able to reach the isthmus, (Taymour et al, 2014), therefore highlighting that sperm motility is not absolutely essential for the first part of the journey. Once into the isthmus sperm bind to oviductal epithelial cells where they will be retained and secretions will help maintain function, and these areas act as reservoirs of functional sperm and inherently lengthen the lifespan of the sperm (Miller, 2018). Sperm are gradually released from the reservoir in the isthmus, so that they can meet with the oocyte in the ampullae, the release is either in response to secretions from cumulus oocyte complexes or follicular fluid (Miller, 2018). There is therefore a relatively constant release of viable sperm that should be capable of achieving fertilisation. However sperm motility is essential for transport within the oviduct and fertilisation, where a state of hyper activated motility is required to achieve release of the sperm from the sperm reservoir on oviductal epithelium (Rodriguez Martinez, 2007) and also to penetrate the oocyte to achieve fertilisation.

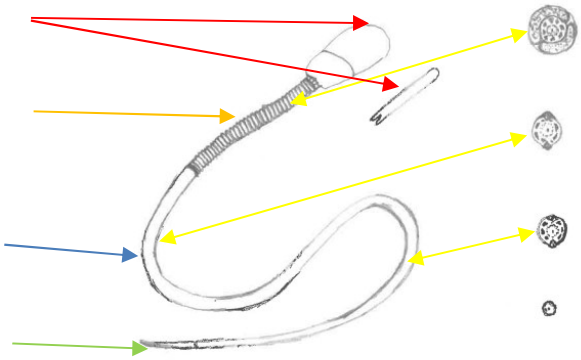


Fig 2 - Normal Sperm structure – parts of the normal structure of the sperm are depicted. Red arrow- Head of sperm (dorsal and lateral views), which is covered in its most anterior aspect with the acrosomal membrane. Orange arrow – Middle piece of the sperm – the mitochondria that provide the energy for movement surround the axonemal complex in this region of the sperm. Blue arrow – Principal piece – contains axonemal complex that contain microtubule doublets required for movement. Green arrow – End piece. Yellow arrows – connect cross sectional area structure with relevant section of the sperm.

Adapted from Chenoweth and Kastelic - Clinical Reproductive Physiology and Endocrinology of Bulls, 2007 in: Youngquist RS, Threlfall WR (eds), Current Therapy in Large Animal Theriogenology 2. Saunders Elsevier, Philadelphia, pp. 221–228

Artificial Insemination (AI)

Having been researched for over 2 centuries and in commercial use for over 75 years, AI is now utilised across the world to breed cattle in large numbers. The huge benefit of AI is that it allows rapid dispersal of valuable genes and allows dairy and beef farmers to rapidly improve the genetic quality of the stock they farm (Vishwanath, 2003). Reduced incidence of disease transmission was readily recognised as a major advantage of AI (DeJarnette, 2004). AI in the UK utilises cryopreserved semen rather than chilled fresh semen, which is used in other parts of the world such as New Zealand, to comply with quarantine periods required for infectious disease control and also breeding tends to be year round in the dairy sector in the UK and therefore semen is required to be stored for longer periods of time.

Collection of semen for AI

Semen collection in the bull will usually take place in Semen Processing Centres that may be owned by genetic companies or privately owned and bulls will reside here whilst semen is collected to supply market demand. In the UK there are two commercial studs in operation, Genus and Cogent, who own all the bulls in their care, and one privately owned stud who will collect and process semen at stud for private clients who will send their bulls to stud to be collected. It is also possible to collect semen on farm under license and send this to be processed by the private stud mentioned.

Bulls will have an appropriate semen collection regimen so that epididymal reserves of semen are depleted on each collection day. Therefore most mature bulls can be optimally collected if 2-4 ejaculates are collected twice per week, whereas in younger bulls 1-2 ejaculates are collected 3 times per week, due to mature bulls having greater epididymal storage capacity. Optimal collection regimes that ensure epididymal reserves are consistently depleted will minimise the number storage induced sperm abnormalities, including bent tails and detached heads in ejaculates (Schenk, 2018).

Semen processing and Cryopreservation

Successful cryopreservation of spermatozoa requires complete arrest or a ‘suspended animation’ in the development of sperm cells that would normally continue post ejaculation (Watson, 1995; Vishwanath and Shannon, 2000). With the best cell recovery of just over 50% following this process (Vishwanath, 2003), it is important to optimise the multiple steps involved in the cryopreservation process in order to maximise the percentage of spermatozoa that survive cryopreservation.

Post collection, semen is processed so that it can be stored in a frozen state for thawing and insemination at a later date. Semen is initially extended in diluent that has some key properties including

ability to maintain osmolarity/ buffer the solution; a source of lipoprotein or high molecular weight material to prevent cold shock; ability to provide cryoprotection, usually using glycerol; an energy source in the form of fructose or glucose, which also help with cryoprotection and antibiotics (Vishwanath and Shannon, 2000). The glycerol is not usually added in the initial dilution and will often be added once the semen is cooled to 5^0C, as it will equilibrate rapidly across membranes at this point (Vishwanath and Shannon, 2000). Cooling of semen from body temperature to 5^0C at a rate of <10^0C/ hour in the presence of protective agents will decrease the effects of cold shock (Medeiros et al., 2002). The process of filling and sealing straws occurs at 5^0C, with a standard dose per straw of between 10 and 20 million per straw, in either 0.25ml or 0.5ml plastic straws (Vishwanath and Shannon, 2000). The optimal rate for further cooling and freezing from 5^0C is 100^0C/ minute (Woelders, 1997), but practically this may not always be the case as static vapour cooling or freezing machines are unable to consistently operate at this rate for all straws (Vishwanath and Shannon, 2000). Once frozen semen is stored at -196^0C, and as long as this temperature is maintained, there should be no deterioration in quality for at least 5 years (Foote, 1978), and it is thought that quality will be maintained indefinitely at these temperatures (Vishwanath and Shannon, 2000).

Representative straws of each freeze code are randomly selected after cryopreservation and, at most AI centres, are thawed and evaluated by specially trained personnel (Amann and DeJarnette, 2012), the aim of which is to identify any substandard batches of semen so that they are not distributed to customers. Spermatozoa must survive the thawing procedure with normal morphology, an intact acrosome, DNA integrity, active mitochondria, and maintain forward progressive motility to traverse the female reproductive tract (Vincent et al., 2012). Some or all of these characteristics, dependent on the centre, are evaluated prior to release by AI centres. There is a significant cost in equipment and time to assess all parameters of every batch of semen produced. There is not complete consistency in how AI centres in the UK analyse their products prior to release (Subjective vs CASA), and which parameters are assessed, therefore purchasers do not always know the exact quality of the product that they are purchasing, just that it has passed minimum standards for release. These standards for UK centres are greater than 70% normal morphology, greater than 40% total motility and 30% progressive motility (if CASA is used) and a total sperm dose of 10-20 M/dose.

Frozen semen distribution

Once the representative straws have been evaluated and a batch classified as suitable for sale then they can be distributed to customers. This stage is critical in maintaining the quality of semen, as repackaging straws into quantities that have been ordered may require removing straws from liquid nitrogen and their exposure to ambient temperatures. This can impact on semen motility if for 15 seconds or more (Senger,

1980). The straws will then be transported to farm, where again they will be removed from liquid nitrogen and transferred to the on-farm storage flask, another potential point of temperature elevation in the straws.

Sexed semen

Sex sorting of bovine sperm has been possible for three decades (Vishwanath and Moreno, 2018), and in modern agriculture being able to determine the sex of the offspring of a mating will enhance the productivity of both dairy and beef herds. Dairy herds require herd replacement females from their genetically superior cows and heifers, but do not require the less valuable dairy bred male calves. Beef herds may benefit from selectively breeding female replacements from certain lines, whilst utilising sorted male semen to produce fattening animals. The sorting process is based on the difference in the DNA content of male and female sperm, and in cattle X sperm have approximately 4% more DNA compared with Y sperm (Seidel, 2014). The nuclear or chromosomal dye Hoechst 33342 allows X and Y sperm to be differentiated (Garner, 2006). The dye diffuses through a viable cell membrane and binds to the A/T base pairs within the minor groove of the DNA helix. Flow cytometry is then used to detect the difference in fluorescence when excited by a laser and sort male and female sperm by DNA content (Vishwanath and Moreno, 2018). The difference results in differing charges being applied to male and female sperm by the jet in air flow cytometer. As the sperm pass charged plates at the discharge point they are separated into differing streams for collection (Vishwanath and Moreno, 2018).

Another benefit of the sex sorting process is that dead and dying sperm, with compromised membranes are discarded in the process.

Sexing machines are capable of differentiating sperm at 95% accuracy but are usually set at 90% as the increased accuracy requires significantly more time (Seidel, 2014). Sexed sperm are currently packed at 2 million sperm per dose as the industry standard (Siedel, 2014). This is approximately a tenfold dilution compared with conventional semen, and this is one factor for the reduced fertility in the field of sexed semen compared with conventional, usually in the region of 10 percentage points, and hasn't been improved by increasing sperm dosage previously (DeJarnette, 2010; DeJarnette et al.,2011). Damage inflicted on the sperm by the sorting process may also lead to reduced fertility (Siedel, 2014); sex sorted semen will have gone through 20 steps compared with 3-4 steps in the production of conventional semen prior to cryopreservation. Causes of extra damage to sexed sperm include staining with Hoechst 33342, laser exposure, high dilution, elevated pressure and forces applied to sort the sperm and centrifugation that occur during the sexing process (Garner, 2006). However, in trials where sperm dosage was increased to 10 million to try and compensate for the drop in fertility, even though there was a small increase in conception rates by 6%, it was not comparable to conventional semen at low or high dosages (DeJarnette et al., 2011).

Therefore, recent work has refined the sex sorting process to minimise the damage inflicted on the sperm and increase the dose to 4 million sperm. This has resulted in a product called SexedUltra4M, which has shown performance comparable with conventional frozen thawed semen at 15 million sperm per dose (Lenz et al., 2016). Two important observations were noted from this trial: firstly, this was the first time a dose response was demonstrated using sex-sorted semen. Secondly, fertility rates between conventional semen and sex-sorted semen approached equivalence despite very different packaging doses. This is extremely promising to reduce the barriers to using sex sorted semen in dairy and beef herds. As well as improved performance in the field, there were also improvements in quality when assessed in the laboratory by CASA and Flow cytometry, where conventional and SexedUltra4M were comparable in terms of CASA motility and progressive motility and acrosome integrity, and in the 24 hour incubation test SexedUltra4M was significantly better than the conventional semen in terms of visual motility and acrosome integrity (Gonzalez-Marin et al., 2018).

On farm semen storage and handling

Frozen AI semen is conventionally stored submerged in liquid nitrogen in vacuum insulated flasks. Semen straws are routinely stored in coloured goblets within up to six cans per flask. The cans have handles attached which allow the cans to be raised to the neck of the flask to allow semen straws to be identified and removed prior to thawing. Following successful cryopreservation in the SPC and delivery to the client the semen should be handled appropriately so that semen quality is maintained. This entails maintaining the low temperatures required to preserve the quality of semen straws and avoid increasing temperatures in the neck of the flask to -79^{o}C, at which injury to the spermatozoa will occur (Diskin and Kenny, 2016). It was demonstrated that the temperature to which semen straws were exposed in tanks containing 'low levels' (14cm) of liquid nitrogen rose to -100^{O}C compared with -172^{O}C in tanks full of liquid nitrogen (Senger, P 1980). Maintaining liquid nitrogen at appropriate levels in the semen storage tank by routine monitoring and topping up with liquid nitrogen is therefore an essential task on cattle farms utilising AI.

Sperm motility and percentage of intact acrosomes were shown to deteriorate significantly in semen straws raised and lowered in a semen storage flask 480 times in a six-month period (Pace, 1978). This data equates to approximately 2.5 services per day, which may be relatively low number for large modern cattle farms, certainly in seasonal calving herds. For example a 350 cow seasonal calving herd could be serving 15 cows per day for the first 3 weeks of the service period. Although there are thoughts that when maintained at -196^{o}C sperm may maintain its fertilising ability indefinitely (Vishwanath and Shannon, 2000), on a working farm this is unlikely to be the case and the number of times that a semen straw is raised to the neck of the

flask and then returned into liquid nitrogen without being utilised may affect semen quality. Semen straws that were stored for between 1951 and 2400 days had more than a 1% decrease in probability of a conception occurring (Haugan, 2007), therefore whilst preserved in liquid nitrogen it may be that sperm do undergo some degree of damage. From this stems a recommendation of only purchasing quantities of semen that can be used in a reasonable length of time to minimise the risk of using semen of reduced quality (Senger P, 1980).

The potential impact of the issues above alongside the actions of the individual responsible for maintaining semen quality in storage can have a significant effect on semen motility and acrosome integrity (Janett et al., 2007). Therefore, ensuring all farm employees responsible for insemination are well trained in flask management and straw handling is important to maintain frozen semen at optimum quality.

Ensuring the semen flask remains efficient in its insulating capacity is important in maintaining temperatures for optimum semen storage. The key factor in maintaining this insulating capacity is the maintenance of a vacuum between inner and outer shells of the flask. Although well cared for flasks that are not exposed to physical damage may maintain this vacuum and performance over extended periods of time, it could be postulated that the risk of the damage occurring to flasks increases with time and therefore the older the flask the greater the potential for failure, as at some point all storage tanks will fail (Nebel, 2007).

Thawing of semen straws for insemination

Although straw thawing practices don't impact on the quality of semen remaining in the tank after that straw is removed they may certainly impact on the likelihood of a successful insemination occurring from the straw itself. The most appropriate straw thawing technique for 0.25ml straws received significant attention in the 1970's and 1980's when artificial insemination with fine French straws (0.25ml) was becoming commonplace. It was concluded that a thawing temperature of 38 (+/- 2^{o}C) for at least 25 seconds was preferable to shorter thaw periods of 8 seconds (Gaillard, 1982). More recent work has compared warm water thawing (33-35^{o}C) for 40 seconds with 'pocket' thaw (straw within paper towel in a pocket for 2 -3 minutes), with no differing effects on fertility rates (Kaproth et al., 2005). Thawing at ambient temperature vs 35^{0}C, did not impact on Non Return Rate in a New Zealand study (Vishwanath and Shannon, 2000). With the advent of sex sorted semen, thawing techniques for this product may be different to conventional semen. Cogent Breeding Ltd recommend a protocol for thawing of sexed semen of 37^{o}C for 40 seconds.

Semen Analysis

Semen analysis is performed in the cattle industry for two main reasons; either the assessment of semen collected as part of a bull breeding soundness evaluation or the assessment of semen in semen collection centres pre- and post-processing. Semen analysis is also performed in a research capacity in an effort to try and identify factors that may be correlated with field fertility. There are a number of tests used in semen analysis for the above reasons and these are discussed below.

Standard light microscopy

Standard light microscopy with the aid of phase contrast can be used to assess a number of properties of individual and populations of sperm. These include assessments of motility, either of undiluted samples where gross motility is evaluated under low power, or phosphate buffered saline diluted samples where individual sperm are assessed for progressive motility. Samples can also be evaluated by light microscopy equipped with phase contrast on smears following staining with nigrosin eosin, or as wet mount preparations using 10% neutral buffered formalin, for the presence of morphological defects. Defects that have resulted from disturbances (thermal stress, infection) in spermatogenesis may have an impact on sperm form, function and subsequent fertility; bulls with high proportions of the pyriform head defect resulted in lower embryo cleavage rates (Thundathil et al, 1999) . Decades of research and observations of sire fertility have demonstrated quantitative positive associations between ejaculate motility, morphology and field fertility (Harstine et al., 2018). These visual assessment methods are subjective in nature and are dependent on level of skill and experience of the operator. Such assessments are therefore prone to within technician and between technician errors (Vincent et al.,2012). It has also been shown that subjective assessments of semen motility are not comparable to measurements by CASA on the same samples (Broekhuijse et al, 2011).

Part of the problem in correlating laboratory results with field fertility results from inherent problems in the laboratory assays themselves. For a laboratory assay to be useful it must be objective, repeatable, accurate, rapid and inexpensive (Graham, 2001). This has led to the desire to develop more objective measures of semen assessment.

Computer Assisted Sperm Analysis (CASA)

CASA systems were first developed in 1986 (Davis and Katz, 1993). CASA systems consist of a microscope attached to a camera, a video frame grabber card and a computer (Kathiravan et al., 2011). Most systems use standard video acquisition rates between 25 and 60 Hz. Computer software algorithms then scan these image sequences to identify individual spermatozoa (typically by sperm heads rather than tails) and trace their progression across the field of view (Holt et al., 2007). Computer-assisted sperm analysis

provides the means for an objective classification of a given population of spermatozoa, usually a minimum of 400 sperm are analysed. Using digital images of each sperm cell's track, CASA machines are able to precisely analyse, by processing algorithms, the motion properties of spermatozoa (Verstegen et al, 2002). The commonly measured parameters are curvilinear velocity (VCL), the point to point track followed by the spermatozoa; the average path velocity (VAP), the average velocity of the smooth pathway that the sperm has taken; the straight line velocity (VSL), the average velocity of the straight line connecting the start and end points of the track. All the parameters are measured in micrometers per second. Amplitude of lateral head displacement (micrometers) and beat cross frequency (Hz) are also measured and routinely reported. Straightness (STR) is calculated from VSL/VAP ratio and Linearity (Lin) is the ratio of VSL/VCL. Individual motility characteristics (percentage motile, VSL and Linear velocity) have shown some correlation ($r > 0.68$) with competitive fertility index, a measure of relative fertility, but increased significance ($r>0.94$) was achieved when several measurements (% motile, % progressively motile, linear velocity, straight line velocity, linearity, track motility %), were combined to correlate with fertility (Budworth et al., 1988). Sperm can then be classified according to the motility data captured and analysed as either static, slowly motile, medium motile or rapidly (progressively) motile, dictated by the low and medium VAP cut off values used (Verstegen, 2002). Total motility and progressive motility evaluated with CASA have both been positively correlated with field fertility (Farrell et al., 1998 and Christenson et al., 1999). Also in an attempt to evaluate functional life-span *in vivo*, many laboratories use a 'stress test' or thermoresistance test (TRT) in which thawed sperm are incubated at 37 °C for 1 to 4 h before re-evaluation for motility (Amann and DeJarnette, 2012), and the need for thermoresistance test was verified (Vincent et al., 2012), when they were performing semen quality assessment in a semen production centre, to ensure only high quality semen is released.

Sample preparation is an important factor in producing reliable and repeatable results; sperm concentration, extender used to dilute semen and loading volume used are all important aspects which need to be maintained constant (Kathiravan et al., 2011). It is often essential to dilute the semen sample to a density where individual sperm tracks can be assessed as too high concentrations can result in false analysis due to multiple collisions of sperm cells (Rijsselaere et al., 2002). The diluent must not contain particles of similar size to sperm heads as this can influence results. Solutions such as Tyrode's albumin-lactate-pyruvate (TALP), are used for this purpose (Farrell et al., 1998). Dilution to a concentration of approximately 25–30 million sperm cells/ml has been determined as optimal for motility analysis using CASA (Farrell et al., 1998); this can be achieved by diluting one part semen to three parts diluent at normal concentrations of conventional sperm (20M/ 0.25 ml straw). The volume of semen loaded is dependent on the chamber used for analysis but is usually between 4 and 7ul (Januskauskas et al., 1999), and the depth of the chamber used for the assessment will vary dependent on models, but Microcell and Leja chambers are 20um deep. The

temperature of the slide for analysis should be set so that there is no impact on motility, and therefore 37^0C is recommended (Tardif et al, 1997).

Flow cytometry

A sperm cell consists of several membrane compartments (i.e. plasma membrane, acrosomal membrane, mitochondrial membrane) and optimal sperm function needs these membranes to be intact and physiologically active. Cryopreservation results in damage to the selectively permeable membranes of the sperm (Vishwanath 2003). Using laboratory techniques to establish the degree of damage and functionality of these membranes is possible, originally performed using fluorescent microscopy (Graham et al., 1990). An advantage of flow cytometry is that large numbers of spermatozoa can be assessed in a very short period of time. A total of 5,000 - 10,000 spermatozoa are normally analysed, which is substantially more than the total of 100 - 200 cells generally observed by microscopic analysis (Gillan et al., 2005). Assays that look at multiple functional aspects of the sperm have been developed that utilise flow cytometry in the analysis. Dependent on the assay being conducted, sperm are incubated with a fluorescent stain and the amount of each stain that is taken up is measured by a flow cytometer (Graham, 2001). The degree of uptake of the stain is assessed when the sperm flow individually within a fluid channel. When these are exposed to laser it will cause any fluorescent stains associated with the sperm to fluoresce. Photomultiplier tubes, associated with filters which allow only specific wavelengths of light to pass through them, permit the determination of whether a specific droplet contains a cell or not, and if the droplet contains a cell, which specific stains that cell is associated with. Not only can the presence or absence of fluorescent stains associated with cells be determined, but the amount of stain associated with each cell can be quantified (Graham, 2001). Flow cytometry allows the rapid and objective assessment of very large numbers of cells, which is preferable compared to fluorescent microscopy which is both subjective and time consuming, and counts are possibly not representative (Gillan et al., 2005). In a few minutes, the flow cytometer can acquire data on all subpopulations within a sample, making it ideal for assessment of heterogenous populations, such as spermatozoa. Flow cytometry is now applied to semen evaluation of traits such as cell viability, acrosomal integrity, mitochondrial function, capacitation status, membrane fluidity and DNA status (Gillan et al., 2005).

Viability assessment – assessment of cell viability is performed through incubation of sperm cells with propridium iodide (PI), which if the cell membrane is damaged will be capable of entering the sperm and binding to DNA. This will then fluoresce red when passed in front of a laser. It is also possible to incubate with a combination of dyes e.g. PI and CFDA or PI and SYBR 14, so that non-viable sperm fluoresce red and viable sperm fluoresce green. The proportion of viable sperm identified with flow cytometry within bull semen has shown to be correlated with field fertility (Januskauskas et al., 2003). More subtle membrane

changes, indicative of early modifications of the position of phospholipids such as phosphatydilserine (PS) can today be examined by Annexin-V staining in bull and boar semen, with a relatively important correlation to fertility (Januskauskas et al. 2003).

Mitochondrial status – a number of stains are available to evaluate the function of mitochondria in the sperm. Rhodamine 123 is actively transported into respiring mitochondria and fluoresces green (Graham, 2001), however, it does not distinguish between mitochondria exhibiting differing respiratory rates. This information can be elucidated if the stain JC-1 is used. As this stain accumulates in the active mitochondria it forms aggregates which then fluoresce orange, thereby distinguish between sperm of high and low mitochondrial activity (Thomas et al, 1998). The proportion of sperm with high mitochondrial activity was correlated with semen motility, but wasn't correlated with fertility (Hallap et al., 2005).

Acrosomal Integrity – plant lectins labelled with fluorescent probes are the common way to evaluate the acrosomal status of sperm. Pisum sativum agglutinin (PSA) is a lectin from the pea plant that binds to a-mannose and a-galactose moieties of the acrosomal matrix. Intact acrosomal membranes do not allow PSA to enter and therefore only disrupted acrosomes take up the fluorescent probe. The peanut lectin, Arachis hypogaea agglutinin (PNA), is an alternative lectin used to evaluate acrosomal integrity. PNA works in a similar manner to PSA, however, PNA exhibits less non-specific binding to other areas of the sperm cell, than does PSA. Acrosome integrity was assessed on sperm stored by different insemination technicians and correlated with the non-return rate of animals they inseminated (Janett et al., 2007).

These tests will only inform the operator of the current status of the acrosome and do not provide any information on the functionality of the acrosome and whether the acrosome is still capable of undergoing the acrosome reaction. The acrosome reacted sperms can be measured by assessing whether the acrosome reaction (AR) can be induced by calcium ionophores (Birck et al., 2010) or glycosaminoglycans (Januskauskas et al., 2000a) among other effectors. The proportion change in acrosome reacted sperm pre- and post- induction of the reaction with the effector is then used to produce an acrosome reaction (AR) index. Concurrent use of the acrosome specific probes alongside the sperm membrane/ viability stains allows four populations of sperm to be identified, live or dead and acrosome-reacted or not. The proportion of live, acrosome reacted sperm, post incubation is the index that yielded the best significant predictive values on field fertility among the 12 indices considered (Birck et al, 2010).

Reactive Oxygen species (ROS) - ROS are functionally important in driving processes associated with sperm capacitation and the acrosome reaction. However, when ROS production exceeds the sperm's limited antioxidant defences, often provided by seminal plasma a state of oxidative stress is induced, characterized by peroxidative damage to the sperm plasma membrane and DNA strand breakage in

the sperm nucleus. The freeze/thaw process involved in AI is known to induce ROS in sperm samples. The level of ROS can be assessed by incubating semen samples with the lipophilic probe C_{11}BODIPY$^{581/591}$ (BODIPY), where the level of lipid peroxidation and exposure to reactive oxygen species (ROS)can be monitored by the shift from red to green fluorescence, detected by flow cytometry (Brouwers and Gadella, 2003).

Sperm DNA integrity – the importance of DNA quality and its resistance to damage and denaturation was demonstrated (Eid et al., 1994) when sperm from low and high fertility bulls could initiate fertilization, but the resulting embryos had differing performance in terms of embryo development rate. It is likely that relatively high numbers of sperm from some low fertility bulls contain genetic abnormalities which impede normal embryonic development. Some of these abnormalities affect the overall structure of spermatozoal chromatin and can be detected using flow cytometry. By evaluating the susceptibility of chromatin to denaturation when a population of sperm are incubated under denaturing conditions, it is possible to detect the level of such abnormalities in the overall structure of spermatozoal chromatin post incubation. This is called the 'Sperm chromatin structure assay' (SCSA) and uses Acridine Orange (AO) as its fluorescent probe. AO will bind with double stranded DNA and fluoresce green, whilst when bound to single stranded (denatured) DNA, it will fluoresce red. Flow cytometry can be used to decipher the ratio of red and green fluorescence and therefore the level of DNA denaturation. SCSA was used to correlate decreasing sperm quality, in terms of the level of DNA fragmentation post incubation demonstrated by increasing red fluorescence, with significantly poorer fertility post AI (Waterhouse et al, 2006).

Other assays of sperm function - There are other laboratory assays that are primarily research tools and would be difficult to use in veterinary practice or even in AI centres for routine semen analysis pre-release and therefore are not discussed in detail, but in name include Hypo osmotic swelling test (HOST), Zona Pellucida penetration assay, In vitro fertilisation outcomes, cervical mucous penetration test.

