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Optimising semen processing procedures of liquid and frozen-thawed bull semen in a commercial artificial insemination centre

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UNIVERSITY of LIMERICK

OLLSCOIL LUIMNIGH

**Optimising Semen Processing Procedures of Liquid and
Frozen-Thawed Bull Semen in a Commercial Artificial
Insemination Centre**

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February 2018



Declaration

I hereby declare that I am the sole author of this thesis and that it has not been submitted for any other University or higher education institution, or for any other academic award in this University. References and acknowledgements have been made, where necessary, to identify the work of others.

Signature: _____

Date: _____

Edel Murphy

Dedication

To my family, without whom, I would not be where or who I am today.

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List of Abbreviations

AF647	Alexa Flour 647
AI	Artificial Insemination
ANOVA	Analysis of Variance
ALH	Lateral Head Displacement
BCF	Beat Cross Frequency
BSA	Bovine Serum Albumin
BSP	Bovine Seminal Plasma
BTS	Beltsville Thawing Solution
CASA	Computer Assisted Sperm Analysis
CR	Calving Rate
CUE	Cornell University Extender
dH ₂ O	Distilled Water
DIM	Days in Milk
DNA	Deoxyribonucleic Acid
EBI	Economic Breeding Index
EBV	Estimated Breeding Value
GEBV	Genomic Estimated Breeding Value
GLM	General Linear Model
ICBF	Irish Cattle Breeding Federation
LDL	Low Density Lipoproteins
LIN	Linearity
NRR	Non-Return Rate
ns	Non-significant

N ₂	Nitrogen
PI	Propidium Iodide
ROS	Reactive Oxygen Species
sem	Standard Error of the Mean
SPSS	Statistical Package for Social Science
STR	Straightness
TSN	Total Sperm Number
VAP	Average Path Velocity
VCL	Curvilinear Velocity
VSL	Straight-line Velocity
WOB	Wobble

Chapter One

Literature Review

1.1 Introduction

Artificial insemination (AI) has revolutionised the dairy breeding industry and is the single most important technique devised to facilitate the genetic improvement of animals (Oliveira et al. 2013, Black 2006). This is evident by the genetic progress achieved within the dairy industry over the past number of decades (Amer et al. 2007). Ireland operates a seasonal grass-based production system (Dillon et al. 2006) which focuses on maximising low cost milk production through the consumption of cheap grass thereby, facilitating a significant cost advantage. This means that the system is highly dependent on excellent reproductive performance, whereby compact calving is co-ordinated to coincide with the start of the grass growing season (Shalloo et al. 2007). In Ireland, the implementation and use of a genomic breeding program for dairy cattle since 2009, has allowed AI centres to reliably identify potential AI sires within weeks of birth as a genomic proof is equivalent to a bull having 40 daughters milking (Berry et al. 2009). Whilst selecting bulls at a younger age has shortened the generation interval (Bouquet and Juga 2013), this presents a number of challenges to AI centres as semen yield from these young bulls is reduced compared to their mature counterparts (Brito et al. 2002a). The pressure on AI centres to produce semen from these young genomically-selected bulls is growing as demands for their semen greatly outweigh production. Thus, it is of the utmost importance to optimise semen processing procedures within AI centres in order to better utilise this valuable semen. Therefore, using a combination of *in vitro* semen quality assessments coupled with large scale field trials, the objective of this thesis was to optimise semen processing procedures of both liquid and frozen-thawed bovine semen in a commercial AI centre in order to maximise production, fertility and profits.

1.2 The Dairy Industry in Ireland

Agriculture is the largest indigenous sector accounting for 7.6% of Ireland's economy-wide gross value added, 12.3% of Ireland's exports and employs 8.6% of the total working population (Bord Bia 2017). At primary production level, approximately 140,000 farm families are involved in production of gross agriculture output valued at €7.12 billion (DAFM 2015) with beef and dairy sectors combined accounting for almost 70% of this output, valued at 39.3 and 29.4%, respectively (FoodWise 2025). The abolition of milk quotas within the European Union in April 2015 presented the Irish dairy sector with the freedom to realise its full potential in terms of output and export earnings with Ireland setting a target of a 50% increase in dairy outputs by the year 2020 (DAFM 2010). Global forecasts estimate the world population to reach 9.1 billion by 2050 (Food and Agricultural Organisation 2013). Due to the expansion of the dairy herd, coupled with modifications in consumer dietary intake resulting in an increased demand for protein, particularly from meat, a potential increase in beef output of 20% is estimated by 2020 (DAFM 2010). While this output increase will be achieved in part by increasing dairy cow numbers of cows, the intensive use of elite AI bulls will produce more robust and profitable cows in order to sustain the population growth.

1.2.1 Implementation of the Economic Breeding Index

Throughout the period of the mid-1980s to late 1990s, a significant decline in Irish dairy herd fertility was recorded (calving rate (CR) of 65% in 1980 compared to 54% in 1998; Mee 2004). Fertility is a multi-factorial trait and its deterioration was caused by a network of genetic, environmental and managerial factors; however, their complex interaction made it difficult to determine the exact reason for this decline

(Walsh et al. 2011). Nevertheless, the reduction in fertility was primarily associated with a large focus on single-trait selection for milk production, as a negative genetic correlation between both milk yield and herd fertility at cow level has been reported (Evans et al. 2002, Pryce et al. 1997). In order to combat this decline, the Economic Breeding Index (EBI), a single-figure profit index comprising information on seven sub-indexes related to milk production, was established by the Irish Cattle Breeding Federation (ICBF). The EBI, implemented in Ireland in 2001, ranks bulls and cow on their daughters milk production, fertility and health traits with the aim of helping farmers identify the most elite and profitable bulls and cows for breeding dairy herd replacement heifers (Berry et al. 2005). These replacement heifers have an increased ability to produce milk solids (fat and protein) with an aim of producing 1 kg milk solids per kg live weight per cow per year as well as having an increased ability to become pregnant and improve health traits resulting in reducing the replacement rate in a herd. The fertility sub-index, which is primarily based on calving interval and cow survival, accounts for approximately 35% of the total EBI and is one of the key components contributing to the increase in reproductive performance (Teagasc 2014). The remaining sub-indexes contributing to the improved performance include production (33%), calving (9%), beef (9%), maintenance (7%), management (4%) and health (3%). The continual increase of AI within the dairy industry, since the development of the EBI, has led to a re-evaluation of the reference population in 2017 so that EBI figures are representative of animals in the current era (ICBF 2017a).

1.2.2 Development of Genomics in Bull Selection

Before the development of genomics, AI bulls were identified, selected and ranked through traditional progeny test schemes involving test matings of approximately 1,000 inseminations with semen collected from these bulls at a peri-pubertal stage (Schaeffer 2006). The generic merit of a bull was consequently determined based upon the performance of the resulting progeny. However, this method of bull selection is time consuming and costly to the industry as progeny testing takes approximately five years beginning at time of AI until the resulting progeny subsequently complete their first lactation (Figure 1.1; Schefers and Weigel 2012). Genomic selection is the selection of bulls based on genomic estimated breeding value (GEBV) which is the estimated worth of the progeny's genotype (Hayes et al. 2009). The utilisation of genomics within the dairy industry as a predictor of breeding value has become a pivotal tool in bull selection as it has the potential to maximise the rate of genetic gain (Figure 1.1; Pryce and Daetwyler 2012, Berry et al. 2009) and reduce financial constraints within AI centres by eliminating the need for progeny testing thus, saving up to 92% in costs (Schaeffer 2006).