Parameter	r/r^2	Reference
Subjective motility	0.52 – 0.59 0.67 0.6	Januskauskas et al., 2003 Gillan et al., 2005 Januskauskas et al., 2000b
Total Motility (TM)	0.53 0.68 0.43-0.61	Correa et al.,, 1997 Budworth et al., 1998 Januskauskas et al., 2003
Progressive Motility (PM)	0.94 0.34 – 0.68	Budworth et al., 1988 Farrell et al., 1998
Viability	0.68 0.58 0.64 0.64	Januskauskas et al., 2003 Christensen et al., 2011 Gillan et al., 2005 Januskauskas et al., 2000b
Mitochondrial Activity	0.07	Sellem et al., 2015
Acrosome integrity	0.48 0.22 0.52	Janett et al., 2007 Christensen et al., 2011 Correa et al., 1997
Morphology	0.59 0.22 -0.76	Correa et al., 1997 Phillips, 2004 Gillan et al., 2005

Table 1 - Summary table of the semen assessment parameters involved in our study and the correlation with field fertility in the literature. For all results $P < 0.05$.

Not all the objective assessments mentioned previously are routinely utilised as pre –release screens of AI semen by all breeding companies; however, a number of institutions do use a combination of CASA and flow cytometry to some degree. Flow cytometry has been implemented in four Danish bull studs to evaluate cell membrane viability, as it was concluded that flow cytometric determination of sperm concentration and viability can be used to improve semen assessment by AI studs and result in a better quality control (Christensen et al., 2005).

The minimum acceptable level of post thaw motility for straws to be released for inseminations was 50% in a Danish stud, assessed by subjective microscopy, where 8.3% of frozen thawed straws were classified as unsatisfactory (Christensen et al., 1999). This is relatively high cut off value compared to other standards in the world, and even if subjective assessment is not totally accurate, they are likely to be discarding the

semen of low motility (nearly 10% of batches), but also some semen of adequate motility. With a more objective assessment of motility such as CASA it may be possible to reduce this threshold slightly so that semen of adequate fertility may be retained and not discarded.

The minimum standards for both CASA and flow cytometry in a North American breeding company were compared to subjective assessments by laboratory technicians and the degree of agreement between them assessed (Vincent et al., 2012). There was agreement between subjective and objective assessments in passing 47.6% of straws and rejecting 29.8% of straws, however in 22% of straws there was disagreement as to the suitability of the straw for use in the field. however if the CASA and Flow cytometry based decisions had been used, the semen released would have been of higher fertility based on discarding more semen with low FERTSOL scores.The values used as minimum standards are shown below.

Parameter	Post Thaw	After 2 hours stress
Total Motility (%)	40	35
Progressive Motility (%)	15	10
Intact Acrosome (%)	66	61
Membrane Intact cells (%)	40	40
Mitochondrial Activity (%)	40	45

Table 2 - Pre-release minimum standard CASA motility and Flow Cytometry parameters for breeding company Semex (Vincent et al., 2012).

Although not published the author has received communication from two UK based commercial studs as to their pre-release standards for semen produced in their studs. Only one of the studs utilised flow cytometry to evaluate membrane intact cells.Personal communications with two breeding companies in the UK are summarised in the table below.

Parameter	Post Thaw
Total motility (%)	40 (both)
Progressive Motility (%)	30 (both)
Membrane intact cells (%)	40 (one)
Normal Morphology (%)	70 (both)

Table 3 - Pre-release minimum standards for UK based breeding companies.

Natural service

As described previously natural service is more widely used than AI in the beef suckler herd in the UK, and therefore the management and fertility of bulls is of paramount importance to these producers (Parkinson, 2004). Targets for suckler herd fertility can vary, but in highly fertile cow herds, ⩾70% of cows should be pregnant after the first 21 days of the breeding season (Barth, 2018). High performing beef suckler farmers in the UK would expect their mature stock bulls to perform with 50 females, and get 90% pregnant within 9 weeks, with 60% of these in the first 3 weeks (Caldow et al, 2005). Therefore, the requirement for optimal bull fertility is essential, however there are obviously female factors involved in achieving good fertility too, and a highly fertile bull will not be able to overcome these, but these are beyond the scope of this discussion. In a given population of bulls it is likely that 20- 40 % of them will have reduced fertility, although few should be classed as infertile (Kastelic and Thundathil, 2008). Accurate identification and appropriate management of these subfertile bulls is key to avoiding poor herd fertility performance. There are a number of factors that influence the performance of a bull in a natural mating scenario, including libido, physical fitness and semen quality (Parkinson, 2004). The latter two assessments are routinely performed in the British Cattle Veterinary Association (BCVA) bull breeding soundness evaluation, whilst tests to assess libido as described (Parkinson, 2004), are not routinely performed in the UK.

Bull Breeding soundness evaluations (BBSE)

Although bull breeding soundness evaluations have been performed for some time - the Society for Theriogenology (SFT) published its first set of guidelines for interpretation of BBSE in bulls in 1983 (Higdon et al., 2000), it is only relatively recently that there has been a standard format for breeding soundness evaluations in the UK. This involves a physical examination of the bull, focusing on the external and internal reproductive organs; the most relevant of which is scrotal circumference as this is highly correlated with paired testes weight, which is correlated with daily sperm production and semen quality (Barth 2007). The amount of tension applied to the scrotal tape by practitioners whilst assessing scrotal circumference can introduce inconsistencies, and therefore this has resulted in the development of spring-scale attachments on scrotal tape (ReliaBull ^{TM}) that mean consistent levels of tension are applied as the testicles are being measured.

The second aspect of the assessment is semen collection and evaluation. Semen is usually collected via electroejaculation (EEJ), as this is a safer method for bulls not halter trained or trained to an Artificial

Vagina (AV), although semen collected with an AV is likely more representative of the semen that cows are inseminated with in a natural mating situation. Semen evaluation has three main components and in the UK, is based on the certificate and guidelines produced by the British Cattle Veterinary Association (BCVA), developed by Penny in 2009. The first assessment is grading the gross motility of undiluted semen on a clean warmed slide under low power on a scale from 1 - 5. This gives an indication of the density and motility of the sample, but is greatly influenced by density (Penny, 2009). Progressive total motility is then assessed on a sample diluted with pre-warmed Phosphate Buffered Saline (PBS) under medium (x 200-400) power, on a heated stage and ideally under phase contrast (Penny, 2009). The result is given as a percentage of progressively motile sperm. A minimum standard for progressive motility is set at 60% (Penny, 2009). The final aspect of semen evaluation as part of the BBSE is the percentage of morphologically normal sperm. A smear stained with nigrosine- eosin is prepared and evaluated using phase contrast at high power (x 1000) under oil immersion. A count of a minimum of 100 sperm is made. The threshold for normal morphology is 70%. In general fertility will be decreased if more than 30% sperm are morphologically abnormal or more than 20% exhibit head defects (Barth 2007).

The assessment of sperm morphology of AI semen post thaw and semen collected as part of bull breeding soundness evaluations is performed in the same manner and categorisation of sperm abnormalities is also the same. Classifications of sperm abnormalities have developed over the years from initially being described as primary defects (those which occur in development within the testicle) and secondary defects (those which occur in storage/ development within the epididymis), to major defects (those which have a significant effect on fertility and include mainly sperm head and midpiece abnormalities, proximal cytoplasmic droplets and single defects present in high percentages) and minor defects (looped tails, detached heads and distal cytoplasmic droplets) (Parkinson, 2004). Currently the classification system is based on whether the defect means that the sperm fails to reach the oviduct or fails to penetrate the zona pellucida, or whether increasing the dose can compensate for defects which are thus termed ‘compensable’ defects. Defects which allow sperm to reach the oviduct and penetrate the zona pellucida but lead to failure of cleavage or the production of a non-viable embryo are termed ‘non-compensable’, as increasing the dose of sperm in these cases does not result in improvements in fertility (Barth, 2007).

The BBSE assessments of sperm quality are subjective and, therefore, operator skill, experience, equipment quality and level of potential bias may all play a part in how the samples are classified. Also, as skill, operator experience and equipment quality will play a key role in how well morphological evaluation of semen is performed, it may be that this is not performed consistently to a high standard amongst veterinary surgeons, as is the experience of Barth (2018), who identified serious deficits in slide preparation,

microscopes and microscopical skills of practitioners. This was based on a number of workshops being run by Barth for cattle practitioners across the globe. In Australia part of the solution to the inconsistencies of the veterinary practitioner's ability and the subjectivity of the assessments has been to set up a semen evaluation service in centralised well equipped semen assessment laboratories. These would utilise more sophisticated technologies such as CASA and differential-interference contrast (DIC) microscopy (Lorton, 2014). Such laboratories could provide services such as BBSE sperm morphology assessments, assessment and quality assurance (QA) for frozen/chilled semen, investigation of infertility problems, collaborative and clinical research and training of A.I. industry personnel (Chenoweth, 2016). Clearly, well-equipped multi species diagnostic laboratories specialising in semen analysis are needed everywhere (Barth, 2018).

All the BBSE's assessments of sperm quality are subjective and therefore operator skill, experience, equipment quality and level of potential bias may all play a part in how the samples are analysed, whereas measures of density and motility, as well as numerous functional tests can be objectified using technology such as CASA and flow cytometry, therefore removing the subjectivity form these assessments.

There are multiple ways in which semen can be assessed, and a number of studies have shown that certain parameters have been correlated with fertility and not in others; therefore, there is not one parameter that consistently predicts fertility with confidence and therefore we will be using multiparametric approach to semen analysis using CASA and flow cytometry in this study.

Study aims

The questions that this thesis aims to address are:

1) How do dairy and beef cattle farmers in North Yorkshire that utilise AI receive, store, handle and thaw frozen AI semen?

   Semen leaves semen production centres (SPC) having satisfied certain pre-release quality criteria, but from that point onwards there may be little control or regulation as to how the semen is handled if farms carry out their own AI (DIY AI). By the time the semen is finally deposited in the cow at insemination it may have been in transit through a number of flasks, been stored for a number of years, and been stored in various degrees of submersion in liquid nitrogen. Thus management practices on the farm where the semen is to be used can significantly impact on the quality of the

semen before and when it is finally used to inseminate a cow. An investigation into the current practices in place on dairy and beef farms in North Yorkshire for semen storage and handling was carried out by use of a questionnaire designed to establish:

a. The semen storage and flask maintenance practices in place to maintain the quality of the semen that had been delivered.
b. The semen handling practices utilised during the insemination process, to establish what risk factors may impact on semen quality.

By investigating the practices above the specific aim was to determine the risk of semen quality being negatively affected through issues with storage and handling post release from the semen production centre. Outputs are descriptive statistics of the storage and management practices employed, to then also determine how consistently farmers treat purchased semen in storage and during the insemination process. This first aim will be covered in experimental chapter 3.

2) What proportion of AI semen tested after transport and storage on beef and dairy farms in North Yorkshire is still above the minimum pre-release standards for conventional semen of North American and United Kingdom cattle breeding companies?

3) Can we define semen quality for insemination more consistently with the use of the combination of a combination of Computer Assisted Semen Analysis (CASA) and Flow Cytometry (FC) in veterinary practice), and which parameters are altered by sex-sorting, bull type or farm type?
The normal ranges for the CASA and FC parameters determined in veterinary practice will be established from testing of both conventional and sex sorted semen collected from storage on North Yorkshire dairy and beef farms. Results will then be compared with existing data available in the literature to begin to 'validate' the laboratory's logistical processes and protocols. Results will also be analysed after categorising the straws by having undergone sex sorting or not (conventional), type of bull (Beef versus Dairy) and farm origin (Beef versus Dairy).

4) Would a laboratory utilising the combination of CASA and FC for semen analysis be of use to practising cattle vets doing on farm BBSE's, therefore do these analyses compare with and enhance the conventional breeding soundness evaluation?

The normal ranges for CASA and FC parameters will be established for samples of fresh extended semen, collected as part of an on-farm bull breeding soundness evaluation. the specific aims of this study are

to determine: a) The relationship between on farm subjective assessments of motility, performed as part of an on-farm bull breeding soundness evaluation, with objective measures of motility established utilising CASA in the laboratory. b) In addition, the level of loss of sperm motility from bull-side evaluation immediately post-collection to CASA evaluation in the laboratory 12-18 hour later will be evaluated subjectively by the laboratory technician and objectively using CASA. And c) the relationship between automated morphological assessment of sperm utilising CASA in the laboratory and the human assessment of sperm morphology using visual examination of stained slides under phase contrast microscopy (PCM).

5) Which CASA and Flow Cytometry parameters are correlated with field fertility?

AI straws from multiple bulls utilised on one seasonal calving North Yorkshire dairy farm will be assessed by CASA and FC and the results will be correlated to the conception rate achieved for each bull using farm data, thus determining relationships of sperm measurements with fertility in the field.

Chapter 2

Materials and methods

1. Farm Recruitment

Farms that were members of either the Bishopton Veterinary Group dairy or beef herd health schemes (pro-active schemes run to maximise health and productivity of beef and dairy herds) were invited to take part in the study. If AI was used in the herd, the AI semen evaluation was offered. If Natural service was the predominant method then at the bull annual/ routine pre-breeding soundness evaluation, semen collected was extended, chilled and transported to the laboratory at Bishopton Veterinary Group for further testing. Veterinary surgeons working at practices that were part of XL Vets were invited to submit semen samples to the study from breeding soundness evaluations performed on their farms. This would help evaluate the process and protocols put in place for submitting fresh, chilled and extended semen.

2. Semen storage and handling questionnaire

Farms who had AI semen straws taken for analysis (the highest use AI bulls in their flask) were asked to complete a questionnaire to provide information relating to on farm semen storage, handling and liquid nitrogen monitoring. The questionnaire was completed at the time of semen straw collection where possible, but if not possible this was completed retrospectively via telephone. The questionnaire is shown below.

FARM DETAILS

Name:

Farm Name:

Farm Type: BEEF ☐ DAIRY ☐ MIXED ☐

Herd status: COMMERCIAL ☐ PEDIGREE ☐ MIXED ☐

Total Number of breeding females:

Breeding Policy: AI only ☐ AI and BULL ☐ if mixed - % of pregs/yr resulting from AI:

Breeding season: ALL YEAR ROUND ☐ SEASONAL ☐

Approximate number of serves per month:

JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC

Number of trained AI technicians:

NAME	DATE OF TRAINING	% SERVICES CARRIED OUT

SEMEN STORAGE AND HANDLING

Location of flask: **Age:** **Make:** **Size:**

Nitrogen top up service in place: NO ☐ YES ☐ if yes by whom:

Do YOU routinely dipstick the flask: NO ☐ YES ☐ if yes, how often:

Number of cans in the flask:

Method of thawing: AIR ☐ WATER ☐ AUTO. STRAW THAWER ☐

If water used, temperature:

Duration of thaw:

Straw splitting practised: YES ☐ NO ☐

SEMEN – Bull 1

Name:

Identification: size: ¼ cc ☐ ½ cc ☐ colour:

Cost: <£10 ☐ £10-£20 ☐ £20-£30 ☐ >£30 ☐

Sexed: yes ☐ no ☐

Proof: young-unproven ☐ young- genomic/unproven ☐ progeny tested ☐

Use: heifers ☐ first line ☐ sweeper ☐ specialist/ET ☐

Location/Can Number: Number of different sires in can:

Number of straws currently in storage:

Date of entry into flask:

Mode of delivery: rep ☐ company courier ☐ other ☐ if other, please state;

Total number of straws purchased:

Company of origin:

SEMEN – Bull 2

Name:

Identification: size: ¼ cc ☐ ½ cc ☐ colour:

Cost: <£10 ☐ £10-£20 ☐ £20-£30 ☐ >£30 ☐

Sexed: yes ☐ no ☐

Proof: young-unproven ☐ young- genomic/unproven ☐ progeny tested ☐

Use: heifers ☐ first line ☐ sweeper ☐ specialist/ET ☐

Location/Can Number: Number of different sires in can:

Number of straws currently in storage:

Date of entry into flask:

Mode of delivery: rep ☐ company courier ☐ other ☐ if other, please state;

Total number of straws purchased:

Company of origin:

Data collected were collated in a master spreadsheet and then categorised for each of the questions asked. The categories were as follows:

- Beef or Dairy Farm: 1= Beef; 2 = Dairy
- Beef or Dairy breed bull: 1 = Beef; 2 = Dairy
- DIY or Technician AI service: 1 = DIY; 2 = Tech
- How many trained DIY AI staff: 1=1; 2=2; 3=3; 4 > 3

As consistency in management of the flask and the handling of semen is important in maintaining semen quality, the greater the number of personnel involved in the handling of the flask and semen within the flask could potentially increase the risk of poor handling practices being utilised, or inconsistencies between the individuals on the farm. Therefore the number of trained AI technicians in use on all farms was categorised.

- Number of AI serves per month: 1 < 50 serves; 2 = 50-99 serves; 3> 99 serves.
- Herd Size: 1< 100 cows; 2 = 100 – 299 cows; 3 > 299

These two questions relate to the frequency of semen straws being elevated to the neck of the flask to allow straws to be removed, and therefore putting other straws at risk of elevated temperatures.

- Age of semen storage flask: 1 < 5 years; 2 = 5-9 years; 3 = 10-14 years; 4 > 14 years.
- How often do farm staff check liquid Nitrogen levels: 0 = Never; 1 = every week; 2 = every 2 weeks; 3 > 2 weeks between checks.
- How frequently do the company responsible 'top up' the flask on farm: 1 ≤ 1 month; 2 1- 2 months; 3 > 2 months.

As a proxy for cumulative damage and potential loss of insulating performance of the flask, we recorded the age of the flask and how often liquid Nitrogen levels were checked by farm staff and by the person responsible for flask top up

- Temperature of water used for thawing straws: 1 = 35 – 35.9 ^{0}C; 2 = 36 – 36.9^{0}C; 3 = 37 – 37.9^{0}C; 4 = 38 – 38.9^{0}C.
- Length of time in water bath when thawing straws: 1 = 0-10 seconds; 2 = 11 – 20 seconds; 3 = 21-30 seconds; 4 > 30 seconds.

Although the method of thawing employed on farms would not affect the semen quality parameters measured in this study, we collected the data for completeness and to assess the variation in thaw method between farms. The temperature of the water used to thaw conventional semen straws was recorded.
The length of time that the straws were left in the water to thaw was also recorded.

3. Collection of AI straws and preparation for analysis

AI bulls for analysis were selected when the farm had bought/used over 25 straws of that batch of semen from a particular bull. Straws (1 straw per bull) were collected from the farm by vets or the veterinary practice breeding technician and were transported back to the laboratory in a semen transport flask following the AI semen collection protocol, so that the cold chain from farm to laboratory was maintained. Semen was stored in liquid nitrogen semen storage flasks prior to analysis and liquid nitrogen levels were monitored daily whilst in storage until analysis was run.

Straws were removed from the liquid nitrogen storage flask and immediately placed in a water bath at 35^0C for 30 seconds, based on the guidelines used in delivery of the DIY AI course run at the practice. On removal from the water bath the straws were dried in paper towel and kept within the paper towel to prevent cold shock. The end of the straw was cut with scissors and a stylet used to dispense the semen into a pre-warmed 1.5ml microcentrifuge tube. Immediately 50ul of the semen was placed into 150ul of pre-warmed solution at 37^0C of Easybuffer B (IMV Technologies) and incubated for 10 minutes at 37^0C. The remaining contents of the straw were kept in the initial microcentrifuge tube and labelled with the bull details. This was kept at room temperature until all the further analysis had been performed.

Thermoresistant stress test - If performing a thermoresistant stress test (TRT), a further aliquot of the post-thaw non-diluted semen sample was incubated in a microcentrifuge tube for 2 hours at 37^oC. Following the incubation 50ul of this sample was added to 150ul of Easybuffer B and incubated for a further 10 minutes.

4. Collection of fresh semen and preparation for analysis

Fresh semen was collected from 70 stock bulls at routine bull breeding soundness examination by electroejaculation using Lane manufacturing Pulsator IV probe and collected into a prewarmed semen collection vessel. Once a sperm rich, presumed to be a representative, ejaculate had been collected and analysed for mass and progressive motility under standard microscopy at bull-side, it was diluted 1:9 in pre-warmed (37^0C) BIOXcell® (IMV Technologies) animal protein free bovine semen preservation medium. The sample was then allowed to cool to ambient temperature for 30 minutes prior to being placed in a polystyrene insulated transport box containing frozen ice bricks. The one sample per bull was then transported back to the laboratory for analysis. XL Vets member practices who were submitting fresh semen samples followed the same collection and processing protocol. The chilled extended sample was posted to arrive at the laboratory by 9 am the following morning. On the submission form the referring veterinary

surgeon recorded volume and appearance of the semen sample, gross motility score (1-5) and progressive motility score.

Once at the laboratory, the extended chilled semen was removed from the polystyrene box. 50ul of the semen was placed into 150ul of pre-warmed (37^0C) Easybuffer B (IMV Technologies) in a micro centrifuge tube and incubated for 10 minutes at 37^0C. This sample was then used for CASA motility analysis and also subjective motility assessment by the laboratory technician. The remaining extended chilled semen was kept at ambient temperature in the laboratory and this sample was utilised for the Flow cytometer analysis.

5. **Computer Assisted Sperm Analysis (CASA)**

The CASA system utilised for motility, density and morphology assessments was the Hamilton Thorne Ceros II, equipped with Animal Breeders Software. The CASA software settings for bull sperm analysis were: Min total count = 400; Camera – Exposure= 8Ms; Gain = 300; Integrate Time (Ms)= 500; Cell Detection – Elongation Max (%) = 70; Elongation Min (%) = 3; Head Brightness Min= 124; Head Size Max (um^2)=100; Head Size Min (um^2) = 5; Static tail filter = False; Tail Brightness Min=74; Chamber – Capillary correction = 1.3; Chamber Depth (um)=20; Chamber type = Capillary; Illumination – Max Photometer = 65; Min Photometer = 60; Kinematics – Progressive STR (%) = 50; Progressive VAP (um/s) = 45; Slow VAP(um/s) = 20; Slow VSL (um/s) = 30; Static Algorithm = Length; Static VAP (um/s) = 4; Static VSL (um/s) = 1; Static Width multiplier = 0.5; Morph – DMR Confidence (%) = 50; DMR Droplet to tail end Max (um) = 7; DMR Tail Length Max (um) = 20; Droplet Confidence (%) = 50; Droplet Distal Distance Min (um) = 4; Droplet Proximal Head Length (um) = 10.5; Min Tail Length (um) = 8; Tail Bend Angle Averaging Length (^{0}) = 5; Tail Bent Confidence (%) = 70; Tail Coiled Angle Min (^{0}) = 180; Tail Coiled Confidence (%) = 80; Tail Confidence (%) = 20; Video Capture – Frame Capture Speed (Hz) = 60; Frame Count = 30.

Following manual agitation of the sample, 3ul of the 10-minute incubated Semen/ Easybuffer B mixture was aspirated by pipette and transferred into a warmed standard count analysis chamber (Leja). CASA analysis was initiated once the sample had stopped 'flowing' across the chamber due to capillary action. Adjustments to the configuration and brightness settings were performed where appropriate to provide the most accurate analysis. Once focus was optimal the protocol for capturing fields was started. Videos were captured (30 frames at 60 frames/second) in a clockwise direction until a minimum count of 400 sperm tracks had been captured. The captured videos were analysed immediately for accuracy i.e. ensuring the computer had been accurate in its identification of sperm and no-sperm particles. If inaccurate

then the data were deleted and the process repeated. The outputs in terms of motility parameters were based on means of the fields captured to provide sufficient sperm tracks.

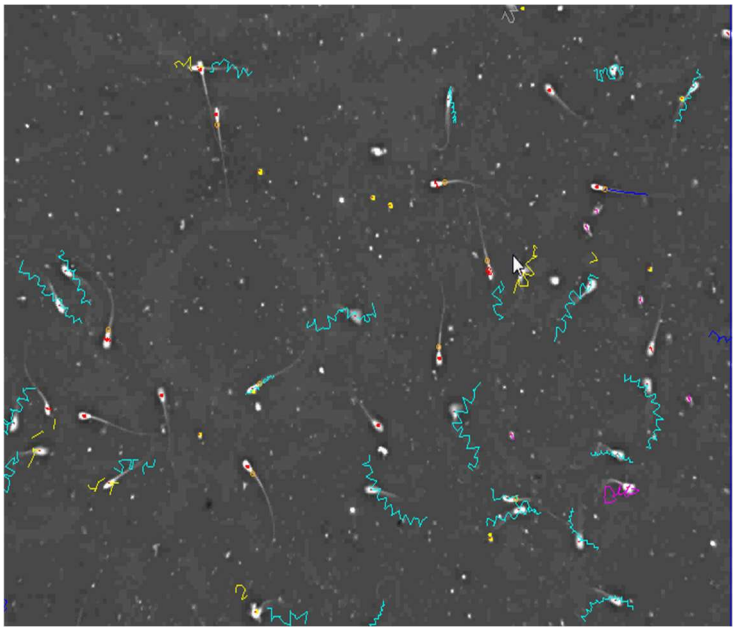


Fig 3 - Screenshot of a video captured as part of CASA motility analysis. The blue tracks indicate Progressive Motility, whilst green tracks indicate slowly motile sperm. Static sperm are identified with a red dot in the head of the sperm.

CASA analysis was utilised to provide an automated assessment of the percentage of morphologically normal sperm, for all samples submitted fresh and frozen. The abnormalities detected were not recorded in the final results spreadsheet. The recorded CASA parameters were total motility (%), progressive motility (%), normal morphology (%). A thermoresistance test was performed for the bulls used on the farm that was to be used for the investigation of field fertility alongside semen parameter results. These bulls also had average path velocity (VAP), straight line velocity (VSL) and curvilinear velocity (VCL) recorded.

6. **Flow Cytometry (FC) analysis**

The semen sample used for flow cytometrical analysis was the undiluted post-thaw sample for AI semen, which was kept at ambient lab temperature post-thaw. When analysing the fresh semen collected at breeding soundness evaluations, analysis was performed on the undiluted extended semen sample that had been stored at ambient temperature since arrival at the lab.

Flow Cytometry analysis was carried out using the Guava Easycyte II mini microcapillary flow cytometer with Cytosoft software (Guava Technologies Inc. Hayward, CA, USA; distributed by IMV

Technologies). The flow cytometer possesses a solid blue phase laser (488nm) and two photodiodes to detect forward and side scatter. Emission properties of the sperm particles were measured using three photomultiplier tubes (green: 525/30nm, yellow:583/26nm, and red:655/50nm). Scatter and fluorescence recording occurred until 5000 sperm had been analysed. The assays used were 'Easykit' supplied by IMV Technologies, which provided single wells containing the appropriate fluorochrome for the assay being run. The protocols supplied with the Easykit were followed. To load the final sample into the flow cytometer for analysis an Eppendorf tube was used, and therefore the semen and fluorochrome mixture was transferred into this immediately prior to analysis. Results from the assays were taken from the analysis screen and transferred to the main results spreadsheet.

Asessment of Viability – the 'Easykit 1 viability' was used to assess the percentage of viable sperm within each sample. This is a kit that contains a dual dye combination to assess live and dead cells. The green stain is a cell permeant nucleic acid stain and therefore will label all sperm heads green. The second dye penetrates only membrane damaged sperm and labels these red. There are therefore three populations of sperm identified; live sperm which will fluoresce green, dead sperm which will fluoresce red and 'dying' sperm which will fluoresce green and red simultaneously as 'orange'. 200ul of Easybuffer B (ref.023826; IMV technologies) was added to the well containing reagents. 1ul were then added into the well and this was then incubated for 10 minutes at 37^{o}C. The sample was then loaded into the Easycyte and a total of 5000 events were counted. The percentage of live (membrane intact) sperm was the population recorded for analysis.

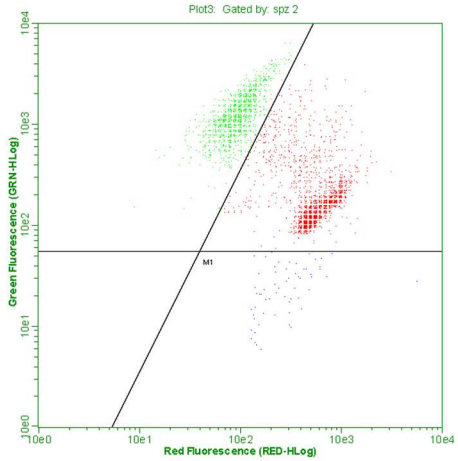


Fig 4- Viability assay results output display – viable sperm showing green fluorescence (upper left quadrant) whilst non viable sperm show as red fluorescence (upper right quadrant).

Assessment of Mitochondrial status – the status of the sperm mitochondria was assessed using 'Easykit 2' (IMV Technologies). 10ul of ethanol was added to the well containing the fluorochrome to suspend the fluorochrome, then a further 190ul of Easybuffer B (IMV Technologies) was added. 0.75ul of semen was added to the well and this was then incubated for 30 minutes at 37^{o}C. Following incubation this was then analysed through the Easycyte. Analysis stopped once 5000 events had been counted. Sperm were differentiated by their fluorescence, either orange if the mitochondrial membranes were still polarised or green if they had low membrane potential/ were depolarised. The percentage of polarised sperm was the population recorded for analysis.

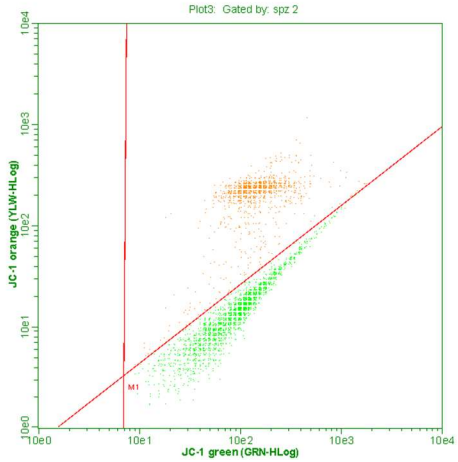


Fig 5 - Mitochondrial activity results output display – sperm with active mitochondria (polarised) show as orange fluorescence (upper right quadrant), and inactive mitochondria as green fluorescence (lower right quadrant).

Assessment of Acrosomal Membrane Integrity – the status of the acrosome in the samples analysed was assessed using 'Easykit 5' (IMV Technologies). This kit utilises two fluorochromes; sperm with disrupted acrosomes were labelled with a green probe and sperm with a damaged plasma membrane (dead sperm) were labelled with red fluorochrome. This allowed identification of four populations of spermatozoa: Live sperm with intact acrosome (Red and Green negative), dead sperm with intact acrosome (Red positive, Green negative), live sperm with disrupted acrosome (Red negative, Green positive) and dead sperm with disrupted acrosome (Red positive, Green positive). The well containing the fluorochrome had 200ul of Embryo Holding Solution (EHS) (IMV Technologies) added, to which 0.7ul semen was added and then incubated protected from light at 37^{o}C for 45 minutes. The sample was then analysed through the Easycyte and 5000 events acquired. The percentage of live sperm with intact acrosome was recorded for analysis.

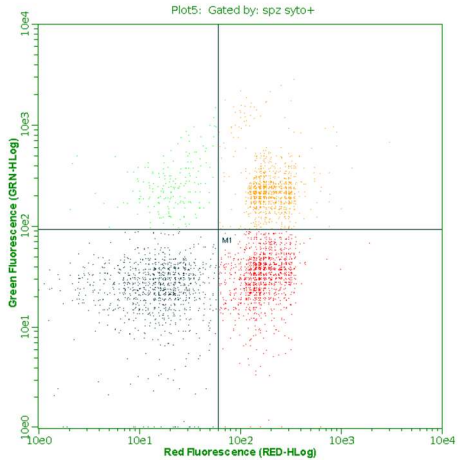


Fig 6 - Viability and Acrosomal integrity results output display – viable sperm with intact cell membrane and acrosome do not fluoresce at all and are represented by black dots (lower left quadrant). Viable sperm with disrupted acrosome fluoresce green (upper left quadrant). Non viable sperm with intact cell membrane fluoresce red (lower right quadrant) and non viable sperm with disrupted acrosome take up both red and green dyes and therefore are represented by orange dots (upper right quadrant).

7. **Manual assessment of morphology**

A manual assessment of morphology was performed utilising phase contrast microscopy of the bull breeding soundness semen samples submitted to the laboratory. For fresh semen collected on farm, Nigrosin-Eosin smears were made using the undiluted sample immediately post collection. Morphology slides for fresh semen were examined in the laboratory using a microscope equipped with phase contrast, under high power (x 1000) oil immersion. For each sample a total of at least 100 sperm were counted and their morphology classified as per Barth and Oko (1989), where results fell between 65 and 70% normal a further count of 100 was performed. The results were recorded as percentage of morphologically normal sperm and the categories of abnormalities were not included in the analysis.

8. **Statistical Analysis**

An overall Excel database included results of all semen samples analysed including fresh extended, conventional AI and sex sorted AI semen. Raw data from the semen storage and handing questionnaire were recorded in a separate Excel spreadsheet, then the main database was updated with categorised data. CASA measurements recorded in the database were % Motile, % Progressively Motile and % normal morphology. Although the CASA analysis produces further measures of motility, these were the only measurements transferred to the database. Flow cytometry results were manually input into the database from the results

displayed on the flow cytometer. Only the relevant data were transferred for each assay. For the viability assay, percentage of cells with intact plasma membrane were recorded. For the mitochondrial activity, percentage of cells with polarised mitochondria were recorded. For the viability and acrosome integrity assay, the percentage of cells with both intact plasma and acrosomal membranes were recorded. All the raw data was stored on the hard drive of the flow cytometer. Once the excel database was complete, data were transferred into a Minitab 18 or Minitab Express database for statistical analysis. Minitab Express was used to generate results for the semen handling and storage questionnaire data.

In order to determine the number of straws that would still pass pre-release standards used by North American and UK based SPCs, these standards were collated from appropriate references and personal communications. For all parameters assessed the results were ranked and the number still greater than minimum standard were counted and displayed as a percentage of the total number of straws that were tested.

For all CASA and Flow cytometry assays performed on fresh, conventional and sex sorted semen the minimum, maximum, interquartile ranges, mean, median and standard deviation were established using Minitab 18/ Express. These data were displayed graphically in boxplots and visually assessed for normality.

To establish whether the differences in CASA and FC assay results for conventional (n =82) vs sex sorted (n = 10)semen, conventional beef (n = 42) vs conventional dairy (n= 40) sires, conventional beef (n=23) vs conventional dairy (n= 59) farms and subjective vs objective assessments of fresh semen were significant for the parameters assessed, the 2 sample students T test was used. The null hypothesis was always that there was no difference between the two groups of results being compared for each parameter. When the P values were <0.05, the difference was considered to be significant.

When evaluating whether any laboratory assay parameters were significantly correlated with bull 'field' performance one farm was selected for further analysis. This was a 380 cow, DIY AI (2 inseminators), seasonally calving dairy herd with accurate fertility records based on veterinary ultrasonography - pregnancy diagnosis at 30 – 37 days post service. One conventional straw per bull was analysed using CASA and FC, however one bull did not have thermoresisitance test performed and no kinematic data (VAP, VCL, VSL) recorded, therefore n=4 for these parameters. The breeding season November 2014 - March 2015 was analysed and the conception rate by bull was assessed. These bulls had been new bulls into the flask at the start of the breeding season and therefore had been in the flask an equal period. For each AI bull utilised, a conception rate was established by dividing the number of pregnancies produced by each bull by the number of inseminations by that particular bull (assuming one straw per insemination). This data was available from farm computer records. Minitab Express was used to perform regression lonear analysis and produce a fitted

line plot of conception rate against each CASA and FC parameter for that bull. All sires selected had 40 or more service results recorded.

For comparison and an initial look at validating our laboratory processes with a research laboratory, we compared the descriptive statistics of CASA and FC result for frozen conventional AI semen with the study by Sellem et al (2015). The respective descriptive statistics were tabulated for comparison. The relevance of the Sellem study is that the same FC assays and protocols were used, and the CASA systems were manufactured by the same company, using the same Leja four chamber slides.

Chapter 3

How do dairy and beef cattle farmers in North Yorkshire that utilise AI receive, store, handle and thaw frozen AI semen?

Abstract

The on farm semen storage and handling protocols of the beef and dairy farms in North Yorkshire that submitted straws for semen analysis were investigated. Information requested included who was responsible for AI/ flask management, herd size, number of inseminations per month, number of trained personnel, thaw temperature and length of thaw. Data was captured through a questionnaire completed at the time of straw collection, or via telephone at a later date. The response rate was moderate and inconsistent, as some questions were answered with 95% completion whilst others only 35% completion. Therefore the power of the results is diminished in those questions with the poorest response rate. Data was collated and then categorised, so that results could be summarised graphically. The results showed DIY AI to be nearly 3 times more popular than using a technician service; 12 % of DIY AI farms relied on one person to do all inseminating, whereas 30% had 2 DIY AI technicians; most common herd size was 300 cows plus; flask age varied from brand new to over 14 years old; the majority of farmers did not monitor liquid nitrogen levels in the flask and relied on a nitrogen top up service to do this. In terms of straw thawing the temperatures most farmers thawed at 37-37.9^{0}C, for between 21 and 30 seconds. The variation in how farmers manage their flasks and handle semen demonstrates that best practice is no being adhered to on farms doing their own AI, and this has the potential to impact on semen quality.

Introduction

Breeding companies put significant effort into producing and delivering a good quality product to the the farmer, and the farmer should have protocols in place to maintain the quality of the product. Appropriate handling of semen straws at point of delivery to the farm, whilst in storage on the farm, and then thawing appropriately prior to use will help to maintain the quality of semen placed into cows at the point of AI, thereby maximising chances of reproductive success. Across dairy and beef farms that utilise Artificial Insemination (AI) in North Yorkshire, we wanted to establish what protocols were in place to maintain semen quality throughout storage, thawing and insemination. Investigation of these storage practices and protocols via a questionnaire will provide us with survey data. This would inform us as to how semen was being received, stored and handled, with the aim of identifying areas that may not being performed optimally and therefore potentially impacting on semen quality.

Materials and methods - Semen storage and handling questionnaire results

The semen storage and handling questionnaire (see chapter 2) requested information as to the storage facilities, personnel involved in storage and semen handling, thawing practices and type of semen stored on the farms where semen was taken from for the study. Not all questionnaires were completed in full by all participants, and follow up contact was attempted to glean more information where possible.

The raw results of the questionnaire were input into a spreadsheet, and then the categorised results as described in chapter 2 were input into the master results spreadsheet. From these categorised data Minitab Express was used to produce Pie charts to describe the results for each questionnaire.

Results

The results of the semen storage and handling questionnaire were categorised as described in materials and methods, allowing the data to be presented in discrete categories. 42 farmers responded to the questionnaire, however not all farmers completed all questions.

There were 23 straws submitted from beef farms and 59 straws from dairy farms, with at least 1 straw per farm and up to 5 straws per farm. These were made up of 42 beef bred bulls and 40 dairy bred bulls.

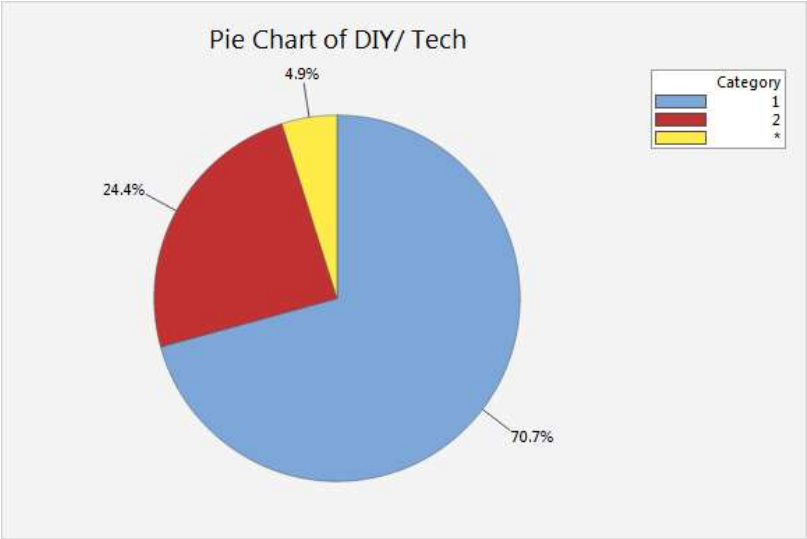


Fig 7 - Herds categorised by method of AI in use on farm. Category 1 = DIY AI; 2 = AI Technician; 3 = No response,

The vast majority of herds in the study were using DIY AI (70.7%); less than 25% of herds were using a technician service. 4.9% of the herds did not respond to this question.

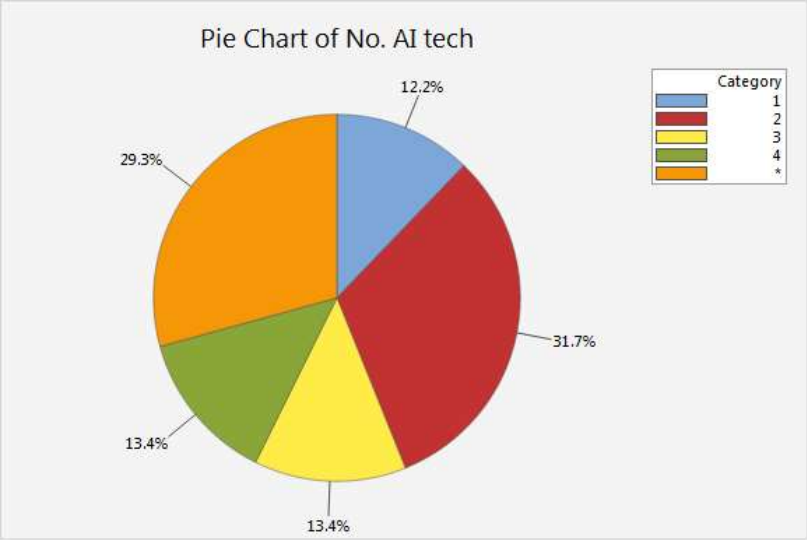


*Fig 8 - Herds categorised by the number of trained AI technicians. Category 1 = 1;2 =2; 3=3; 4 = >3; *= no response.*

The most common situation was to have 2 technicians per farm (31.7%), with 12.2% of farms only having one person responsible for all AI tasks on that farm. Similar proportions of farms had 3 trained technicians or 4 plus technicians. 29% of submissions did not have any trained staff members and utilised the technician service or did not respond.

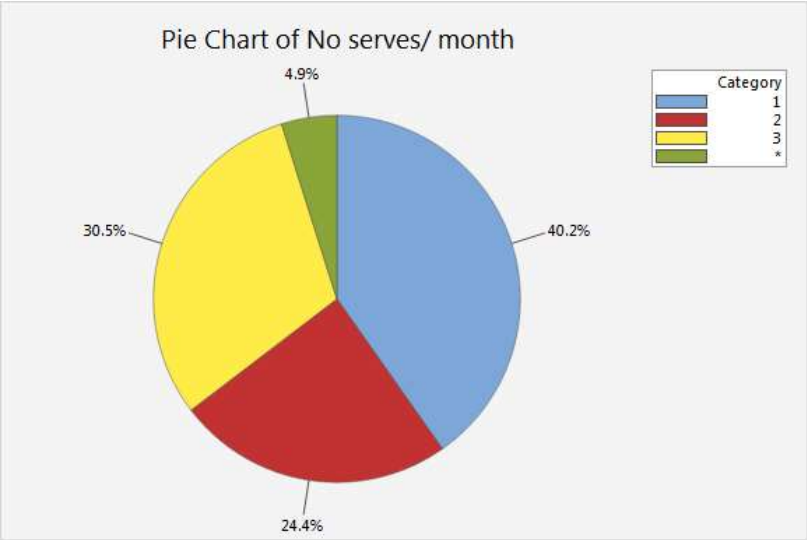


*Fig 9 - Herds categorised by number of serves/month; 1 < 50 serves; 2 = 50-99 serves; 3> 99 serves; * = no response.*

40.2% of herds were carrying out less than 50 serves/month. 30.5% of herds were serving more than 99 times per month. The service numbers were not provided by 4.9% of submissions.

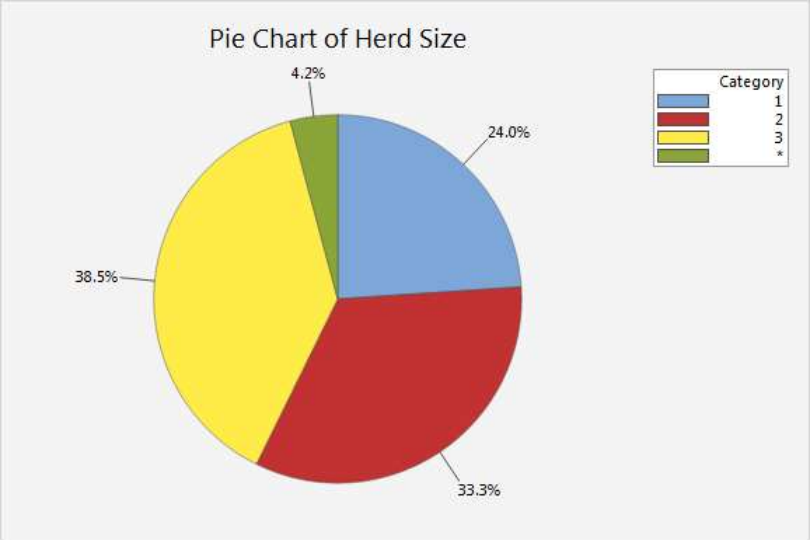


*Fig 10 - Herds categorised by number of cows per herd; 1< 100 cows; 2 = 100 – 299 cows; 3 > 299; * = no response.*

The category greater than 299 cows was the largest with 38.5% of herds in this group. Exactly one third of submissions came from medium sized herds i.e between 100 and 299 cows, and small herds (<99 cows) provided 24% of straws for testing.

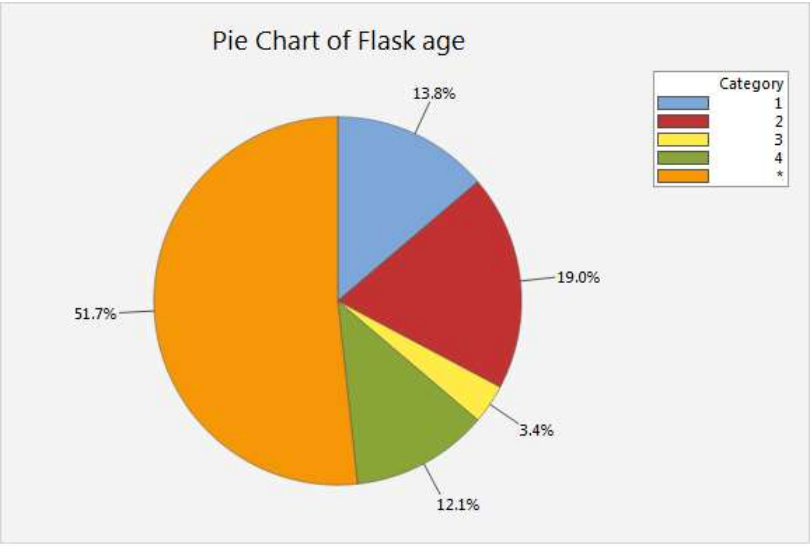
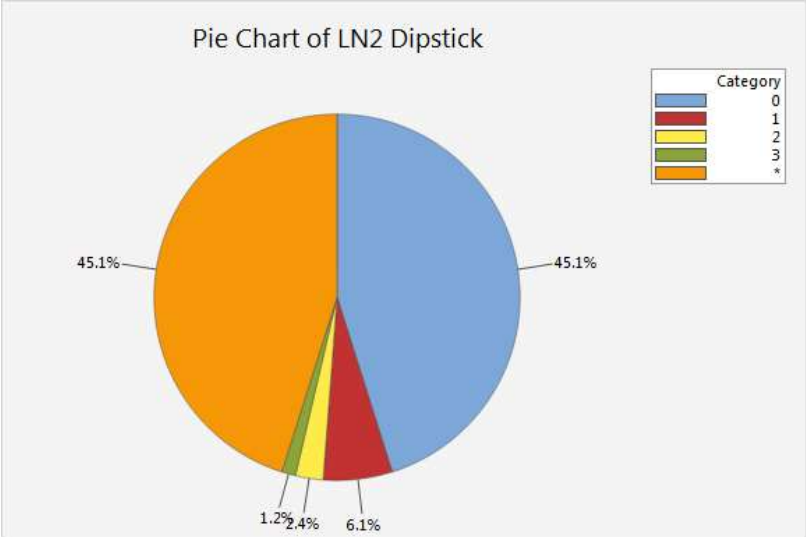


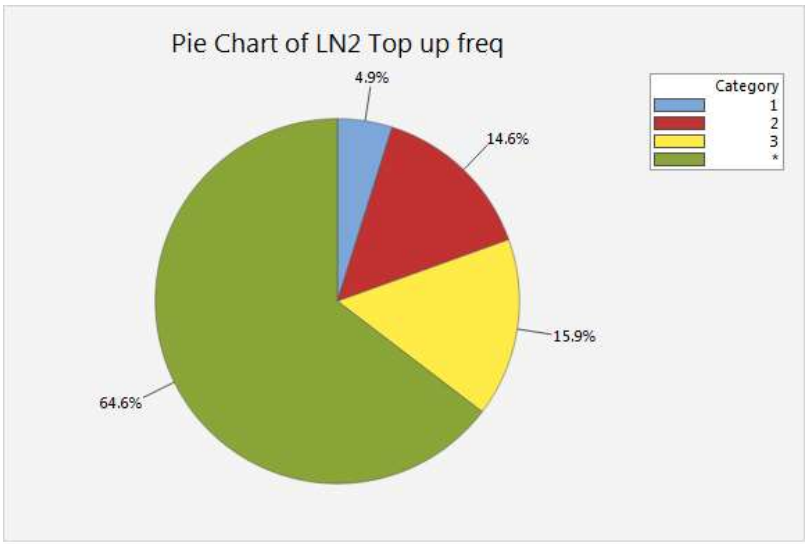
*Fig 11 - Herds categorised by age of flask; 1 < 5 years; 2 = 5-9 years; 3 = 10-14 years; 4 > 14 years; * = no response.*

12.1% of flasks in use that were over 14 years old. Over half of the herds surveyed (51.7%) did not respond with the age of flask. One third of submissions had bought their flask in the last nine years, with 13.8% being new in the last 4 years.



- *Fig 12 - Herds categorised by frequency of checking liquid nitrogen levels. Category 0 = Never; 1 = every week; 2 = every 2 weeks; 3 > 2 weeks between checks; * = no response.*

Of the herds that submitted an answer, the most common response (45.1%) was that liquid nitrogen levels were not checked at all utilising a dipstick. 6.1% herds checked their liquid nitrogen levels weekly; 2.4 % and 1.2% of herd checked flasks fortnightly and less frequently than fortnightly respectively.



- *Fig 13 - Herds categorised by frequency of topping up the semen storage flask; Category 1 ≤ 1 month; 2 1- 2 months; 3 > 2 months; * = no response.*

The most common response (15.9%) was greater than 2 months between flask top ups; with the minority (4.9%) topping up more frequently than monthly. 14.6% of herds received a monthly tank top up. 64.6% of herds did not provide details of their topping up service.

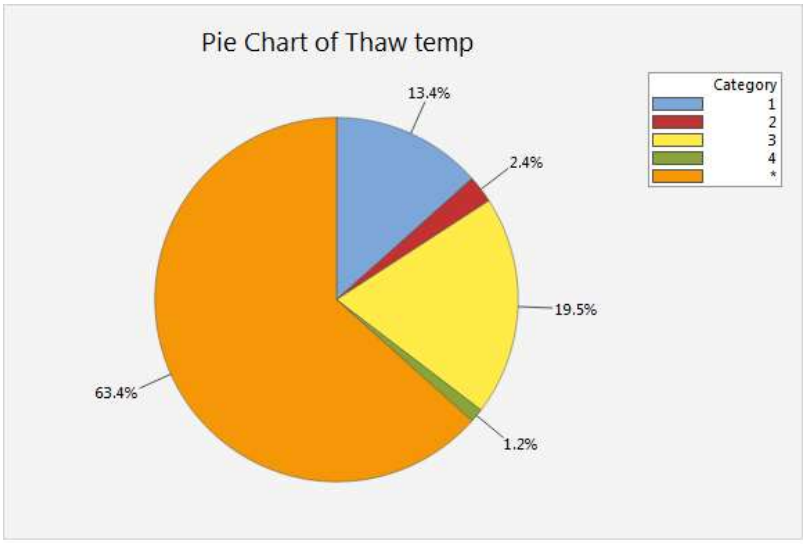
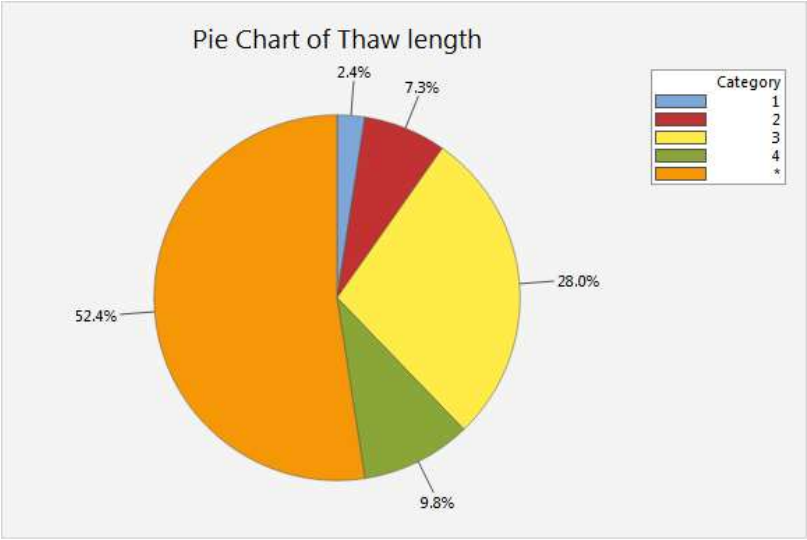


*Fig 14 - Herds categorised by temperature of thaw water; Category 1 = 35 – 35.9 ^{0}C; 2 = 36 – 36.9^{0}C; 3 = 37 – 37.9^{0}C; 4 = 38 – 38.9^{0}C; * = no response.*

Of the farms that submitted a result, the majority (19.5%) used a temperature between 37^{0}C and 37.9^{0}C to thaw their AI straws. Next most common thaw temperature was 35 – 35.9^{0}C. 36 -36.9^{0}C accounted for 2.4% of farms, and >38^{0}C was least common temperature, with 1.2% of farms using these temperatures.



- *Fig 15 - Herds categorised by length of time for thawing; Category 1 = 0-10 seconds; 2 = 11 – 20 seconds; 3 = 21-30 seconds; 4 > 30 seconds; *= no response.*

The majority (28%) of farms that responded thawed straws for between 21 and 30 seconds. Some farms (2.4%) were using short thaw times of less than 10 seconds. Only 9.8% of farms thawed straws for longer than 30 seconds, and 7.3% using thaw times of between 10 and 20 seconds.

Discussion

Semen storage and handling

The responsibility for semen handling and insemination was held by the farmer on 70% of farms that used AI. Technicians had responsibility for the flask management and insemination on 24% of farms, and these may have accounted for some of the 64% of farms who did not respond to the frequency of top up service as it was left to the responsibility of the technician. These farms are likely ones that utilise a breeding company to perform heat detection and AI on the farm, and semen is kept in a flask on the farm rather than being transported in the technician's vehicle. As these technicians take responsibility for the maintenance of the flasks, and they should be well trained and have strict protocols, there should be less risk of mishandling resulting in damage to semen in the flask.

According to results from the questionnaire on semen storage and handling 40.2% of herds performed fewer than 50 serves per month. Greater than 99 serves was the second most common response, and these herds are likely to be the ones lifting cans of semen in to the neck of the flask more frequently and therefore risking elevating the temperature of the semen straws. Repeated lifting of straws into the neck of the semen storage flask, and therefore exposing the straws to warmer temperatures whilst retrieving straws, and then returning into liquid nitrogen can have negative impacts on semen quality in terms of motility and acrosomal integrity. Significant reductions in sperm motility and acrosomal integrity were detected in straws that were lifted up and replaced in liquid nitrogen on average 2.5 times per day over a six-month period (Pace and Sullivan, 1980). If a herd was performing 100 serves per month, with straws all stored in one can, then that can would be elevated on average 3 times per day and therefore the potential to significantly impact on the motility and acrosomal integrity of the sperm within those cans. It therefore makes sense to split the in-use straws across the 6 cans in the flask to reduce the number of times a batch of straws will be elevated to the neck of the flask before they are all used. This information was not gathered in the questionnaire but would have been useful to know. These herds performing high numbers of services per month would need to be careful to not elevate the straws above the neck of the tanks and retain the straws below the frost line to minimise the risk of reducing semen quality.

Herd size was used as another proxy for the likely frequency of services performed. The greatest proportion of herds had at least 300 cows (these were mainly dairy herds) and based on average conception rates of 34% (Hanks and Kossaibati, 2017), then this will require at least 900 services per year, which equates to 75 serves per month. If the straws were all in one can then this would mean 2.5 services per day, and therefore this could impact negatively on semen quality (Pace and Sullivan, 1980).

There was a wide range in age of flasks in use on the farms that were sampled. The proportion of flasks in use that were over 14 years old was 12.1%, thus demonstrating that farmers were willing to keep using flasks for long periods of time and were not replacing flasks based on age alone. An efficient semen storage flask that maintains levels of liquid nitrogen for long periods of time, with minimal loss of vacuum is required for optimal semen storage. As long as the flask is cared for appropriately through minimal movement and physical impacts to the flask, good function should be long lasting. The age of the flask itself doesn't appear to directly influence when farmers choose to replace them. Standard observations of performance e.g. liquid nitrogen level monitoring, performed weekly as recommended (Nebel, 2007) should provide an early warning system of when a flask may be losing its functionality and require replacing.

Of the farms utilising DIY AI, the most common situation was to have 2 technicians per farm (31.7%), with 12.2% of farms only having one person responsible for all AI tasks on that farm. It is likely that having at least two people capable of performing DIY AI is most common because if only one person is trained in DIY AI, they are unlikely to be available to perform AI every day of the year and therefore this may result in suboptimal fertility performance in the herd. It has been demonstrated that there is between technician variability in the quality (CASA and flow cytometry parameters) of semen in storage (Janett et al., 2007) and also between technician variation in performance in the field in terms of non-return rate (NRR) (Janett et al., 2007). As the number of people involved in DIY AI per farm increases, the potential increase in risk of semen quality and fertility issues relating to semen storage and flask management is likely to increase, unless strict protocols are in place and are complied with.

Straw handling and thawing practices should be relatively consistent within herds, but also between herds if best practice is being utilised in across cattle herds in North Yorkshire. When thaw water temperature data was captured, it demonstrated that not all farms were utilising the same protocol for straw thawing. Although the majority of farms used a temperature between 37^0C and 37.9^0C, the range was between 35^0C and 39^0C. The author recommends thawing conventional AI semen in 0.25ml straws at 35^0C

on the DEFRA approved XL Vets Farmskills DIY AI training course that is run by the veterinary practice. There has been a number of experiments to establish optimal temperature and length of thaw for 0.5ml and 0.25ml straws (Gaillard et al, 1982), with temperatures ranging from 0 to 40^0C, and from 7 seconds to 75 minutes. Experiments by Muino et al., (2008), have assessed the effect of short thaw at high temperatures (5s at 70^0C) versus longer thaw at lower temperatures (40s at 37^0C) on post thaw semen quality and found no difference in terms of motility or acrosomal integrity, but the study did not assess impact on field fertility. The majority (57.1%) of farms thawed straws for between 21 and 30 seconds although some farms (4.8%) were using short thaw times of less than 10 seconds, which may be based on historical advice where thawing at higher temperatures for a shorter period of time was advised. Gaillard et al. (1982) showed a benefit in field fertility performance of thawing for 25 seconds versus 7 seconds. DeJarnette et al. (2000) states that when no specific instructions are given with a straw of semen, thawing should be performed at 33-35^0C for a minimum of 40 seconds. With the increased use of sex sorted semen, breeding companies will provide thawing guidelines for these straws. Currently in the UK thawing at 35-37^0C for 30 seconds is recommended by one breeding company (http://www.genusbreeding.co.uk/wp-content/uploads/2012/07/Semen-Handling-Protocol.pdf), whilst another company recommends 37^0C for a minimum of 40 seconds (https://www.cogentuk.com/sexed-ultra/handling-sexed-semen).

Although the length of time of thaw and thaw temperature may not independently impact on final semen quality, in combination they may. Variations in methods utilised on farm may demonstrate that personnel are not all using up to date information on the optimal way to handle semen or that not all personnel on each farm are handling semen consistently, which may demonstrate protocol drift. These findings should prompt a review of the protocols in place across all farms within the practice that utilise AI. There is not always consistent behaviour between farms and potentially within farms, and therefore all would benefit from being updated with current best practice, as there may have been a significant period of time since initial training. Current thawing protocols based on the literature should advise placing straws in a water bath at 35^0C for 30 – 60 seconds, and to only thaw straws that will be used within 10 minutes (Diskin, 2018).

Chapter 4:

Objective Semen Analysis using CASA and Flow Cytometry in Veterinary Practice.

Abstract

92 straws of AI semen (82 conventional and 10 sex sorted) stored on beef and dairy farms in North Yorkshire were evaluated for motility and morphology using CASA, and physiological parameters using Flow cytometry, in a semen assessment laboratory based at Bishopton Veterinary group. CASA % motile, % progressively motile and % normal morphology were established and flow cytometer assessments were % viable, % active mitochondria and % viable and intact acrosome. For conventional straws between 32% and 90% of straws were still above breeding company pre-release thresholds dependent on the assay and for sex sorted straws between 0% and 70% passed the thresholds.

Categories of semen were then compared using students t test, following visual assessment of normality.

Comparisons between sex sorted and conventional straws showed significant differences in CASA motility ($p=0.0182$), progressive motility ($p=0.0024$), mitochondrial activity ($p<0.0001$) and morphology ($p=0.0257$).

Straws from dairy sires had significantly greater viability ($p=0.0432$) and morphology ($p<0.0001$) than beef sires, whereas straws from dairy farms had significantly greater acrosome integrity ($p=0.0043$), CASA motility ($p=0.0129$), progressive motility ($p=0.0243$) and morphology ($p=0.0271$) than straws from beef farms. There was no significant difference in motility ($p=0.1001$) or progressive motility between dairy and beef sires ($p=0.0804$). There was no significant difference in acrosome integrity between beef and dairy sire ($p=0.1959$).

Investigation of field fertility outcomes on one dairy farm showed significant positive correlations between the flow cytometry viability assay and conception rate ($r^2 = 85.3\%$ and $P = 0.025$), and also the CASA % motile at 2 hours post thaw and conception rate ($r^2 = 90\%$ and $P = 0.05$).

Semen samples (n=70) collected by electroejaculation from 70 bulls subjected to a bull breeding soundness examination were evaluated in the same manner, as well as having manual assessment of morphology performed. Significant differences were present when on farm subjective motility assessments were compared with laboratory based assessments (CASA and subjective), ($p<0.0001$) but not between CASA motility assessment in the lab and subjective assessment in the lab. There was no difference between morphological evaluations assessed by CASA or manually.

Multiparametric objective semen analysis in a Veterinary practice-based laboratory can offer additional information on the semen in use on farm (AI and natural service), to complement traditional subjective

methods. This may allow a more consistent methodology in investigations, and also allow meta-analysis of the relationship between these parameters and fertility results from different investigations. This should help identify which parameters may therefore be most important when selecting semen to use on farm.

Introduction

Objective semen analysis utilising CASA and flow cytometry has historically been utilised for research purposes or by semen production centres to assess semen quality prior to release and to decide whether to sell or discard the batch (Utt, 2018). The conventional assessments of semen quality including concentration, motility and morphology are of limited value in predicting potential of semen to perform in the field (Rodriguez Martinez, 2006), and these are the methods that have historically and are currently used by veterinarians in practice to perform semen assessments of bulls in breeding soundness evaluations. However a combination of laboratory tests can be predictive of fertility when evaluating bulls for breeding soundness (Kastelic and Thundathil, 2008), and in AI the use of combinations of tests has been reported to be more reliable predictors of field fertility (Sellem et al, 2015). This is due to the fact that spermatozoa must possess a large number of properties to fertilise an oocyte, and any laboratory measuring a single sperm attribute will produce results that do not correlate well with fertility (Graham, 2001). If a veterinary surgeon wants to be proactive and help their clients achieve optimal performance in their herd, they must look at optimising male factors as well as female factors of fertility. To evaluate the potential of the males impact on fertility they could utilise technologies that will help achieve that, this would include objective measures of semen quality such as CASA and Flow cytometry. Barth (2018) described the importance of specialist andrology laboratories, such as those that operate in Australia (Lorton, 2014), and therefore a specialist laboratory in the UK would likely provide a service that veterinary surgeons in practice would find useful. Potential indications for practising veterinary surgeons to refer samples to the laboratory could include investigation of poor performance in AI and natural breeding situations, but more proactive practitioners may want to know the quality of semen (AI or natural breeding) prior to use in order to mitigate any potential poor performance, and potentially select bulls who will likely perform better and disregard bulls with poor semen evaluation results.

Materials and Methods

Chapter 2 contains the materials and methods for collection of samples, processing of samples and capture of results and describes the statistical analysis performed to evaluate the results.

Results

1) What proportion of AI semen tested after transport and storage on beef and dairy farms in North Yorkshire is still above the minimum pre-release standards for conventional semen of North American and United Kingdom cattle breeding companies?

The following table summarises the mean values of all the parameters assessed when analysing both conventional (n=82) and sex sorted (n=10) AI semen. This enables direct comparison between conventional and sexed semen for all parameters at a glance. Included in the table is the 'minimum standard' for North America and United Kingdom (UK) for the parameters measured (where they exist) and the percentage of straws tested in the study that satisfy that standard.

Parameter	Conventional	Sexed	N. America Standard	UK Standard	% straws above standard - conventional	% straws above standard – sexed
% Viable	43.45	45.60	40	40	65% UK/US	70% UK/US
% Polarised Mitochondria	38.56	17.92	40	N/A	46% US	0% US
% Viable and intact acrosome	35.30	36.45	N/A	N/A	N/A	N/A
CASA % Motile	37.44	26.12	40	40	44% UK/US	10% UK/US
CASA % Progressively Motile	26.11	14.51	15	30	32% UK 86% US	10% UK 40% US
CASA % Morph Normal	81.62	75.08	70	70	90% UK/ US	70% UK/ US

Table 4 – Summary results table of mean values for CASA and Flow Cytometry assays for conventional and sex sorted semen, pre-release standards for North America and United Kingdom and the percentage of straws tested that would still pass respective pre-release standard.

Both conventional and sex sorted semen exhibited similar mean values for the viability and viability/ acrosome integrity Flow Cytometry assays in this study. Sex sorted semen was found to have a mean value approximately half that of conventional semen for the Mitochondrial activity assay. Conventional

semen had greater mean values for Motility, Progressive motility and Morphology than sex sorted semen.

When comparing to pre-release standards, the proportion of conventional straws tested that would pass pre-release standards varied from 32% for progressive motility (UK), to 90% of straws passing the UK/US morphological assessment standard of greater than 70% normal.

Of the sex sorted semen straws evaluated 0% of the straws would pass the US mitochondrial activity assay cut off. 70% of straws would pass the viability flow cytometry assay and the CASA morphology evaluation.

2) Can optimum semen quality for insemination be better and more consistently achieved with the use of the combination of Computer Assisted Semen Analysis (CASA) and Flow Cytometry (FC)?

	Sellem et al. (n = 153)	Sellem et al. (n = 153)	Study (n=82)	Study (n = 82)
Parameter	Mean +/- SD	Range	Mean +/- SD	Range
FC % Viable	52.2 +/- 12.4	17.9 – 75.5	43.45 +/- 15.12	0 – 67.64
FC % Polarised Mitochondria	41.2 +/- 12.6	17.6–62.8	38.56 +/- 15.22	0.26 – 72.5
FC % Viable & intact acrosome	45.4 +/- 12.8	10.7–69.3	35.30 +/- 15.56	0.06 – 68.82
CASA % Motile	58.8 +/- 10.3	25.0–81.0	37.44 +/- 15.00	0 – 66.9
CASA % Morph normal	92.9 +/- 3.6	82.8–98.1	81.62 +/- 8.83	51.4 – 93.9

Table 5 - descriptive statistics of results of a study by Sellem et al., 2015 alongside equivalent descriptive statistics from our study.

These two sets of data were captured using the same flow cytometry assays equipment and assays, therefore enabling assessment of how similar the outputs were for conventional semen analysis.

Viability assay – the mean value for viable sperm from Sellem was 8.7% greater than the mean value from this study, and the range of results were greater in this study by 10%, with a minimum value of 0%.

Mitochondrial activity assay – the mean values were very similar between the two studies, only differing by 2.64%. Min value was just above 0 in this study, with a maximum value of 72.5% which was 10% greater than the Sellem study; therefore the range was greater by 10% again.

Viability and Acrosome Integrity Assay – the Sellem study had a mean value of 45.4, which was 10.1% greater than this study, and a standard deviation of 12.8, which was smaller than this study. Both studies had similar maximum values, but this study had a minimum value of 0.06, which was 10% less than the Sellem study.

CASA % Motile – mean values differed by almost 20%, with the Sellem study average greater than in this study. Although the maximum value of the Sellem study was only 14% greater than our study, there was 25% difference between the minimum values, as in this study the minimum value was 0%.

CASA Morph Normal – mean value of the Sellem study was 11% greater than this study. The range of results was much greater in this study than the Sellem study, 42.5% vs 15.3%.

The ranges for the CASA and FC parameters of both conventional and sex sorted semen collected from storage on North Yorkshire dairy and beef farms were established. 82 conventional and 10 sexed semen straws were tested. Sex sorted data was only used when comparing to conventional semen; the rest of the comparison only included conventional semen (N=82)

a) Flow Cytometry Viability assay

The results are reported as percentage of the sperm cells analysed still with an intact plasma membrane, and therefore considered a viable sperm cell.

i) Conventional vs sex sorted semen

Variable	N	N*	Mean	SE Mean	St Dev	Minimum	Q1	Median	Q3	Maximum
% Viable Conventional	79	3	43.446	1.701	15.119	0.000	33.580	47.580	55.440	67.640
% Viable Sexed	10	0	45.593	4.799	15.176	17.080	34.778	47.605	58.008	66.310

Table 6 – Descriptive statistics of the FC Viability assay results for conventional and sex sorted semen.

The overall range of results for conventional semen is greater due to a minimum value being 0 for conventional semen. The rest of the descriptive statistics show very similar results for conventional and sex sorted semen. There are 3 values missing for conventional semen (N*).

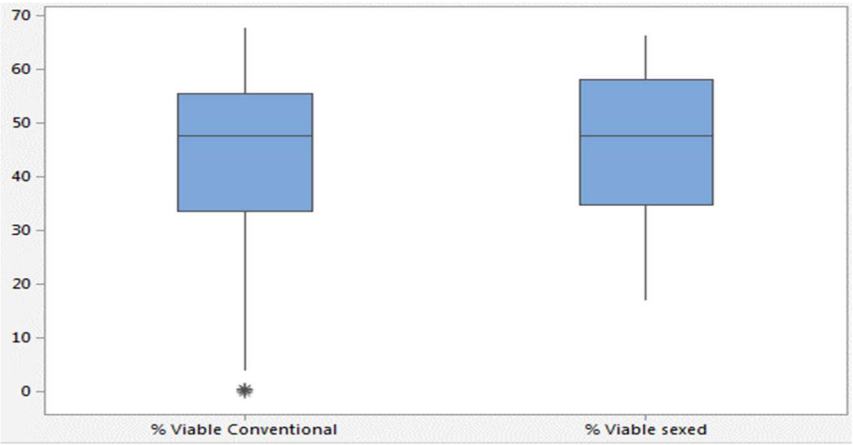


*Fig 16 - FC Viability assay results for conventional and sex sorted semen. *= values missing.*

T-Value	DF	P-Value
-0.42	11	0.6814

Table 7 – Student 2 value T Test comparing FC Viability results for conventional and s sorted semen

The results of the analysis show that the difference in the mean % viable between conventional and sex sorted semen is not significant as P value is 0.6814.

(ii) Beef vs Dairy Semen

Variable	N	N*	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
% Viable Beef	39	3	39.954	2.752	17.184	0.000	30.580	47.560	52.780	64.080
%Viable Dairy	40	0	46.851	1.906	12.057	19.760	36.430	48.830	56.900	67.640

Table 8 - descriptive statistics of the FC Viability assay results for conventional straws categorised as Beef and Dairy breeds.

Dairy breeds have superior values across all the statistics used to describe the viability assay results for Beef and Dairy breeds of bull.

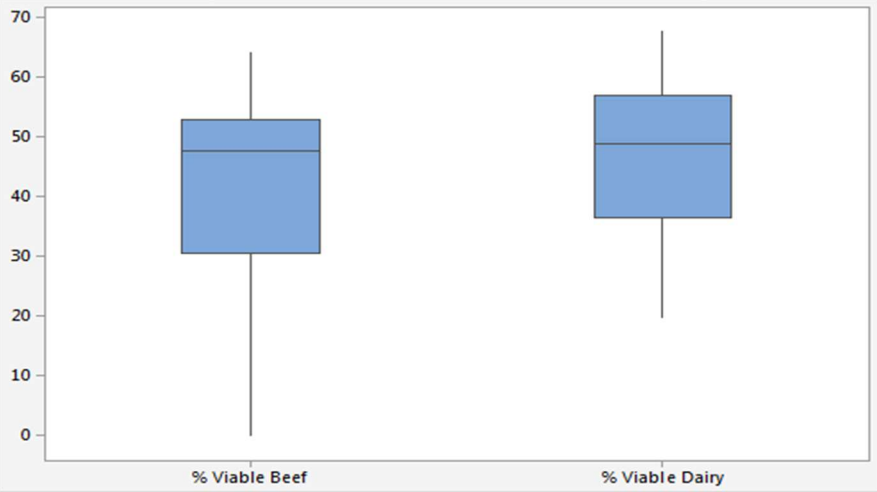


Fig 17 – FC Viability assay results for Beef and Dairy breed of bull

T-Value	DF	P-Value
-2.06	67	0.0432

Table 9 - Student 2 value T Test comparing FC Viability results for Beef and Dairy breed of bull.

The P value is less than 0.05 and therefore the difference between viability assay results for Beef and and Dairy breeds of bull is significant.

(iii) Semen in use on Beef vs Dairy farms

Results were grouped according to whether the straws tested were to be used on Beef or Dairy farms.

Variable	N	N*	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
% Viable Beef	21	2	33.934	4.331	19.847	0.000	24.270	32.680	49.320	63.920
%Viable Dairy	58	1	41.185	1.759	13.400	9.710	31.698	43.740	49.180	72.500

Table 10 - Descriptive statistics of the FC Viability assay results for conventional straws categorised as for use on Beef vs Dairy farms.

Semen straws for use on Dairy farms have superior values across all but one value used to describe the viability assay results.

1)

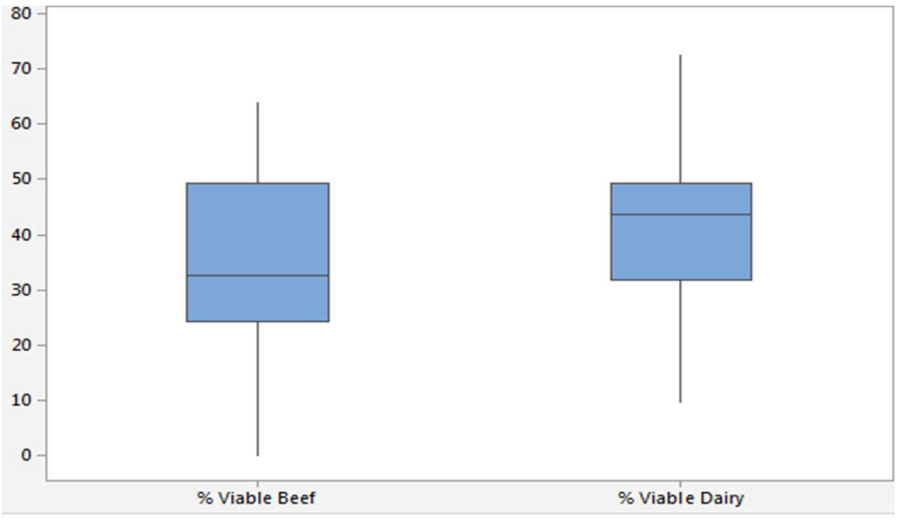


Fig 18 – FC Viability assay results for type of farm, Beef vs Dairy.

T-Value	DF	P-Value
-1.55	26	0.1329

Table 11 - Student 2 value T Test comparing FC Viability results for type of farm, Beef vs Dairy.

The P value is greater than 0.05 and therefore the difference in viability assay depending on whether semen is for use on beef vs dairy farms is not significant.

b) Flow Cytometry Mitochondrial Activity assay

The results of the mitochondrial activity assay are reported as a percentage of the analysed sperm that still have polarised mitochondria and are therefore still capable of performing their function.

(i) Conventional vs sexed semen

Variable	N	N*	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
% Polarised Mitochondria Conventional	82	0	38.563	1.681	15.224	0.260	28.760	39.190	48.235	72.500
% Polarised mitochondria Sexed	10	0	17.915	1.944	6.146	6.940	13.190	18.340	22.948	26.740

Table12– descriptive statistics of the FC mitochondrial activity assay results as categorised by conventional vs sex sorted semen.

Conventional semen had higher values for all statistics looked at apart from minimum value of 0.26% polarised mitochondria, where sex sorted semen was higher at 6.94%.

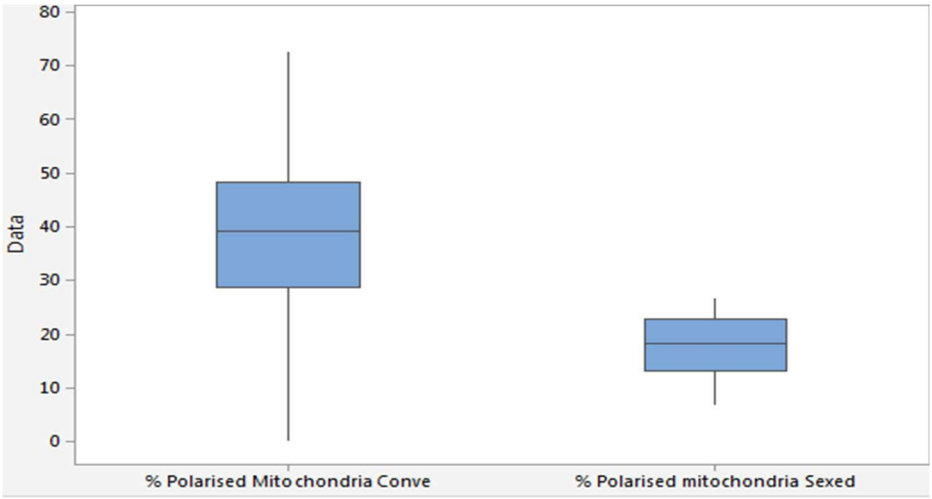


Fig 19 - FC Mitochondrial activity assay results for conventional and sex sorted semen.

T-Value	DF	P-Value
8.03	25	<0.0001

Table 13 – Student 2 value T test for conventional vs sexed semen.

With a P value of <0.0001, the difference between %polarised mitochondria in conventional and sex sorted semen was significant.

(i) Beef vs Dairy semen

Variable	N	N*	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
% Polarised Mitochondria Beef	42	0	38.425	2.587	16.765	0.260	28.005	39.150	50.865	67.980
% Polarised Mitochondria Dairy	40	0	38.708	2.155	13.632	9.710	29.415	40.560	46.480	72.500

Table 14 - descriptive statistics of the FC mitochondrial activity assay results for conventional beef and dairy breed semen.

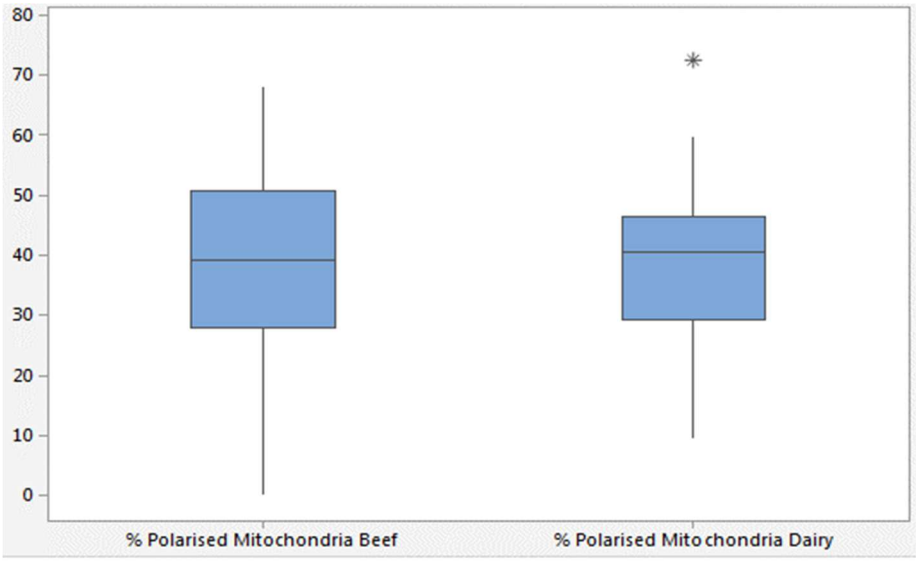


Fig 20 – FC Mitochondrial activity assay results categorised by beef or dairy breed of bull.

T-Value	DF	P-Value
-0.08	78	0.9332

Table 15 – Student 2 value T test for beef or dairy breed of bull.

As the P value is greater than 0.05 the difference in mitochondrial activity results between Beef and Dairy semen was not considered significant.

(ii) Semen in use on Beef vs Dairy farms

Variable	N	N*	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
% Polarised Mitochondria Beef	23	0	36.098	4.035	19.351	0.260	23.200	41.840	51.300	62.080
% Polarised Mitochondria Dairy	59	0	39.523	1.738	13.352	9.710	30.960	38.840	46.580	72.500

Table 16 -The results for the FC mitochondrial activity assay grouped according to whether the conventional straws tested were to be used on Beef or Dairy farms.

Beef farms provided the lowest mitochondrial activity results and had a greater interquartile range. Dairy farms provided the best mitochondrial activity result at 72.5%.

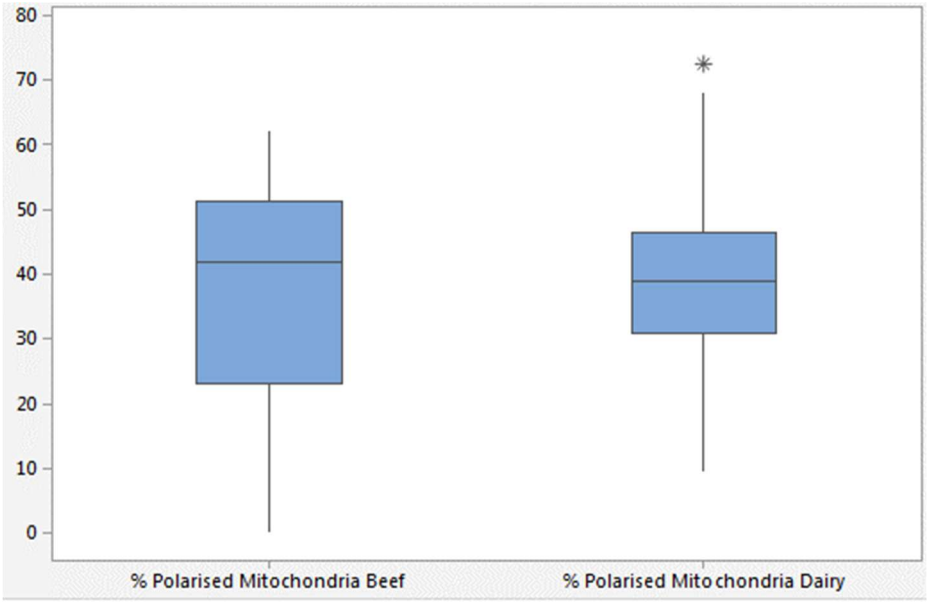


Fig 21 – FC mitochondrial activity assay results grouped by beef or dairy farms.

T-Value	DF	P-Value
-0.78	30	0.4418

Table 17 – Student 2 value T test for beef or dairy farm.

To establish whether the difference between mitochondrial activity assay results for beef and dairy farms was significant, the 2 value T test was used, and with a P value greater than 0.05 this is not significant.

c) Flow cytometry Viability and Acrosome Integrity assay

The results of this assay are reported as the percentage of sperm cells analysed that have both an intact plasma membrane and intact acrosome; therefore giving an indication of the population of live sperm that are still capable of completing the acrosome reaction.

(i) Conventional vs sex sorted semen

Variable	N	N*	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
% Viable & intact acrosome Conventional	82	0	35.298	1.719	15.562	0.060	23.655	36.330	46.353	68.820
% Viable & intact acrosome Sexed	10	0	36.450	3.214	10.163	15.340	31.953	35.125	43.703	52.620

Table 18- descriptive statistics of the FC viability and acrosome integrity assay results for conventional and sexed semen.

The mean value for viable and intact acrosome of both populations are very similar; but the range of values for conventional semen is again greater at 68.76, compared to 37.28.

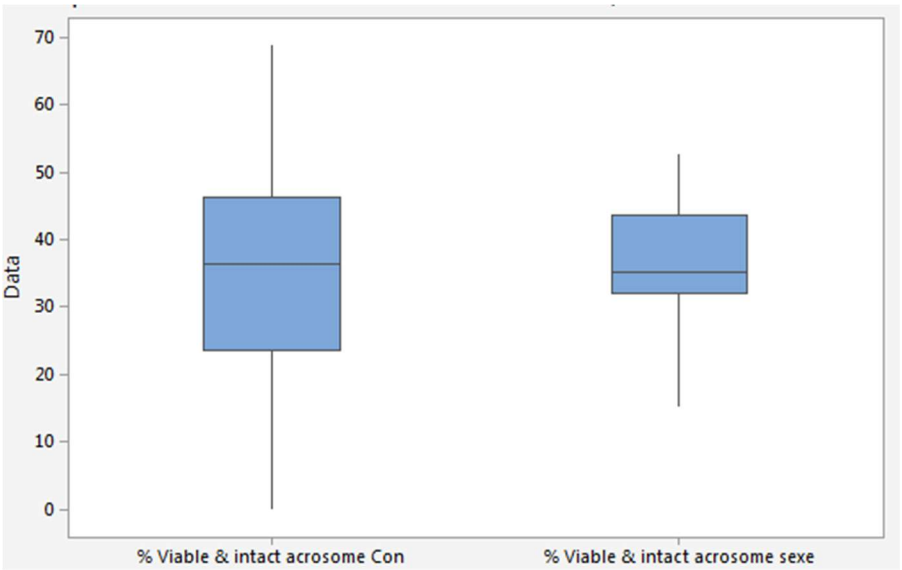


Fig 22 – FC viability and acrosome integrity results when straws were categorised as conventional or sexed semen.

T-Value	DF	P-Value
-0.32	14	0.7565

Table 19 – Student 2 value T test for conventional vs sexed semen

The results of the statistical analysis show that there is no significant difference between the mean values of viability and acrosomal integrity of conventional and sex sorted semen.

(ii) Beef vs Dairy semen

Variable	N	N *	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
% Viable & intact acrosome Beef	42	0	33.129	2.599	16.842	0.060	22.400	34.560	43.205	64.200
%Viable & intact acrosome Dairy	40	0	37.575	2.205	13.943	10.340	27.450	37.925	48.075	68.820

Table 20 - Descriptive statistics to describe the results of the FC viability and acrosome integrity assay categorised by Beef and Dairy breed for conventional straws.

The lowest value was recorded for a beef breed straw, and the highest value reported for a diary breed straw. The interquartile range for beef and dairy breeds was very similar.

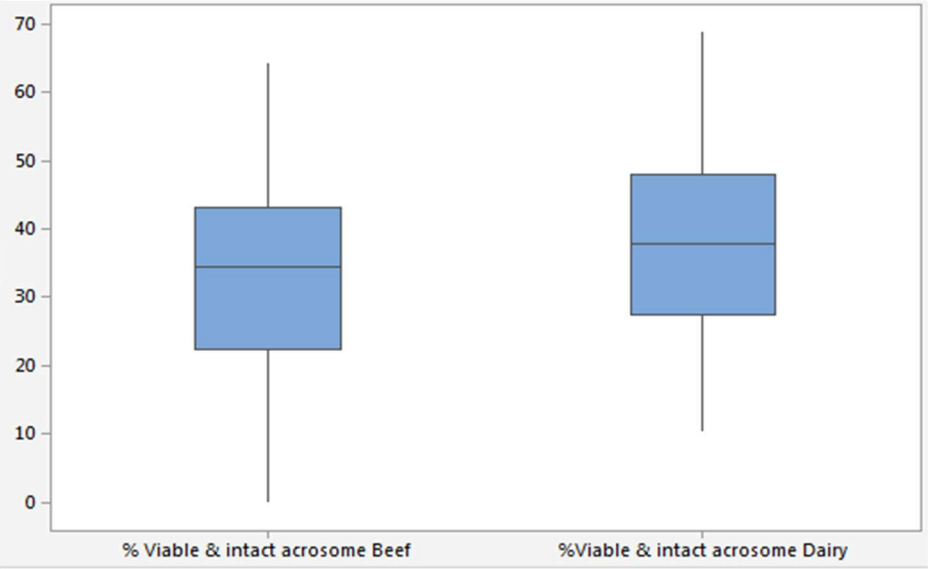


Fig 23 – FC Viability and Acrosome integrity results for beef or dairy breed of bull.

T-Value	DF	P-Value
-1.30	78	0.1959

Table 21 – Student 2 value T test for beef vs dairy breed of bull

There was no statistical difference between viability and acrosome integrity assay results for Beef and Dairy breeds.

(iii) **Semen in use on Beef vs Dairy farms**

Variable	N	N*	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
Dairy Farm	59	0	38.775	1.743	13.387	10.340	28.760	39.180	48.300	68.820
Beef Farm	23	0	26.378	3.633	17.425	0.060	14.340	23.660	40.680	56.540

Table 22 - The FC viability and acrosome integrity results were grouped according to whether the conventional straws tested were to be used on Beef or Dairy farms.

The mean, minimum, Q1, median, Q3 and maximum values were greater in dairy farms compared with beef farms.

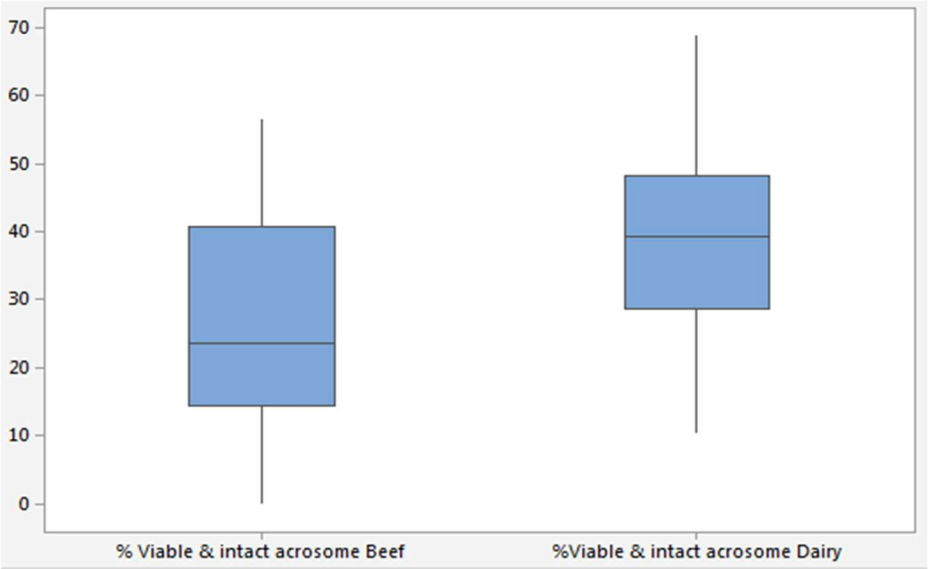


Fig 24 – FC Viability and Acrosome integrity results for beef or dairy farms.

T-Value	DF	P-Value
-3.08	32	0.0043

Table 23 – Student 2 value T test for beef vs dairy farm

The results of the statistical analysis show a statistically significant difference (p=0.0043) between the mean values of viability and acrosomal integrity of beef and dairy semen in this study.

d) CASA Evaluation of % Motile semen

The results displayed for the CASA analysis of motility are the percentage of sperm cells classified as 'motile' expressed as a percentage of all the sperm cells analysed.

(i) Conventional vs sex sorted semen

Variable	N	N*	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
% Motile Conventional	79	3	37.443	1.688	14.999	0.000	29.100	38.400	50.000	66.900
% Motile Sexed	10	0	26.120	3.784	11.966	9.400	15.600	24.300	37.325	46.300

Table 24 – The descriptive statistics of the CASA motility results when straws were categorised by conventional vs sexed semen.

The CASA results for motility are consistent with some of the flow cytometry viability assay results, in that the range of results for the conventional semen is 66.9, versus 30.7 for sex sorted semen. The mean motile value of the conventional semen analysis is greater than the sex sorted.

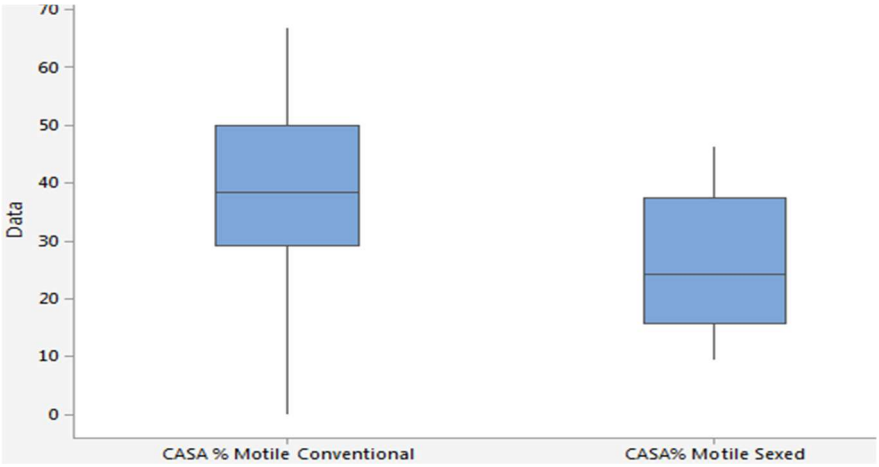


Fig 25 – CASA % motile results for conventional vs sexed semen.

T-Value	DF	P-Value
2.73	12	0.0182

Table 25 – Student 2 value T test for conventional vs sexed semen

The 2 sample T test confirms that the difference in the percentage of motile sperm between conventional and sex sorted semen is significant (p=0.0182).

(ii) Beef vs Dairy semen

Variable	N	N*	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
% Motile Beef	41	1	34.783	2.472	15.831	0.000	27.450	39.000	46.800	57.300
% Motile Dairy	38	2	40.313	2.218	13.676	12.400	30.475	37.150	52.350	66.900

Table 26-The descriptive statistics of the CASA motility results when conventional straws were categorised by Beef and Dairy Breed Sires.

Both mean and median values are similar for beef and dairy breed sires, despite the minimum values for beef and dairy being 0% and 12.4%, and the maximum values for beef and dairy being 57.3% and 66.9% respectively.

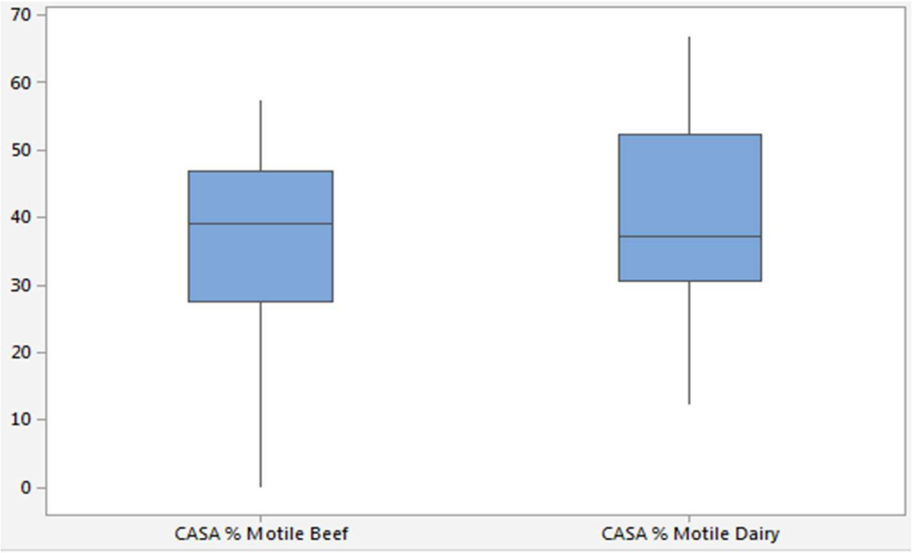


Fig 26 – CASA % motile results for beef and dairy breed of bull.

T-Value	DF	P-Value
-1.66	76	0.1001

Table 27 – Student 2 value T test for Beef vs Dairy breed of bull.

The 2 sample T test was used to establish whether the difference in mean % motile sperm between beef and dairy sires was significant, and with a P value of 0.1001 the difference was not significant.

(iii) Semen in use on Beef vs Dairy farms

Variable	N	N*	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
% Motile Beef	22	1	29.277	3.945	18.505	0.000	13.550	28.050	50.225	53.000
% Motile Dairy	57	2	40.595	1.615	12.191	12.400	31.300	39.500	49.100	66.900

Table 28 – descriptive statistics of CASA motility results categorised by whether the conventional straws were being used on beef or dairy farms.

Mean and median values for dairy sires were greater, and the highest motility result was a straw from a dairy farm. The lowest motility value was 0%, and that was being stored on a beef farm.

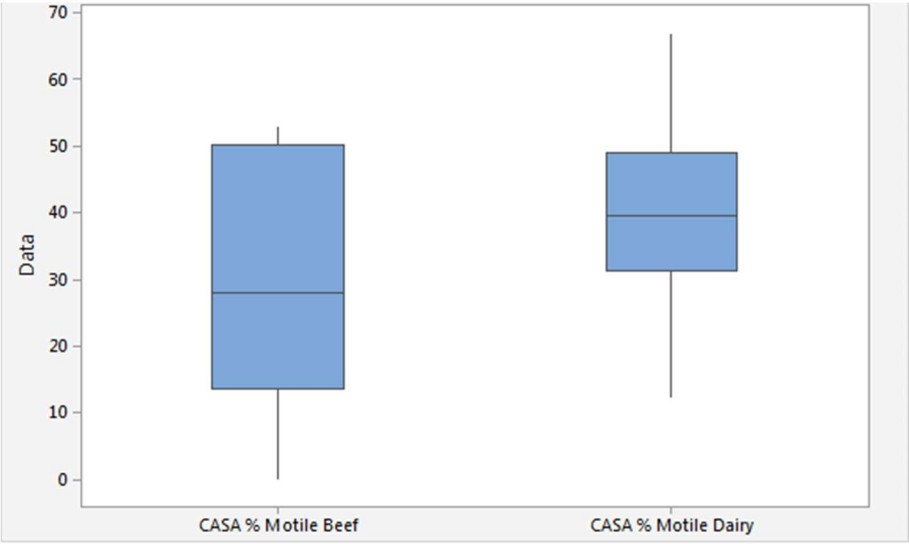


Fig 27 – CASA % motile results for beef or dairy type of farm.

T-Value	DF	P-Value
-2.65	28	0.0129

Table 29 – Student 2 value T test for beef farm vs dairy farm.

The difference in mean motility between beef and dairy farms was significant; with a P value of 0.0129.

e) CASA % Progressively Motile

The results displayed for the CASA analysis of progressive motility are the percentage of sperm cells classified as 'progressively motile' as a percentage of all the sperm cells classified in- that analysis.

(i) Conventional vs sex sorted semen

Variable	N	N*	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
% Progressively Motile Conventional	79	3	26.105	1.430	12.710	0.000	18.300	25.300	33.900	59.000
% Progressively motile Sexed	10	0	14.510	2.798	8.849	3.900	7.950	11.150	21.450	31.500

Table 30 - The descriptive statistics for CASA progressive motility categorised by conventional and sex sorted samples.

These are consistent with some of the flow cytometry assay results in that the range of results for the conventional semen is 59%, versus 27.6% for sex sorted semen. The mean value of the conventional semen analysis is greater than the sex sorted.

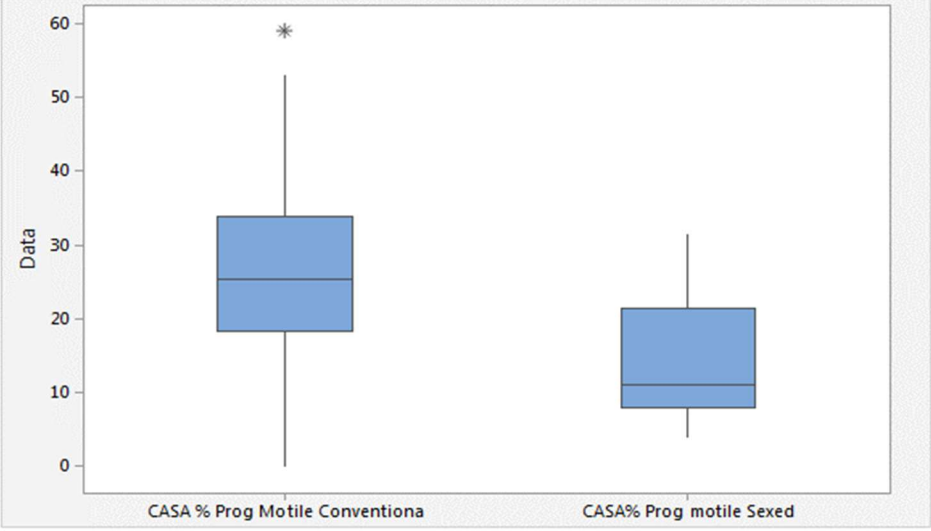


Fig 28 – CASA Progressive motility results for conventional and sex sorted semen.

T-Value	DF	P-Value
3.69	14	0.0024

Table 31 – Student 2 value T test for conventional vs sexed semen

The P value of the 2 value T test of 0.0024 shows that the difference in mean progressive motility between conventional and sex sorted semen is significant.

(ii) Beef vs Dairy semen

Variable	N	N*	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
% Progressively Motile Beef	41	1	23.693	1.901	12.172	0.000	18.050	25.900	31.850	45.100
% Progressively Motile Dairy	38	2	28.708	2.096	12.922	6.300	18.175	25.050	38.600	59.000

Table 32 - The descriptive statistics for CASA progressive motility of conventional straws categorised by beef or Dairy breed of sire.

Dairy sires had greater minimum, mean and maximum values than beef sires.

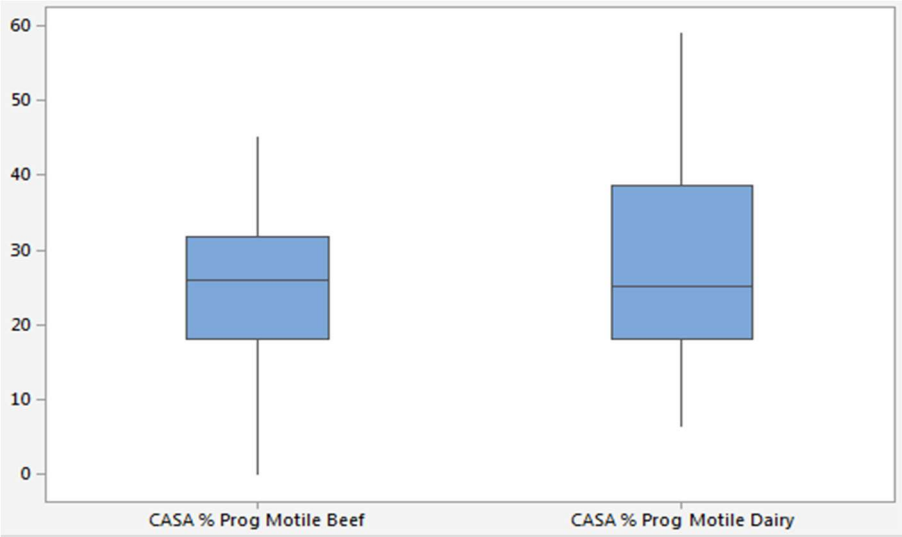


Fig 29 – CASA progressive motility results for dairy and beef breed of sire.

T-Value	DF	P-Value
-1.77	75	0.0804

Table 33 – Student 2 value T test for beef vs dairy breed of bull.

The P value of 0.0804 shows that there is not a significant difference between beef and dairy sires in CASA progressive motility.

(iii) **Semen in use on Beef vs Dairy farms**

Variable	N	N*	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
Progressively Motile Beef	22	1	20.232	3.095	14.518	0.000	3.450	20.150	31.300	45.100
Progressively Motile Dairy	57	2	28.372	1.494	11.278	6.300	20.300	26.700	34.250	59.000

Table 34 – the descriptive statistics of CASA progressive motility of conventional straws categorised by beef or dairy farms.

Samples from dairy farms had higher mean, minimum, median and maximum values than beef farms.

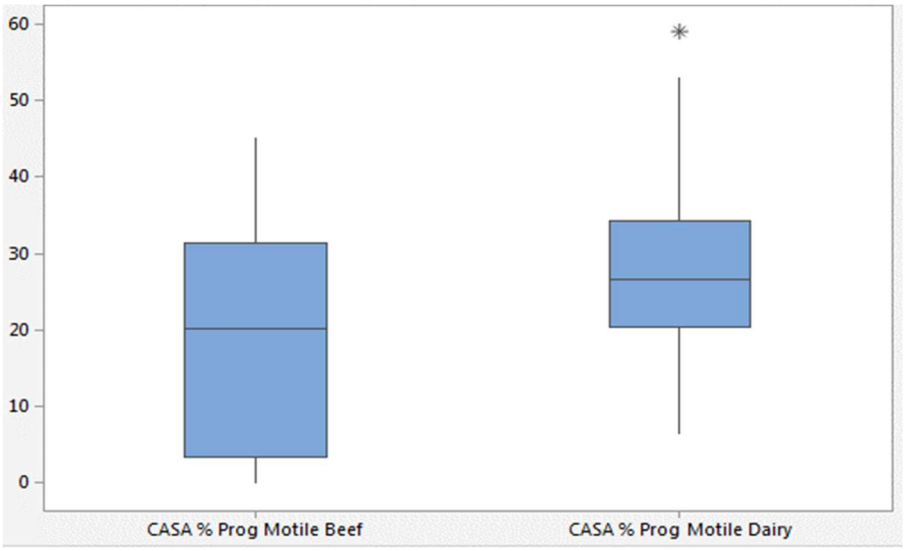


Fig 30 – CASA progressive motility results for dairy and beef farms.

T-Value	DF	P-Value
-2.37	31	0.0243

Table 35 – Student 2 value T test for beef vs dairy farms.

The P value of 0.0243 shows that the difference in progressive motility between these two populations is significant. This data therefore suggests that the AI semen in use on Beef farms in North Yorkshire was poorer in terms of motility.

f) CASA % Morphology Normal

The results displayed for the CASA analysis of morphology are the percentage of sperm cells that are morphologically normal as a percentage of the sperm cells classified in that analysis.

(i) Conventional vs sex sorted semen

Variable	N	N*	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
% Morphology normal conventional	77	5	81.623	1.006	8.830	51.400	77.600	83.300	87.650	93.900
% Morphology normal sexed	10	0	75.080	2.365	7.480	64.900	68.000	74.200	83.300	84.800

Table 36 -The descriptive statistics for CASA percentage normal morphology categorised by conventional or sex sorted semen.

The mean value of morphologically normal spermatozoa in the conventional population is greater than for sex sorted semen, and the values for conventional semen have a greater range.

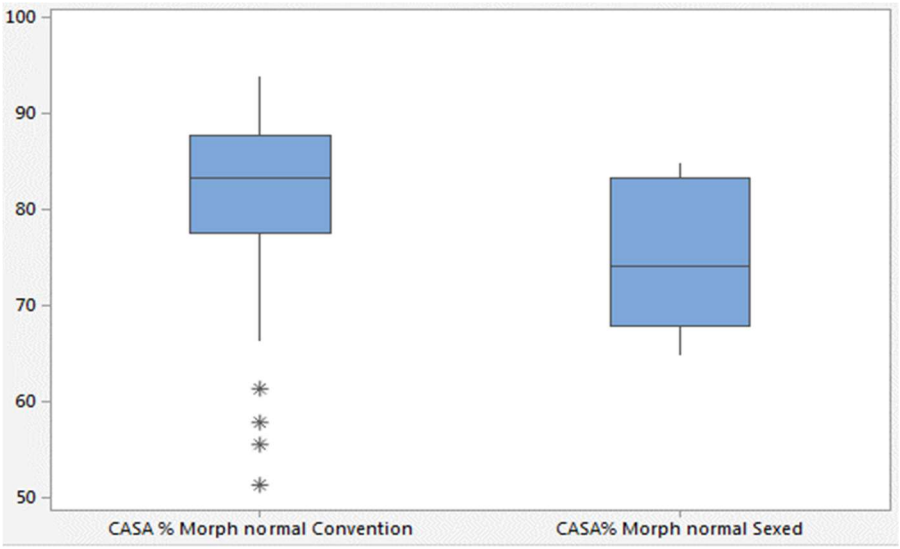


Fig 31 – CASA morphology results for conventional and sex sorted semen.

T-Value	DF	P-Value
2.55	12	0.0257

Table 37 – Student 2 value T test for conventional vs sexed semen

The P value of 0.0257 supports the alternative hypothesis that there is a significant difference between the percentage of normal sperm in the conventional and sexed semen samples.

(ii) Beef vs Dairy semen

Variable	N	N*	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
% Morphology normal Beef	38	4	77.679	1.409	8.683	51.400	72.825	79.850	83.675	88.600
% Morphology normal Dairy	39	1	85.467	1.152	7.196	57.900	82.700	86.700	90.600	93.900

Table 38 - The descriptive statistics for CASA morphology of conventional AI semen categorised by beef or dairy breed.

Dairy breed semen had greater mean and median values as well as greater min and max values compared with beef breed.

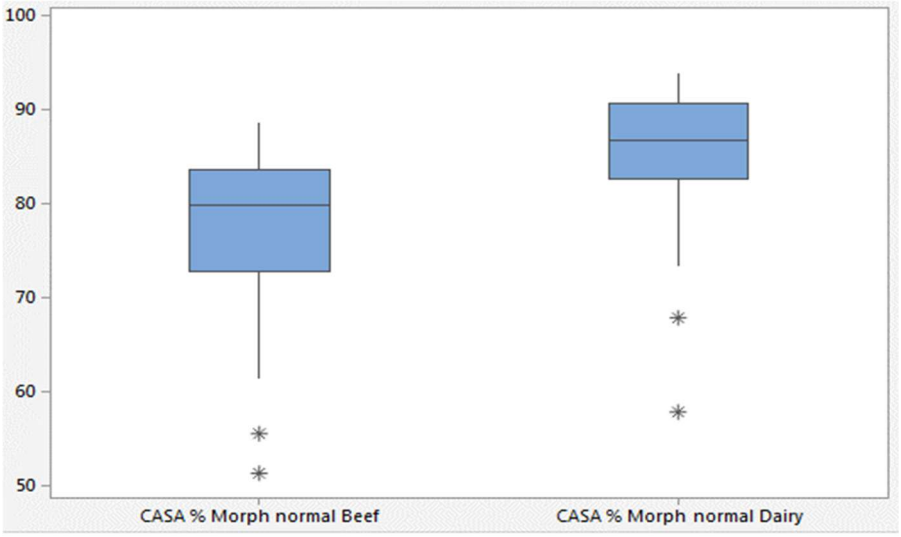


Fig 32 – CASA Morphology results for beef and dairy breed of bull.

T-Value	DF	P-Value
-4.28	71	<0.0001

Table 39 – Student 2 value T test for Beef vs Dairy breed of bull.

The P value of <0.0001 means that the difference in percentage of morphologically normal sperm between beef and dairy breeds was significant.

(iii) **Semen in use on Beef vs Dairy farms**

Variable	N	N*	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
% Morphology normal Beef	19	4	77.568	2.025	8.828	55.600	72.600	79.900	84.300	87.700
% Morphology normal Dairy	58	1	82.952	1.115	8.489	51.400	79.675	84.750	88.675	93.900

Table 40 – the descriptive statistics of CASA morphology results of conventional straws categorised by whether the straws were being stored/ used on beef or dairy farms.

Mean and median values were similar, although the range of results for dairy farms was greater by 10%.

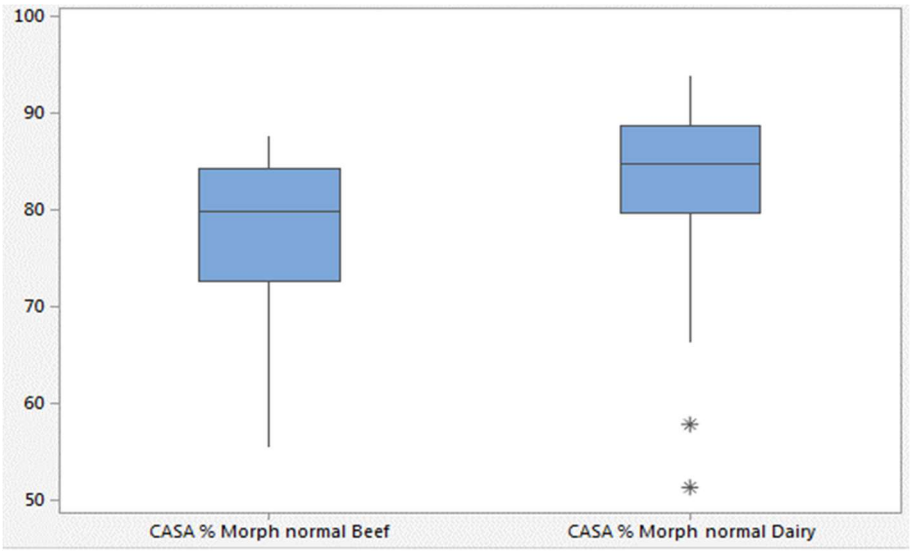


Fig 33 – CASA Morphology results for beef or dairy farms.

T-Value	DF	P-Value
-2.33	29	0.0271

Table 41 – Student 2 value T test for beef vs dairy farms.

The P value of 0.0271 shows that the difference is significant, and that semen stored on beef farms has a greater percentage of morphologically abnormal sperm.

3) Would a laboratory utilising a combination of CASA and Flow Cytometry for semen analysis be of use to practising cattle vets doing on farm Bull Breeding soundness evaluations? What may this service add to the conventional breeding soundness evaluation?

a) CASA analysis

The normal ranges for CASA parameters were established for 70 samples of fresh extended semen, collected as part of an on-farm bull breeding soundness evaluation. The relationship between on farm subjective assessments of motility performed as part of an on-farm bull breeding soundness evaluation (N=47, as not all practitioners completed motility evaluation on the submission forms), with objective measures of motility established utilising CASA in the laboratory were determined. In addition, the level of loss of sperm motility from bull-side evaluation immediately post-collection to CASA evaluation in the laboratory 12-18 hour later will be evaluated subjectively by the laboratory technician (N=31, this was not recorded for all submissions by the laboratory technician) and objectively using CASA. The table and boxplot show assessment of motility of fresh semen samples collected on farm. Results are displayed for subjective assessment by the submitting veterinarian (BBSE % Prog motile), following transport to the semen analysis lab and reassessment subjectively by the laboratory technician (RAFT Prog Motile%) and objective assessment using CASA (CASA % motile and CASA % Prog motile).

Variable	N	N*	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
CASA % Motile	70	0	52.141	2.959	24.759	1.400	37.100	54.900	72.425	90.800
CASA % Progressively motile	70	0	38.100	2.725	22.802	0.000	19.750	40.200	55.725	76.600
BBSE % Progressively motile	47	23	71.957	1.838	12.601	30.000	65.000	75.000	80.000	92.000
RAFT % Progressively motile	31	39	46.452	3.853	21.455	5.000	30.000	45.000	65.000	80.000

Table 42 – descriptive statistics of the motility assessments of fresh extended semen collected at bull breeding soundness evaluations (N= missing values).*

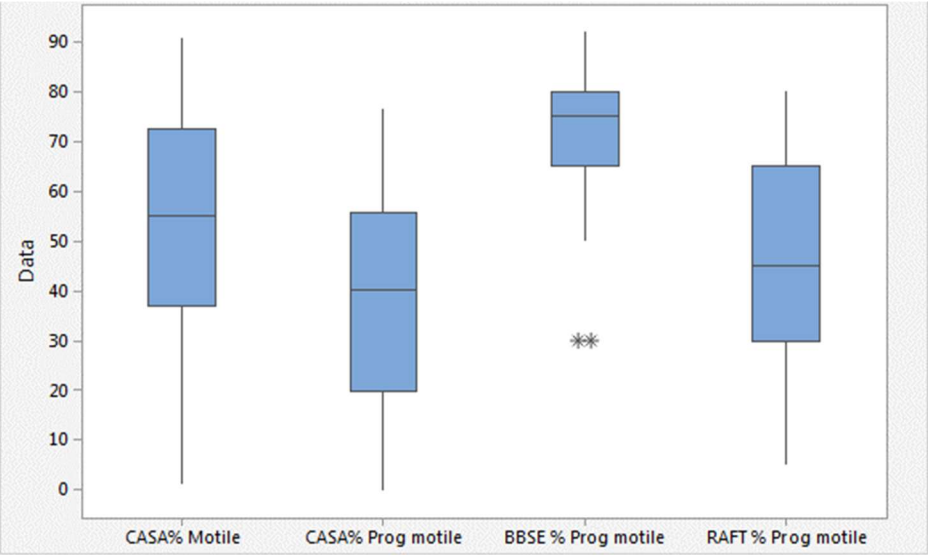


Fig 34 – Fresh semen motility evaluation results.

Subjective assessment on farm showed a much smaller range in motility results, compared with the other three methods. The greatest motility value was achieved on farm immediately post collection. The mean subjective progressive motility immediately post collection was 71.957%; the mean subjective progressive motility on reassessment at the lab was 46.452% representing a drop of 25.505%. RAFT and both CASA assessments had similar interquartile ranges. There was 0% progressive motility on some samples when evaluated by CASA.

i) Comparison of subjective on farm assessment vs subjective assessment at the laboratory

Sample	N	Mean	StDev	SE Mean
BBSE % Progressively motile	47	71.957	12.601	1.838
RAFT % Progressively motile	31	46.452	21.455	3.853

T-Value	DF	P-Value
5.97	43	<0.0001

Table 43 – Student 2 value T test comparing on farm subjective assessment of motility and subjective assessment of motility in the laboratory.

With a P value of < 0.0001, it can be said that the difference in assessment of progressive motility on farm and subjectively in the laboratory is significant.

ii) Comparison of subjective on farm assessment with CASA % motile results

Sample	N	Mean	StDev	SE Mean
BBSE% Progressively motile	47	71.957	12.601	1.838
CASA % Motile	70	52.141	24.759	2.959

T-Value	DF	P-Value
5.69	108	<0.0001

Table 44 – Student 2 value T test comparing on farm subjective assessment of motility and objective assessment of total motility in the laboratory.

The difference between the mean values of motility is 19.816%; therefore the mean deterioration in motility between collection and arrival at the laboratory is 19.816%. The P value of <0.0001 confirms that there is a significant difference between the means of progressive motility on farm assessed subjectively and the objective assessment of motility in the practice using CASA.

iii) Comparison of subjective assessment at the laboratory with CASA % motile results

Sample	N	Mean	StDev	SE Mean
RAFT% Progressively motile	31	46.452	21.455	3.853
CASA% Motile	70	52.141	24.759	2.959

T-Value	DF	P-Value
-1.17	65	0.2458

Table 45 – Student 2 value T test of subjective and objective assessment of motility in the laboratory.

There was no statistical difference between the subjective assessment of motility in the laboratory and objective CASA assessment of % motility.

b) Flow cytometry analysis

The normal ranges for Flow Cytometry parameters were established for 70 samples of fresh extended semen, collected as part of an on-farm bull breeding soundness evaluation.

Variable	N	N*	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
% Viable	68	2	52.088	2.855	23.544	1.030	32.150	57.090	70.568	92.200
% polarised mitochondria	66	4	51.851	3.130	25.431	0.780	30.108	54.630	71.435	91.560
% viable & intact acrosome	65	5	40.066	2.402	19.365	4.980	23.300	42.200	54.250	87.440

Table 46 - Descriptive statistics of flow cytometrical assay results for fresh semen.

The results for viability and mitochondrial activity assay are very similar in terms of mean, minimum, median and maximum values. Viability and acrosome integrity results are lower than the other two assays in terms of mean and median, Q1 and Q3 values, and has a lesser range in results.

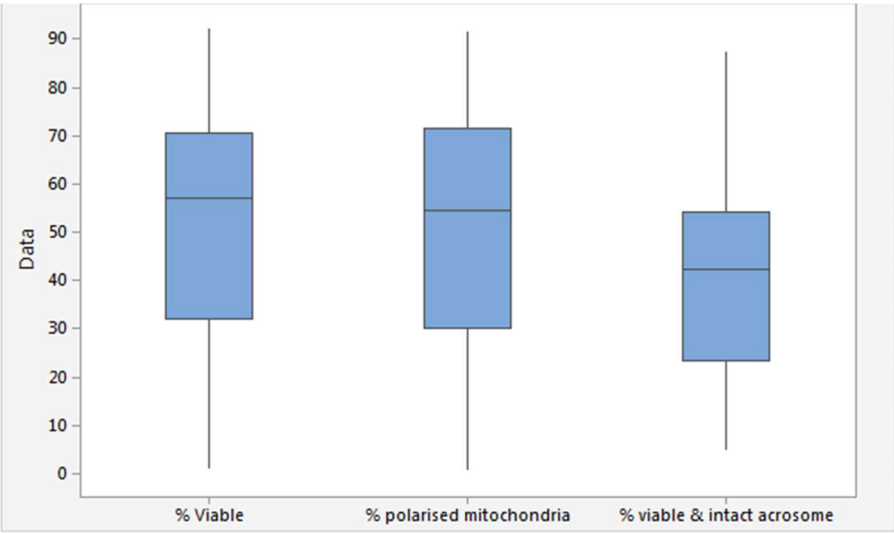


Fig 35 – Fresh semen flow cytometry assay results.

c) Sperm morphology evaluation

Sperm morphology evaluation results are reported as a percentage of normal sperm, determined by CASA or subjectively by RAFT technicians using Phase Contrast Microscopy (PCM).

Variable	N	N*	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
CASA Morph	70	0	82.389	1.299	10.869	37.500	77.825	83.700	91.075	97.100
PCM Morph	49	21	78.463	1.723	12.062	28.000	74.600	80.000	85.500	96.000

Table 47 – Descriptive statistics of sperm morphology evaluation results of fresh semen performed by CASA and phase contrast microscopy (PCM).

The mean percentage of normal sperm is greater when samples are analysed by CASA compared with PCM; Minimum, Q1, Median, Q3 and Maximum values are all greater when analysis was done by CASA.

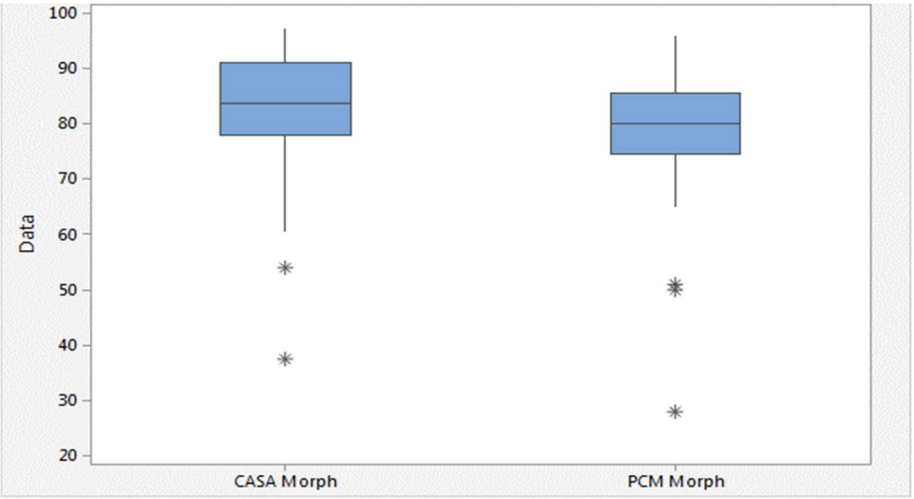


Fig 36 - Fresh semen morphology results produced by CASA and PCM.

Sample	N	Mean	StDev	SE Mean
CASA Morph	70	82.389	10.869	1.299
PCM Morph	49	78.463	12.062	1.723

T-Value	DF	P-Value
1.82	96	0.0720

Table 48 – Student 2 value T test of CASA and PCM derived morphology evaluations of fresh semen.

The difference in the mean percentage of normal sperm when evaluated by CASA and PCM is 3.925%, but with a P value of 0.072 this is not significant.

4 Which CASA and Flow Cytometry parameters for deep frozen conventional semen straws are correlated with field fertility?

Variable	N	N*	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
% Viable	5	0	51.568	5.850	13.080	32.920	40.300	50.200	63.520	67.640
% Polarised Mitochondria	5	0	46.744	3.710	8.295	37.230	40.455	44.690	54.060	59.720
% Viable & intact acrosome	5	0	41.202	5.741	12.836	29.350	30.515	36.600	54.190	60.080
% Motile	5	0	43.380	7.245	16.200	29.800	30.350	35.900	60.150	66.900
% Prog motile	5	0	32.700	7.884	17.630	17.300	19.700	22.500	50.800	59.000
% Morph normal	5	0	79.920	7.381	16.504	57.900	62.300	88.500	93.250	93.900
% Motile TRT	4	1	33.93	10.52	21.04	8.60	14.65	33.50	53.63	60.10
% Prog motile TRT	4	1	14.800	6.969	13.937	0.000	1.975	13.600	28.825	32.000
Curvilinear velocity (VCL) um/s	4	1	189.66	17.75	35.49	155.95	160.64	181.92	226.42	238.85
Average Path Velocity (VAP) um/s	4	1	97.923	8.405	16.811	81.210	83.853	94.760	115.155	120.960
Straight line velocity (VSL) um/s	4	1	77.365	8.224	16.448	56.700	61.848	77.910	92.338	96.940
Conception rate (%)	5	0	40.640	7.176	16.046	13.500	28.450	44.700	50.800	56.400

Table 49 – Descriptive statistics of the Semen analysis results and conception rate data for all the bulls used in the field fertility investigation. For one of the bulls evaluated there was no thermoresistance test performed, and kinematic properties not recorded, therefore N=4 for those results.

Bull	% Viable	% Polarised Mitochondria	% Viable & intact acrosome	% Motile	% Prog motile	% Morph normal	% Motile TRT	% Prog motile TRT	Conception Rate
1	59.4	48.4	36.6	53.4	42.6	92.6	34.2	7.9	45.2
2	67.64	43.68	60.08	66.9	59	93.9	60.1	32	56.4
3	32.92	44.69	31.68	29.8	22.5	57.9	8.6	0	13.5
4	50.2	37.23	29.35	35.9	17.3	66.7	32.8	19.3	43.4
5	47.68	59.72	48.3	30.9	22.1	88.5	*	*	44.7

Table 50 – Mean CASA and FC and conception rate results for the conventional AI bulls used in the field fertility investigation.

Bull 3 was the poorest performing bull in terms of conception rate, and ranked lowest out of the bulls in the viability assay, CASA % motile, CASA Morph normal, CASA % motile and % Prog motile after thermoresistance test (TRT). Bull 2 was highest performing in terms of conception rate, and ranked as the best bull in the viability, viability and acrosome integrity assay and all CASA parameters.

Results generated from CASA and Flow Cytometry analysis were plotted against conception rates, and regression analysis performed. Results are displayed in the fitted line plots below.

a) Conception rate and CASA % motile

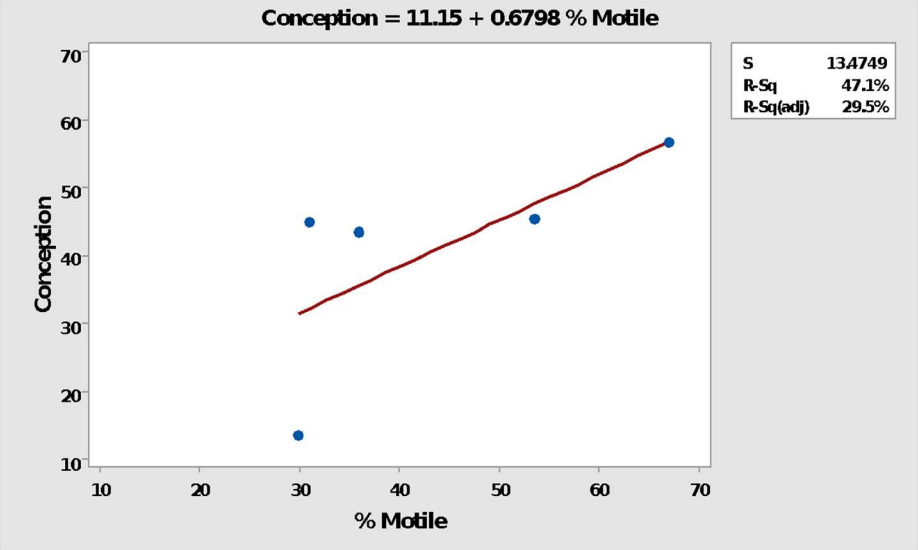


Fig 37 – Conception rate against CASA motility post thaw.

There is a small positive linear relationship between CASA % motility and conception rate, although the tightness of fit is not good, demonstrated by a large S value. P= 0.2007 therefore this is not a significant correlation.

b) Conception rate and CASA % progressively motile

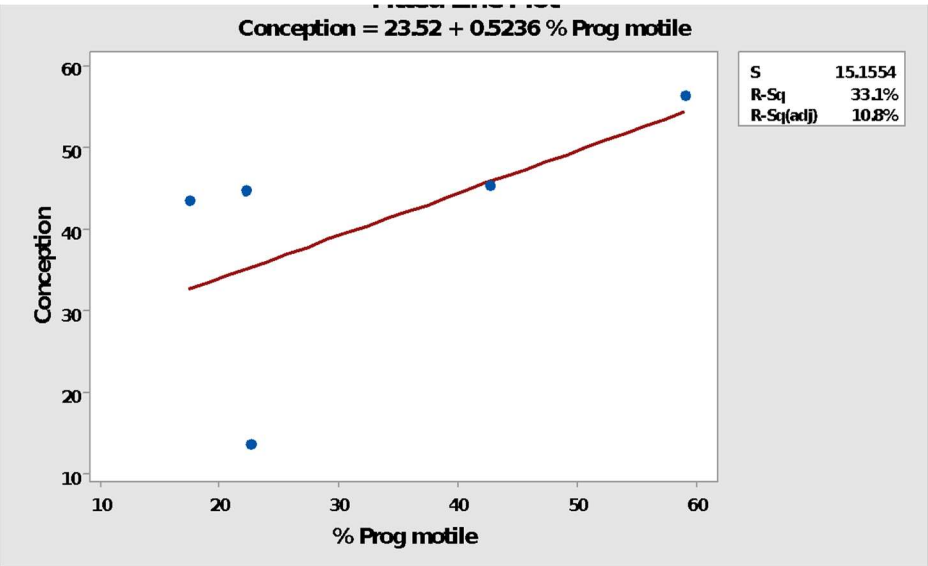


Fig 38 - Conception rate against CASA Progressive Motility (PM)

The relationship demonstrated above is weakly linear with an R squared value of 33.1% and an S value of 15. The low conception rate bull as an outlier. There is no significant correlation between CASA progressive motility and conception rate (P= 0.3102).

c) Conception rate and CASA % morphologically normal

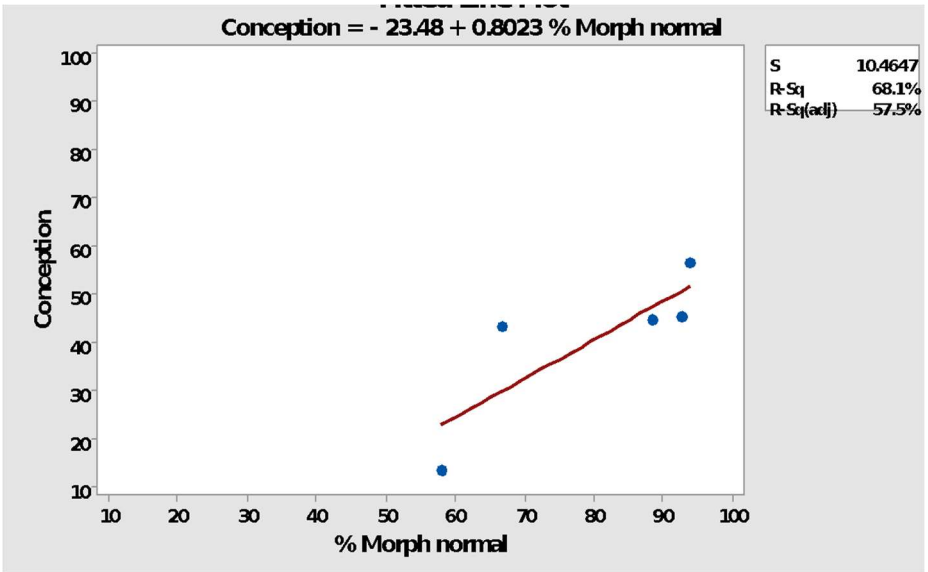


Fig 39 – Conception rate against % CASA morphologically normal sperm

The relationship demonstrated is positive, and there is a tighter fit of data points around the line of best fit for this parameter (S= 10.4647). This relationship is not significant (P= 0.0854).

d) Conception rate and Flow Cytometry % viability

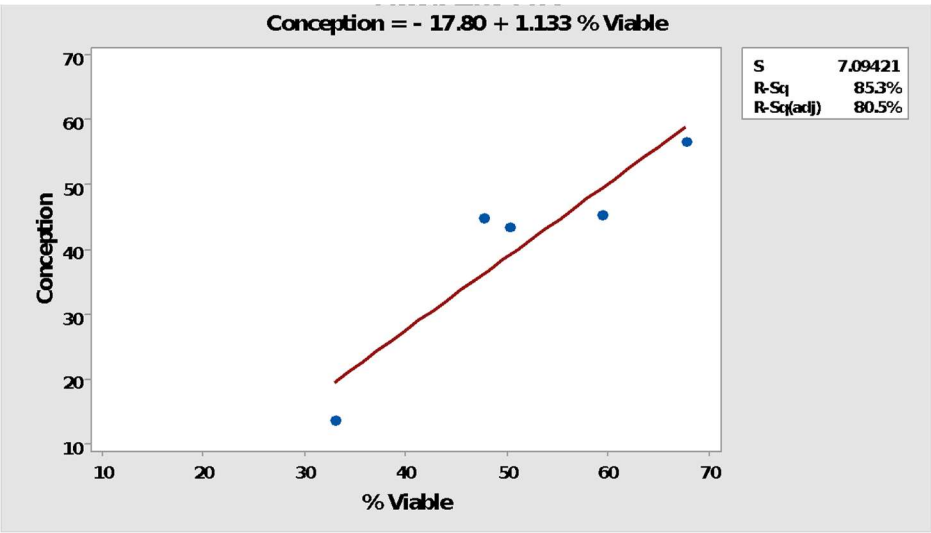


Fig 40– Conception rate against FC % viable sperm.

The data points fit well around the line of best fit, which shows a strong positive correlation (R sq = 85.3%), and P = 0.025, therefore the relationship between viability and conception rate is significant.

e) Conception rate and Flow Cytometry % polarised mitochondria

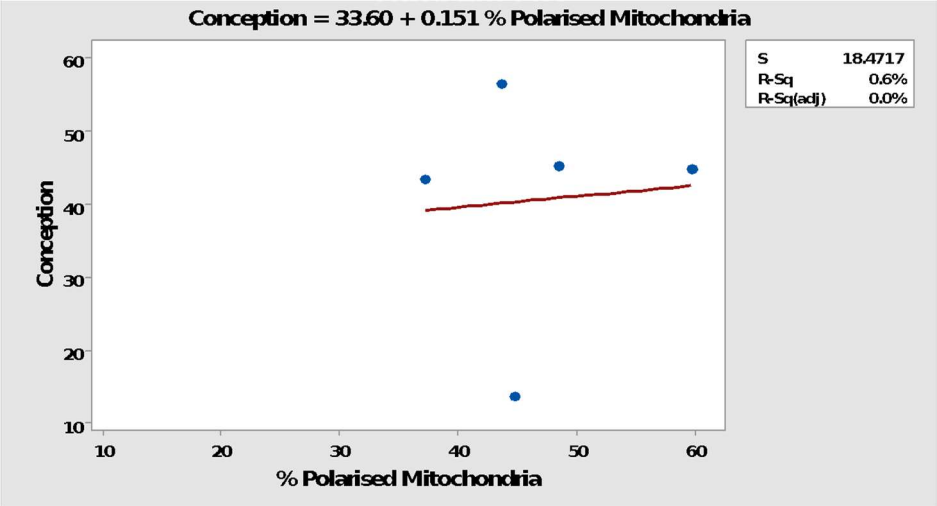


Fig 41 – Conception rate against FC % polarised mitochondria.

There is no relationship evident between these two variables in this situation.

f) Conception rate and Flow Cytometry % viable and intact acrosome

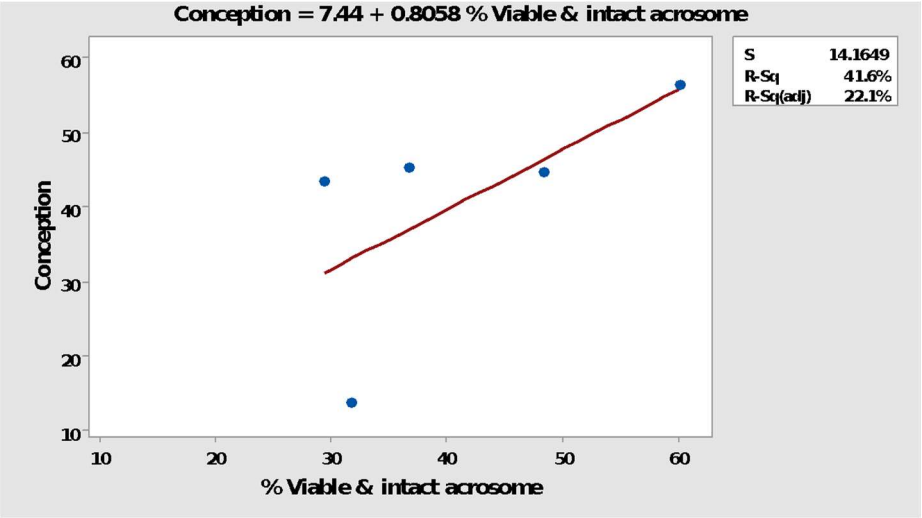


Fig 42 – Conception rate FC % viable and intact acrosome.

The low conception rate bull is a distinct outlier in this evaluation, and thus the correlation is extremely weak.

On samples from four of the bulls further analysis was performed. The samples were re-evaluated for % motile and % Progressively Motile following incubation at 37^{o}C for 2 hours, i.e. following thermoresistance testing (TRT). The results were plotted against conception rate also and are displayed below:

g) Conception rate and CASA % mobile after TRT

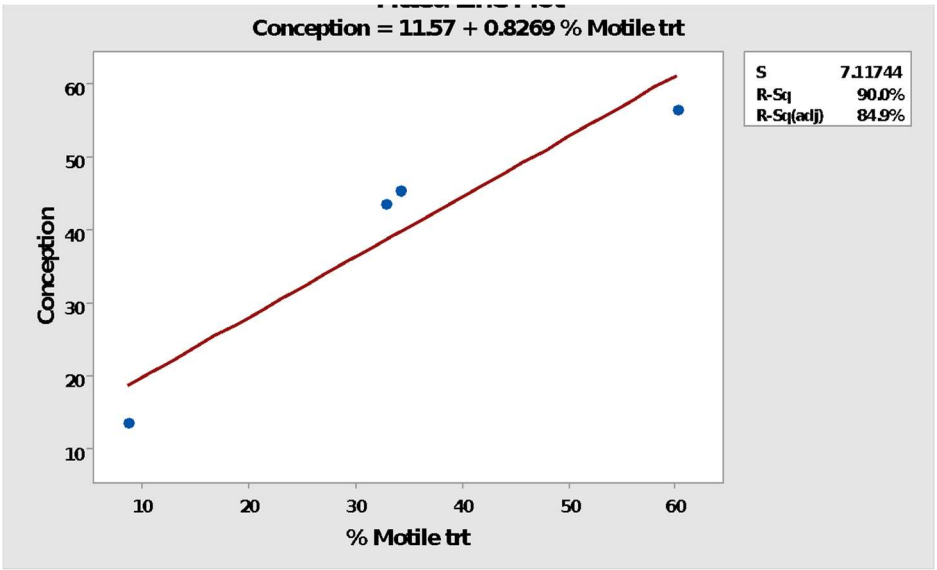


Fig 43 – Conception rate against CASA % motile after TRT.

This relationship is strongly positive (R Sq = 90%). There is good fit of data points to the regression line (S = 7.12). P= 0.05 in this relationship, therefore % motile TRT can be considered a significant indicator of conception rate.

h) Conception rate against CASA % progressively motile after TRT

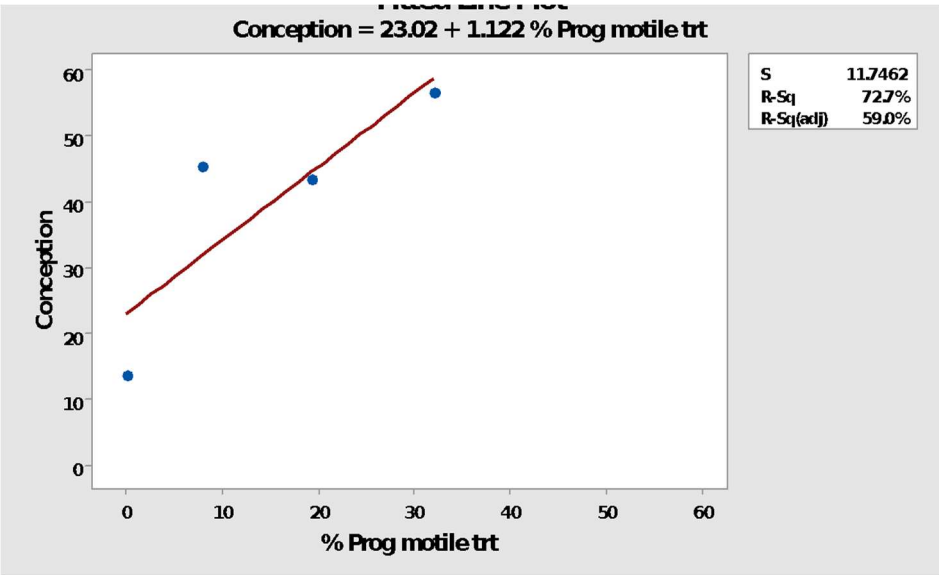


Fig 44– Conception rate against CASA % progressively motile TRT.

There is a positive correlation (R Sq= 72.7%), however the S value is greater than % motile TRT and P = 0.15, therefore the relationship is weak and not significant.

i) Conception rate against CASA VCL (um/s)

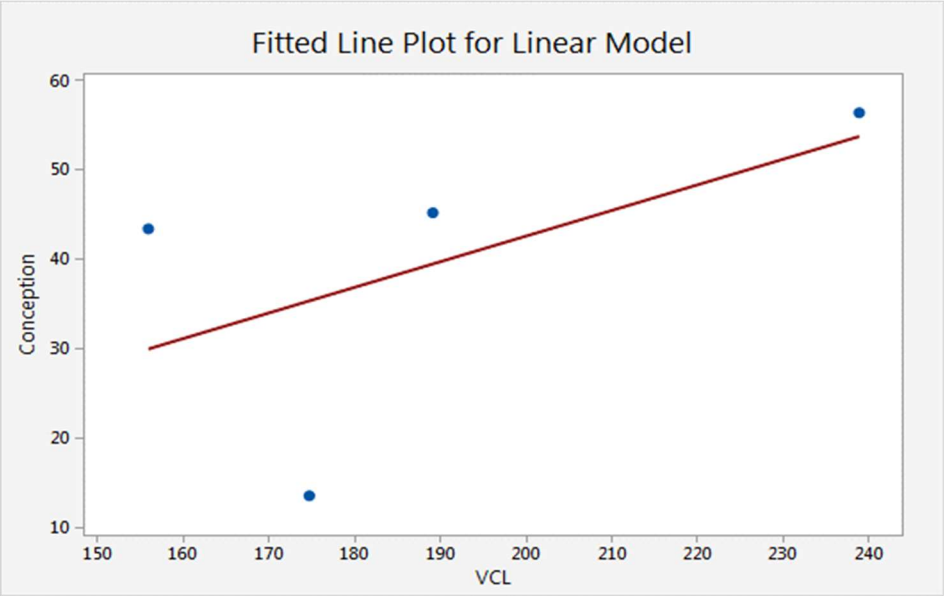


Fig 45 – Conception rate against CASA VCL

There is only a mildly positive correlation between VCL and conception rate (R Sq = 30.87%), and P = 0.44, therefore no significance to the relationship. There is an obvious outlier which will affect outputs with a small number of bulls analysed.

j) Conception rate against VAP (um/s)

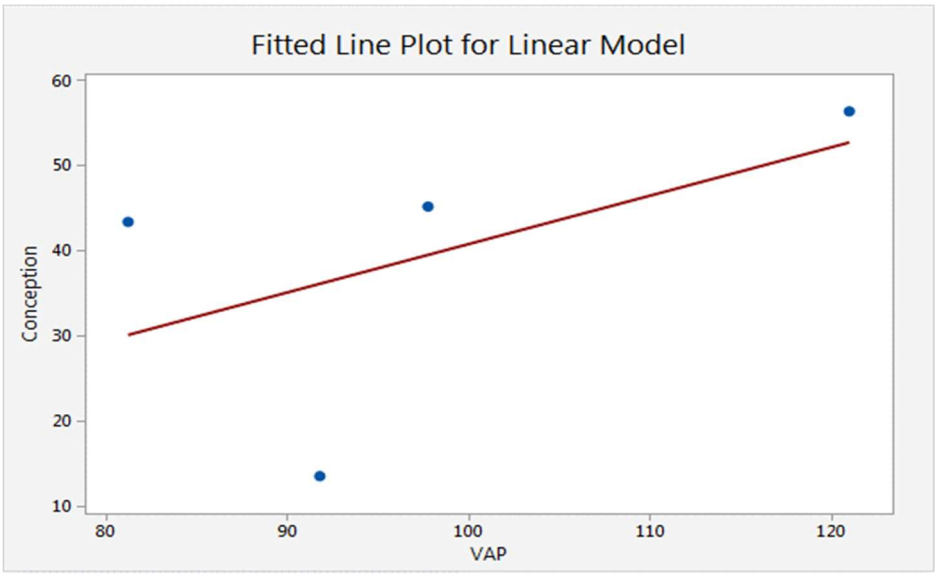


Fig 46 – Conception rate against CASA VAP

There is a mildly positive relationship between VAP and conception rate (R Sq = 27.24) and P = 0.48, therefore no significance to this relationship. Again there is a notable outlier in this dataset.

k) Conception rate against VSL (um/s)

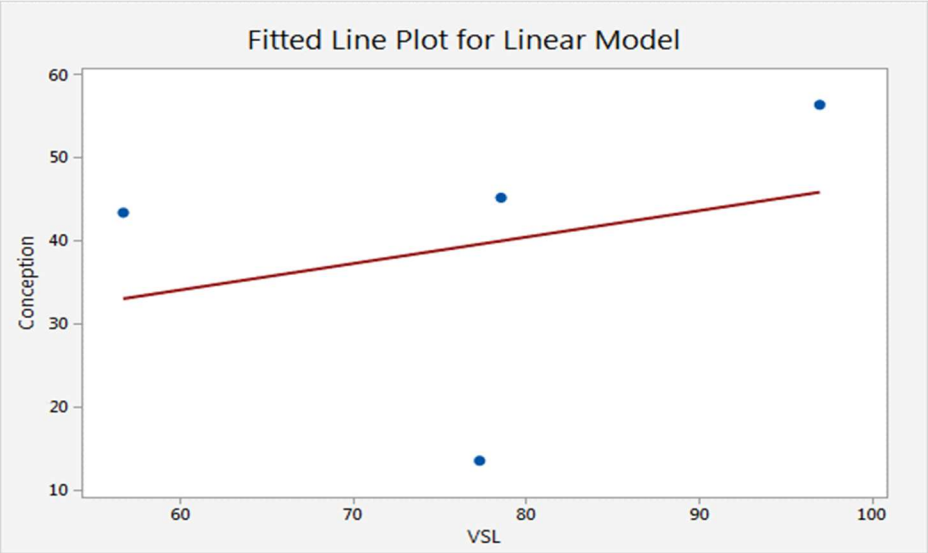


Fig 47 - Conception rate against CASA VSL

There is no obvious relationship between VSL and conception rate (R Sq = 8.18) and P = 0.71.

Discussion

AI Semen Quality assessment using CASA and flow cytometry

It is important that customers have confidence in the product that they are buying, and the breeding companies need to be confident in the products they are selling. Therefore, ensuring that the semen meets minimum standards pre-release is important. The standards are not uniform across all breeding companies in the UK, or internationally and will vary in their complexity from subjective motility assessment alone to multiparametric objective assessments of semen motility utilising CASA, flow cytometric assessments and morphological assessments. AI semen, if it has been stored correctly at -196^{0}C should retain fertilising potential indefinitely (Vishwanath and Shannon, 2000), and therefore we would expect all straws retrieved off farm to still be above the minimum release standards. However these minimum standards are not

entirely consistent between North America and UK. There is no industry standard for all parameters; not all studs have the same equipment to be performing all analyses.

Flow cytometry assays – both the North American and UK recommendations used the same cut off values for the viability assay. Only a North American stud provided cut off values for Mitochondrial activity, and no values were available for acrosome integrity assay. It is of concern that for any of the assays used to evaluate the semen taken from farm storage in this study, there was always a proportion of straws that failed to meet the cut off value.

Of greatest concern was that none of the sex sorted straws tested managed to achieve the minimum standard. Currently there is not an agreed UK pre-release standard for Mitochondrial activity, and therefore it is unlikely to have been measured in the straws evaluated prior to release. We therefore cannot say whether the mitochondrial activity has been negatively affected during storage on farm. The correlation between mitochondrial activity and sperm motility has been demonstrated by Garner et al. (1997) and Hallap et al. (2005) but no consistent relationship demonstrated with field fertility and therefore currently the mitochondrial activity assessments should not be looked at with too much significance in isolation, altough there was a small correlation between mitochondrial activity and field fertility demonstrated by Sellem et al (2015) and this may need to be looked into further. However, it appears in this experiment that the sex sorting process using flow cytometry, may affect the membrane potential of mitochondria compared with conventionally produced semen, as also described by Holden et al., (2017).

Some 70% of sex sorted straws passed the viability assay, compared with 65% of conventional straws, and the mean viability was greater for sex sorted semen. The viability of sperm within both conventional and sex sorted straws has therefore apparently deteriorated post release from the stud, either through handling of semen and the movement from flask to flask, or through poor storage practices on farm. Motility parameters used to screen the semen would only allow release of 44% of conventional straws and 10% of sex sorted straws if total motility was being used, representing a loss in motility below the 'standard' for 56% of conventional and 90% of sex sorted semen straws. This is of concern as over half of straws in use on dairy and beef farms in North Yorkshire were suboptimal for a parameter that accounts for a small fraction of variation of fertility in the field (Farrell et al,. 1998). The standard for progressive motility in North America is only 15% versus 30% in the UK, therefore more straws pass this test on the North American standards; 86% of conventional straws achieved this standard, compared with only 32% if the UK standard was used. Progressive motility is defined by the CASA machine evaluating the sample, but it generally requires sperm to be swimming with greater velocity and linearity, and therefore sex sorted semen potentially struggles to

achieve these values as the sorting process can impact on the motility of sperm (Carvalho et al, 2009). The pre-release standard that was satisfied by most straws that were evaluated in this experiment is the percentage of morphologically normal sperm as evaluated by CASA; with 90% of conventional and 70% of sex sorted straws still above standard. As the morphology of sperm will not be impacted greatly by any issues with storage or transport then it is logical that this is the parameter that still has the greatest percentage above the standard.

The proportion of straws that fail to meet pre-release standards when tested from farm storage does prompt concern that the quality of semen being used in AI may potentially be negatively impacting on fertility outcomes. It therefore may be a logical approach for farmers using AI to screen the quality of the semen in their flasks prior to using it, using a laboratory with CASA and flow cytometry, especially if they do not know the transport history of that batch of straws. This would allow suboptimal batches not to be used and only batches that are still above pre-release standards to be used. In the UK it is standard practice for Porcine liquid AI semen to be routinely screened by an independent laboratory where results are compared to a standard prescribed by AHDB Pork (formerly BPEX) - 'Standard for Porcine Semen quality in AI centres'; therefore the Levy body and industry have a constant knowledge of the quality of liquid AI semen being used on pig farms in the UK. In the cattle industry this is not the case, and quality is assumed to be above minimum standards. However, our results indicate that quality cannot be assumed and a similar Levy board led initiative may be appropriate to help identify sub-standard batches of semen or bulls.

Comparisons of results with Sellem et al., 2015

The laboratory that was used for this study was new. Validation and verification of the outputs generated, through comparison with a recent study that utilised the same flow cytometry equipment and assays and a CASA system manufactured by the same company, was carried out. Although the semen samples that were evaluated were different, as both studies analysed large numbers of straws it could be expected that there would be normal distribution of results with similar outputs. However, the sourcing of straws was different; Sellem et al. (2015) sourced straws from one stud and analysed them prior to distribution to farms, whereas this study utilised straws from multiple studs after they had been stored on farm for variable lengths of time. The Sellem study had mean results greater than this study for all parameters assessed, not always because the maximum results were greater, but also potentially because results in this study were skewed by some straws having results close to 0% for the flow cytometry assays and CASA motility. Such straws would not have been analysed in the Sellem study as pre-release screening would have meant that they were not distributed to farm.

Maximum values for all FC assays were comparable between studies, with the difference less than 10% for all parameters. This study had a greater maximum value for the Mitochondrial activity assay, whereas for all other assays the maximum values were greater in the Sellem study.

CASA evaluation of motility in this study had a much lower (20%) mean % motile than Sellem. This again is partly due to the minimum value being 0%, however in addition, straws analysed in this study did not exhibit maximum values as high as the Sellem study.

CASA morphology evaluation was of greatest concern as our minimum value was 52.4%. As morphology is not influenced by how semen is stored or handled, either there were some bull semen collections with substandard morphology or the CASA system was misdiagnosing morphological defects. However when settings used for analysis were checked between the two CASA systems they were comparable e.g. distal midpiece reflex droplet to tail end max = 5 (Sellem) vs 7 (this study), tail length max = 20 vs 20, droplet distal distance min = 5 vs 4, droplet proximal head length = 15 vs 10.5, elongation max = 79 vs 70 and min = 1 vs 3, tail bend angle averaging length = 5 vs 5, tail bending angle rate min = 20 vs 20, tail coiled angle min = 180 vs 180. The settings in this study were provided by the supplier and validated, therefore it is probable that there were some straws with high degree of morphological abnormalities that should not have been released by studs. As the CASA system does not have the ability to detect abnormalities of the head of the sperm, morphological evaluation is likely to overestimate the percentage of normal sperm, and therefore is not relied upon to evaluate sperm morphology in our laboratory currently.

The comparison with the Sellem study provided some confidence that our protocols in the laboratory were providing accurate and reasonable results reflective of the quality of the semen being analysed.

Flow cytometry - Viability assay

The viability assay results for % viable sperm in conventional and sex sorted semen were similar and no significant difference was found between the means of the two populations. Therefore, these results suggest that the process of sex sorting semen via flow cytometry prior to placing into straws and cryopreserving, does not adversely affect cell membrane integrity any more than conventional semen processing that does not undergo sex sorting. This is not in agreement with DeJarnette et al., (2011), who found post thaw viability to be greater in sex sorted semen, but it does suggest that the sex sorting is not

adversely affecting sperm viability. Ting-xi et al, (2016) found sex sorted semen to have lower percentage of viable sperm post thaw compared with conventional semen.

When viability assay results for dairy or beef breeds of bull were compared, the dairy breeds of bull showed a significantly larger percentage of viable sperm post thaw. There was approximately the same number of beef and dairy breed straws, so both populations are equally represented in the study. The dairy breeds were primarily represented by Holstein bulls, whereas the beef breeds consisted of a more diverse range. These results are in agreement with Morrell et al., (2018), who found a difference (P =0.053) in flow cytometry derived viability assay results in favour of dairy versus beef sires. This study did not look at individual breed variations within the beef and dairy groupings, but there has been shown to be individual breed variations in semen quality parameters, (Morrell et al., 2017). The breed variations may be as a result of selection for characteristics not related to fertility e.g. extreme muscling in Belgian Blue cattle. Also the desire to collect from pedigree beef bulls on farm, and therefore suboptimal conditions compared with stud collection for the vast majority of dairy bulls.

No significant difference was found in viability when assessing the origin of the straw as dairy vs beef farm, irrespective of the breed of bull. The aim of this analysis was to assess whether beef or dairy farmers were initially buying semen of differing qualities, which may be influenced by where the semen was produced i.e. on farm collection vs stud collection (private) vs stud collection (commercial) or whether they were storing or handling the straws in a way that impacted on the viability of the sperm. Although the type of farm was found to have no influence on the viability of the straws in the flask on that farm, if a dairy farm is using primarily dairy breed bulls, it is more likely to be using semen with better viability characteristics than a beef farmer using solely beef breed genetics.

Flow cytometry - Mitochondrial Activity assay

When the percentage of polarised mitochondria post thaw for conventional and sex sorted semen were compared, the results of the statistical analysis showed the difference to be highly significant, with a P value of <0.0001. This is in agreement with Ting-zi et al, (2016), who found numerical differences in mitochondrial membrane potential between conventional and sex sorted semen straws of the same bulls. These findings suggest that the flow cytometry process of sorting the female sperm, prior to placing into straws and

cryopreservation, results in changes to the membrane potential of the mitochondria within the female sperm. Smith et al (1993) found evidence of mitochondrial damage when using bisbenzimide stain Hoechst 33342 and UV light to visualise oocyte chromosomes and commented this maybe a biologically diverse attribute and so it is perhaps not surprising that this was shown here.

When the mitochondrial activity of beef and dairy breeds were compared, there was no difference between the groups, with means of 38.43% and 38.71% respectively. When Morrell et al (2018) looked at mitochondrial activity between beef and dairy breeds, there was a difference of 22%, which was significant ($P < 0.001$). In this study a greater number of bulls were evaluated, N= 82 vs N=37, and therefore these results may be more representative than the Morrell, 2018 study.

Whether the straws were being stored on beef or dairy farms did not have any effect on mitochondrial activity, with mean values of 36.10% and 39.52%, and a P value of 0.4418. As the mitochondrial activity is unlikely to be influenced by the location of storage, then this is an understandable finding. If the way in which the semen was being handled and stored on dairy and beef farms was going to impact on semen quality then the viability assay results would have likely been significant too.

Flow cytometry - Viability and Acrosome integrity assay

The changes that occur in the sex sorting process have been shown to have an effect on the status of the acrosome. Moce et al. (2006) showed that sex sorted semen had a higher percentage of acrosome reacted sperm in the live population than non-sorted sperm and concluded the sorting process induces changes in the sperm membranes that accelerated the capacitation and acrosome reaction processes of sperm after cryopreservation. Carvalho et al. (2010) also demonstrated a significant difference in post thaw percentage of live intact acrosome sperm between conventional and sex sorted semen in favour of conventional semen.

In this study there was no apparent effect of the sex sorting process on plasma membrane or acrosomal status, therefore the straws that were assessed had minimal acrosomal changes relative to the conventional semen. Based on this small population (n=10), the sex sorted semen should be just as capable of performing the acrosome reaction as conventionally produced semen, and therefore perform well in field fertility. The number of sex sorted straws analysed was small, and therefore this conclusion should be

treated with caution. Further studies to analyse more sex sorted semen samples should be carried out, including the newer sexed semen products SexedUltra4M (Cogent) and Sexcel(Genus).

The difference in percentage of viable acrosome intact sperm between beef and dairy sires did not show any significance in this study, however in the Morell et al study (2018), there was a significant difference in favour of beef sires for the percentage of live, intact acrosome sperm post thaw. This was in contrast to other measures of semen quality in that study, where dairy sires tended to be superior in quality.

Straws taken from flasks on dairy farms in this study had a significantly greater proportion of live intact acrosome sperm than straws taken from beef farms. Therefore, dairy farms may be sourcing better quality semen with regard to this parameter in the first instance or may be handling the semen better to maintain the quality of the semen in the flask.

CASA Evaluation of AI semen – CASA % Motile

This study showed a significant difference in total motility between conventional and sex sorted semen, which is consistent with the work of Carvalho et al. (2010), and Kurykin et al. (2016) who demonstrated significant differences in post thaw motility between conventional and sex sorted semen. This would suggest that the sex sorting process appears to result in a smaller percentage of motile sperm in the population prior to cryopreservation, or a smaller number of sperm that are progressively motile after cryopreservation as a result of changes occurring during the sorting process. The reduced motility may due to alterations caused by the stain, the laser or the electric charges and physical forces applied to droplets of sperm in the sexing process (Carvalho et al., 2010). DeJarnette et al. (2011) also found conventional semen to have a numerically greater total motility post thaw compared with sex sorted semen.

Morrell et al., (2018) showed a numerical difference in total motility of sperm between beef and dairy sires, with beef sires having greater motility, however this was not significant. This is not consistent with the data from this study, that showed dairy sires to have numerically greater motility. However, this difference was not found to be significant. Another study by Hoflack et al. (2007), comparing a specific dairy breed, Holstein, with a specific beef breed, Belgian Blue, demonstrated that the Holstein bulls had significantly greater total motility. Our study will have mainly consisted of Holstein sires as the dairy breed and Belgian Blue as the beef breed, but with a number of other breeds in addition. This confounding factor may have resulted in the lack of significance in this study compared with Hoflack et al. (2007). The same study

suggested that the kinematic differences between Holstein and Belgian Blue sperm may be related to the shape and size of the head of the sperm, with a larger head making the movement slower and less fluent.

Semen taken from flasks on beef farms had significantly poorer total motility, compared to flasks on dairy farms. As the difference cannot be stated to be due to the breed difference being stored, we can ask if the beef semen being stored on beef farms is either of poorer quality when purchased compared with semen purchased for dairy farms e.g. privately collected pedigree beef semen vs commercially collected dairy or beef semen stored on dairy farms, or that the beef farms are not storing and handling the semen adequately compared with dairy farms. Further work is required to evaluate potential reasons for lower motility of semen between beef and dairy farms, as this may be a barrier for some beef farmers to utilising AI.

CASA Evaluation of AI semen – CASA % Progressively Motile

The progressive motility results from this study followed the same pattern as total motility when looking at conventional vs sex sorted, beef vs dairy breed and beef vs dairy farm. The same reasons that were described for the differences in total motility results are likely to be valid for progressive motility.

CASA Evaluation of AI semen – CASA Morphology

The morphological evaluation performed by the CASA system produces an output figure of % normal morphology, and then classifies the abnormal sperm dependent on the defect. However, the CASA system is unable to identify abnormalities affecting the head of the sperm and detached heads. It is therefore likely to be an underestimate of the percentage of abnormal sperm in the straws being evaluated. The evaluations were therefore checked by technician to ensure that the midpiece and tail defects that were identified were being correctly classified.

When the mean percentage of morphologically normal sperm of conventional and sex sorted semen were compared in this study, the conventional semen had a significantly greater percentage of morphologically normal sperm. This was not consistent with Carvalho et al. (2010), or Holden et al. (2017), where no difference was detected between sex sorted semen and conventional semen. There was a significantly greater number of acrosomal abnormalities detected in conventional semen when Kurykin et al., (2016) compared sex sorted and conventional semen, as well as a numerically greater number of abnormal midpieces (P=0.06) in conventional semen. As these abnormalities were assessed using microscopy at 1000x, these defects were being picked up, whereas in our study these would have been

missed by the CASA morphological evaluation. As a result of these findings and the CASA systems' inability to identify head defects, the decision has been made to utilise phase contrast microscopy at x1000 and under oil immersion to evaluate morphology in the future. Also, in this study the same bulls were not compared in each population (sexes vs non sexed), therefore there will have been some individual bull variation impact on the results. It may be that the sex sorted bulls in our study had greater number of morphological defects compared with the bulls in the conventional group, and as the population was small (N= 10), results could be more influenced by any single 'substandard' bulls.

The findings in this study relating to morphology of beef sires vs dairy sires, where the dairy breed had significantly greater proportion of normal morphology compared with beef sires, was consistent with Morrel et al., (2018) and Hoflack et al., (2007) who found beef sires to be significantly inferior relative to dairy breed of sire with respect to morphology. Hoflack et al., (2007) and Morrel et al., (2018) suggested that the breed difference may be due to the continuous selection for fertility in Holstein AI sires, whereas this is not the case in the Belgian Blue breed, where selection is usually for other traits. Even though morphology may not always directly be selected for, due to its impact on sperm motility and therefore potential performance in the field, morphology is indirectly selected for. Morrel et al., (2018) also suggested that factors such as increased scrotal circumference, Holstein vs Beef breeds, and adaptation to the environment may be factors in different breeds producing semen with greater percentage of morphologically normal sperm. Although analysis of morphology was evaluated with CASA in this study, and therefore no head defects were able to be detected, findings were consistent with studies that used conventional microscopy. However, the defects that are not detected by CASA are non-compensable and can have significant impacts on fertility; therefore for both commercial work and research at this laboratory all morphological examinations will be performed by an experienced technician using phase contrast microscopy.

In summary, the best quality semen in this study was conventional semen of dairy breed and stored on dairy farms. This semen is therefore more likely to perform better in the field than the sex sorted, beef breed semen stored on beef farms. If a semen screening service was available through an andrology laboratory then although of benefit across all sectors, it may be even more relevant to beef farmers using beef breed semen. Beef farmers may also benefit from further training on semen storage and handling to minimise damage in storage/ transit so that the semen they purchase remains as good a quality as possible.

Bull breeding soundness evaluations and semen assessment

Bull breeding soundness evaluations are performed in the field and the semen evaluation aspect of the assessment is generally performed subjectively utilising microscopic examination of the semen sample under low power for gross motility and medium power to evaluate progressive motility. Subjective assessment of sperm motility has been shown to have between and within operator variation in motility assessments (Vincent et al., 2012). Farrell et al. (1998) showed that CASA analysis provided more repeatable and more discriminating estimation of motility of a sample compared with the subjective assessment of experienced technicians. When looking at CASA vs subjective assessment of motility, Broekhuijse et al (2011) showed that CASA results are preferable as accurate continuous motility data are generated rather than discreet motility percentages in increments of 5-10%, as it is with motility estimation by laboratory technicians. Therefore, when looking at fresh semen evaluation as part of breeding soundness evaluations in this study, on farm subjective assessments of motility were compared with subjective assessments in the laboratory by an experienced technician and also an objective CASA assessment. Both CASA total motility (CASA % motile) and progressive motility (CASA % Prog motile) are reported, however the value that is most directly comparable to the bull side motility assessment is the CASA % motile, as it is challenging for a subjective assessment to differentiate motile sperm from progressively motile sperm as this is defined by kinematic properties of the sperm track that is not measurable subjectively. Subjective assessment is limited to sperm that are swimming in a progressive manner and static or non-progressive sperm. From this work it was proposed that results would inform practitioners of the benefit of having access to a semen assessment laboratory for fresh sample submission.

The difference between the two subjective assessments is of interest, as there was a significant difference between the means of the two measurements. This may be partly due to the fact that there is a time lag of up to 18 hours between the measurements taking place. However, the protocol that was used to transport semen from farm to the lab is consistent with protocols that a number of commercial studs use to transport semen from farm to stud (collected by AV and EEJ), and between collection centres and processing centres, such as Singleton (1970), therefore this is unlikely to be the only factor involved. The fact that the experienced technician in the lab operates the CASA machine on a regular basis may help to 'calibrate' their subjective assessment; they are perhaps more likely to be more objective in their approach to motility assessment. Therefore, the difference may be partly due to the time delay but also potentially partly due to overestimation of motility by the practitioners in the field, as was demonstrated demonstrated

when subjective and objective assessments of porcine semen motility were compared by Broekhuijse et al.(2011).

The benefit of CASA is that motility data is generated that is accurate and continuous, whereas subjective assessments result in discreet motility percentages in increments of 5-10% (Broekhuijse et al, 2011). In this study the median value for practitioner evaluated (BBSE) Prog motile was 75%, with Q1 at 65% and Q3 at 80%, therefore exhibiting a narrow interquartile range. The data distribution is skewed to the right slightly. The borderline for a bull to pass a bull breeding soundness evaluation is 60% progressive motility, and the mean value in this data set is 70%. This could reflect the subjectivity of the assessment, and that the practitioners evaluating the samples have this figure in their head when attempting to quantify the percentage of motile sperm and their assessments may be biased. The same samples analysed through the CASA produced data (CASA % motile) with a greater range, interquartile range and a more normal distribution, therefore more 'continuous and accurate'. The greater interquartile ranges for both CASA measurements and the subjective assessment by the laboratory technician (RAFT Prog motile%), may indicate that the subjective assessment in the laboratory is more 'objective' than the field assessment of motility (BBSE Prog motile %); this is consistent with Farrell et al. (1998), who found that the ranges of motility results of the same samples analysed by CASA were more accurate and discriminating than subjective assessments. The relative objectivity of the experienced laboratory technician is demonstrated by the fact there is no significant difference in the mean values of RAFT % Prog motile and CASA % motile. Therefore, it could be said that the practitioners performing semen evaluation on farm, could benefit from training that would increase the objectivity of their assessments, or from being able to send samples to a semen laboratory for motility assessment by CASA. If practitioners did routinely submit samples, and strict handling and transport protocols were adhered to, then motility evaluation performed by CASA could provide more accurate motility values, with no bias. It may be necessary to alter the boundaries for pass/ fail because, as discussed above, the mean motility value for CASA % motile was 19% lower than the BBSE Prog motile %.

If practitioners were submitting samples to a laboratory for motility assessment it may also be of benefit to perform flow cytometric analysis on those fresh extended samples, as it may give greater information on the semen quality of the bull, that may not otherwise be picked up on a standard breeding soundness evaluation. Further analysis would also provide verifications of the subjective assessments made. Kastelic and Thundathil (2008) concluded that cell biology approaches, e.g. plasma membrane viability, sperm capacitation, may serve as supplementary tests to a standard breeding soundness evaluation and

improve the reliability of fertility predictions. If the flow cytometry assays were shown to add power to predictions of field fertility of breeding soundness evaluations using fresh extended semen (Motility and morphology) then they could become part of standard breeding soundness evaluation. The flow cytometry data for fresh extended semen when compared with Gonzalez-Marin et al. (2018), showed mean viability to be 17.5% lower at a similar time post collection e.g. 24 hours. This may indicate that the processing on farm and transport to the laboratory is reducing the viability, compared with the viability results at 24 hours post collection when incubated at 18^{O}C but not transported, as in the Gonzalez-Marin et al. study. The total motility evaluated by CASA in the same study after 24 hours of incubation, and our fresh semen evaluation on arrival at the lab was 18% greater, and subjective assessments bigger by 23%. Therefore, alternative transport/ incubation protocols, or potentially different semen extenders may need to be investigated if laboratory analysis is to be validated further, and therefore offered as a commercial service without impacting on motility results. The transport protocol for our submissions used similar systems to those described by Singleton (1970), who was developing procedures for receiving submissions from bulls being kept on private farms for processing for AI. Bespoke polystyrene boxes were utilised, containing ice bricks, whereas Singleton (1970) utilised vacuum flasks. In both protocols the sperm were insulated from ice bricks by cotton wool or bubble wrap. Motility evaluation prior to processing was 66% in the Singleton (1970) study, whereas in this study mean total motility on arrival at the lab was 52%. The use of storage and transport at more ambient temperatures (Vishwanath and Shannon, 2000), should be investigated as an alternative protocol.

The morphology evaluation aspect of bull breeding soundness is usually performed by the veterinary practitioner under high power oil immersion phase contrast microscopy. There is likely to be variation in operator experience, skill and also microscope quality which may impact on the reliability of the results. Therefore, if practitioners were submitting fresh semen samples to a laboratory for motility analysis, it may be worthwhile performing morphology analysis too. If this could be performed on the CASA without the need for phase contrast microscopy it would be a swifter process. When the results of the morphology assessments produced by CASA and Phase contrast microscopy in our study were compared, the mean value generated by CASA was greater than the figure produced by phase contrast microscopy, but the difference was not significant. The phase contrast microscopy was performed by an experienced veterinarian. As CASA is unable to detect abnormalities of the head, and only identifies abnormalities of the midpiece and tail it is likely that the CASA will underestimate the number of defects in a sample. Also, by only being able to analyse the midpiece and tail the CASA system will not detect the important primary defects in the organelles that are known to be involved in fertilisation, such as acrosome (Garner, 1997). Due to the inability of the CASA

system to evaluate these defects CASA is not relied upon in this laboratory to perform morphology assessments.

The work in this study provides some further indications that a specialist andrology laboratory, as discussed by Lorton (2014), could be a useful service to veterinary practitioners that are performing bull breeding soundness evaluations in the field in the UK. As long as semen handling and transport protocols are adhered to, sending semen samples into the laboratory for objective assessment of motility will provide an accurate evaluation of the motility, rather than a subjective estimate to the nearest 5 or 10%, which may potentially be influenced by the operator knowing where the 'cut-off' for the pass/ fail decision is on the BCVA breeding soundness evaluation. The similarity of our trained laboratory technicians' assessment of motility with the objective assessment by CASA provides some indication of the benefit of being able to utilise CASA to 'calibrate' one's own subjective assessments. Therefore, even if practitioners did not routinely submit semen samples for motility evaluation, they may benefit from training sessions in the andrology laboratory, utilising CASA to cross reference with their own subjective assessment of motility of the same sample and thereby refining their process for evaluating semen motility. The experiences of Barth (2018) highlight the inconsistencies in how well practitioners are equipped to perform morphological evaluation and also how well they are performed. If practitioners do not have available the appropriate equipment to perform analysis optimally, then perhaps they should consider submitting semen samples to an andrology laboratory where there is high quality equipment and experienced, highly trained individuals to perform the evaluation.

CASA and Flow Cytometry parameters and field fertility

The eventual aim of performing multiparametric analysis of semen using CASA and Flow cytometry is to provide a semen quality index that is predictive of fertility (Sellem et al., 2015). In this study all the parameters measured were examined individually through simple regression analysis to establish any correlation between those results and the known conception rate of particular bulls used on one farm. The assessments that showed a significant correlation were the percentage of viable sperm post thaw assessed by flow cytometry (R^2 = 85.%; $P<0.05$), and the percentage of sperm motile after 2 hours thermoresistance test (TRT) (R^2 = 90%; P= 0.05). CASA motility has been correlated with fertility in the field by a number of authors (Christensen et al., 2011, Farrel et al 1998, Januskauskas et al., 2000b) but this is not a consistently demonstrated relationship. The post thaw total and progressive motility in this study did not show strong correlations with conception rate (R^2 = 47.1%, P=0.2 and R^2= 33.1%, P=0.3 respectively), and therefore the

value in performing the thermoresistance test was demonstrated. The thermoresistance test is run routinely as part of the laboratory standard CASA analysis, as it may provide more information as to how a bull will perform in the field rather than just looking at post thaw motility. Thermoresistance testing was used in an investigation of CASA results of 3 bulls and the relationship to fertility by Oliveira et al. (2012); the 4 hour CASA motility differences were significant between 2 bulls (1 and 3) and bull 2; however this did not correlate with any difference in conception rate. The same author demonstrated that total motility and progressive motility after 2 hours of thermal incubation were important predictors of conception rate (Oliveira et al., 2013).

There was a positive but not significant correlation (R^2 = 68.1%; P=0.09) between CASA morphology and conception rate in this study. Sperm morphology is a routine screening test for semen quality and its relationship to field fertility has been confirmed previously (Fitzpatrick et al., 2002), and should therefore be included in standard laboratory semen analysis. As previously discussed the utilisation of CASA to evaluate sperm morphology is likely to overestimate the proportion of normal sperm, as it is unable to detect defects in the midpiece and head of the sperm. In an Australian study the parameter that was most predictive of conception rate was the percentage of morphologically normal sperm assessed by phase contrast microscopy immediately post-thaw in the AI sires (Phillips et al, 2004).

Sperm viability post thaw has also been correlated with field fertility (Januskauskas et al., 2000b; Januskauskas et al., 2003, Christensen et al., 2011) and therefore data from this study is consistent with these previous studies. Because sperm viability is performed with flow cytometry, it enables large numbers of sperm to be analysed e.g. 5000 or more, and therefore the analysis is reliable and precise (Christensen et al., 2011). This is therefore an essential parameter to assess as part of standard laboratory semen analysis.

There was no apparent relationship between mitochondrial activity and conception rates in this study based on the five bulls assessed. Mitochondrial activity has been correlated with sperm motility (Garner et al., 1997, Hallap et al., 2005), and with field fertility (Sellem et al., 2015) but there was no correlation between motility and mitochondrial activity in this study, (R^2 = 4.5%; P=0.72).

Acrosomal status and the ability to undergo the acrosome reaction in frozen thawed sperm can impact on fertility (Birck et al, 2010), and therefore the percentage of sperm that were viable and had an intact acrosome post thaw was assessed. This would indicate the level of cell and acrosome specific damage caused as a result of cryopreservation. When these values were correlated with conception rate there was only a mildly positive but insignificant correlation with conception rate. The study did not assess whether

sperm with intact acrosomes were capable of undergoing the acrosome reaction; this investigation may be performed in the future as it is this process which is crucial to successful fertilisation and conception.

Average Path Velocity (VAP), curvilinear velocity (VCL) and straight line velocity (VSL) were evaluated in four bulls and those values were assessed relative to conception rate. There was no correlation with fertility for any of these parameters in this study. This is not in agreement with other studies (Farrell et al, 1998), who found these factors increased the ability to predict fertility when more than one parameter was assessed. These parameters are always recorded when CASA is used to assess semen, so can always be looked at independently or in conjunction with other parameters to help increase completeness of the analysis and increase prediction of fertility.

Although this was a retrospective study and only based on a small number of bulls in one herd, it is still possible to see that if the screening of the semen had been performed prior to the breeding season then the poorest performing bull in terms of conception rate would have been highlighted as suboptimal. With this information prior to the breeding season this bull may not have been used, and if possible more straws of bulls that ranked highest on the tests used instead.

Chapter 5

General Discussions and conclusions

Questionnaire

The aim of the AI Semen Flask management and semen thawing protocols questionnaire was to establish how well semen storage and straw handling at point of thaw & AI were being managed by farmers. The potential risk of mishandling of flasks and semen resulting in deterioration in semen quality whilst on farm could then be considered. It can't be assumed that all farmers are following evidence-based semen storage, handling and insemination guidelines, as described by Diskin and Kenny, (2016). It is likely that farmers performing DIY AI will have attended an approved training course that includes the relevant training on flask management and semen storage, such as the 'Farmskills' Defra approved training delivered by the author. However not all farmers surveyed will have completed their DIY training on this particular course. Some farmers may have been performing DIY AI for a number of years, and it is possible that over time they have 'drifted' from the protocols that are regarded as best practice, even if they were appropriately trained originally. It is advised that liquid nitrogen levels in semen flasks are monitored weekly by the farmer (Nebel,

2007), so that any issues in flask performance are picked up early, however only 6% of the farmers surveyed performed this, and 45% of farmers did not monitor at all. It is likely that a reliance on the personnel who provided the liquid nitrogen top up service existed, however the majority of farms only received a top up on a monthly basis at best & were therefore not monitoring weekly. It can also be concluded that thawing practices were not optimal or consistent with current best practice as half the farmers that responded were thawing at temperatures of over 37^0C, and current recommendations are 35^0C (Diskin, 2018) and 33-35^0C and (DeJarnette et al., 2000). Thaw times were also not in line with current guidelines of 30 - 60 seconds (Diskin, 2018); only 10% of farmers were thawing for more than 30 seconds. Flask handling, semen storage, as well as straw thawing practices were not optimal in this study; it is possible that the farms surveyed were not maintaining semen in the same state that it was delivered in and this could be contributing to the poor quality of semen seen in the analyses. All farmers performing DIY AI could benefit from regular refresher training sessions to keep up to date with current protocols, especially as sex sorted semen becomes more common, and therefore correct handling becomes more important. Flask management questions not answered in this study include the number of bulls in total in storage, the number of bulls per can, the length of time they have been in storage and the age of the straws? These would have been interesting extra results to discuss.

Comparison with pre-release standards

The number of straws that failed to achieve the minimum pre -release standards is of concern. From this it can be concluded that farmers in north Yorkshire are not all using AI semen of suitable quality and this could be impacting on fertility performance. This level of drop is possibly due to a drop in semen quality in transport and storage as discussed previously, but may also be due to inconsistent pre-release standards being employed when comparing breeding centres. Some centres may be using solely subjective assessments to assess pre -release quality rather than objective measurements as in this study. Vincent et al. (2012), compared the use of subjective and objective methods to screen the quality of semen. There was agreement in 77% of cases that semen was fit or unfit for purpose, but in 11% cases subjective assessment passed the semen that was rejected by objective assessment (CASA and Flow cytometry). A similar level of disagreement may be expected if some of the straws analysed had been assessed subjectively. However, 10% or more straws failed standards on all parameters looked at; it is unlikely this is solely due to discrepancies between subjective and objective assessments. The cause of the high prevalence of poor quality semen is unclear, but to help mitigate against using poor quality semen, pre-screening prior to use may help ensure suboptimal batches are discarded. Conversely if pre-screening semen, the bull or batch that has the best performance in the multiple objective assessments should be selected, to help achieve good fertility performance. Alternatively monitoring the pregnancy rate by bull extremely closely, and testing any

bulls that is more than 2 standard deviations below the pregnancy rate mean my identify sub fertile bulls. However, by the time these issues are detected there has already been losses due to poor fertility.

Comparing categories of semen

Differences in conventional vs sex sorted, beef breed vs dairy breed and beef farm vs dairy farm may potentially explain how straws had failed the pre-release standards. Significantly poorer quality semen with regards four of the assays was found on beef farms vs dairy farms. This could be explained by either mishandling in transport to the farm and on the farm resulting in a drop in quality, or that the semen was of poor quality when it began its journey to the farms. Poorer quality semen arriving on a beef farm could be due to it being from a beef breed of bull, which we showed to be inferior to dairy in a number of traits in agreement with Morrell (2017 and 2018), but also could be due to the source of the straws. It is the author's opinion that the majority of 'on farm' collected semen is from pedigree beef bulls. As this collection is performed on farm, often in suboptimal conditions, and not processed as per semen collected on stud, it is possible that quality post thaw will be less. Even though it may pass pre-release standards (subjective or objective), the amount of deterioration required to fall below satisfactory standard is potentially less. There needs to be more work done to establish breed impacts on semen quality, and whether method and location of collection can have significant impact on semen quality. For beef farmers in particular to have more confidence in utilising AI, they should have access to use better quality semen than that which was analysed in our study. Dairy, but beef farmers especially may well be advised to test at least one straw per batch of semen prior to use, utilising objective assessments including CASA and flow cytometry.

Sexed semen

The small number of sex sorted straws that was examined in this study showed significantly poorer motility and progressive motility, as well as poorer mitochondrial activity. Due to the small number of straws assessed, it cannot be conclusively stated that sexed semen is inferior to conventional semen. The advent of new sexed semen technologies such as SexedUltra 4M (Cogent) and Sexcel (Genus), warrants much further work to establish how these new processes are improving the laboratory assay results, and if they are consistent with Gonzalez Marin et al., (2018).

BBSE

Utilising CASA and flow cytometry in assessment of semen collected at BBSEs would increase the objectivity of the semen quality assessments compared with the subjective assessments traditionally performed by the practitioner on the farm. This was demonstrated by the greater & more normal range of results produced by objective assessments compared with narrow on farm subjective assessments in our study. It also reduces

any bias that may be present in the mind of the practitioner when making a decision to pass or fail a bull. The benefit of CASA was demonstrated by Broekhuijse et al., (2011), who compared CASA with subjective assessments of motility in pig studs, where technicians were performing assessments of motility daily. The benefit of CASA over subjective assessments may be even more pronounced for practitioners who are only performing semen motility assessment on a weekly or monthly basis. The breeding soundness diagnosis can be a contentious one, and one that bull owners may try and influence by applying pressure to veterinary surgeons, or having the bull re-tested elsewhere until the bull is passed (Barth, 2018). Therefore having an objective measure of motility utilising CASA can remove this awkward aspect of passing or failing a bull on a subjective motility assessment. It is also appreciated that although the standard BBSE will identify bulls with substantial deficits in fertility, it will not always identify sub fertile bulls, and a more comprehensive approach is required including assessment of sperm function (Kastelic and Thundathil, 2008). Flow cytometry assays may therefore play a part in this. Further work is still required to evaluate the relevance of some of the flow cytometry assays performed in this study with fertility in the natural service situation. It should be noted that CASA is unable to identify morphological defects affecting the head of the sperm, and therefore some of the non-compensable defects that have significant impacts on fertility. However the importance of high quality equipment and a knowledge of good equipment maintenance and operation is important (Barth, 2018), and a number of suboptimal setups are present in practice. Therefore providing a service to evaluate morphology utilising high quality, well maintained equipment by a highly trained veterinary surgeon may provide a solution, as is the case in Australia where specialist andrology laboratories exist for this purpose as described by Lorton (2014). An andrology laboratory could be extremely beneficial to veterinary surgeons performing BBSE in the UK as it could provide objective assessments of motility to high accuracy, and offer supplementary flow cytometry tests (dependent on further work identifying relevant assays) as well as providing morphology evaluation to a high level of skill and accuracy.

Correlations with field fertility outcomes

The parameters identified as having significant correlations with field fertility in this small scale study (Viability, Morphology, Motility post TRT) have previously been described, and so the relevance of those assays when looking to predict fertility or mitigate against poor fertility is recognised. The ability to predict fertility relies on a multiparametric approach (Vincent et al., 2012), which includes other flow cytometry assays that were not all performed in this study, such as oxidation status and the SCSA that can increase the predictive power of semen analysis (Sellem et al., 2015). To be able to more confidently identify bulls that are likely to perform well or not in the field, these assays should also be run and the algorithm developed by Sellem et al. (2015), used to rank bulls by fertility index. To provide more power to these predictions of fertility, it would be ideal if all breeding companies performed all the CASA and FC assessments on batches

of semen prior to release, and then followed these through to pregnancy data produced on farm, so that the algorithm becomes more strongly predictive of fertility. An organisation such as XL Vets may be able to provide sufficient pregnancy data, from semen with known CASA and FC results, to enhance the predictive ability. It will then be possible to test a group of AI bulls prior to use and select the bulls that are more likely to perform well in the field, and also discard the low ranking bulls who are likely to negatively impact on the herd fertility. It would also be possible to re-test straws that have been in storage for a period of time, to assess whether there has been any deterioration in quality, and therefore whether it is worth using that batch of semen still.

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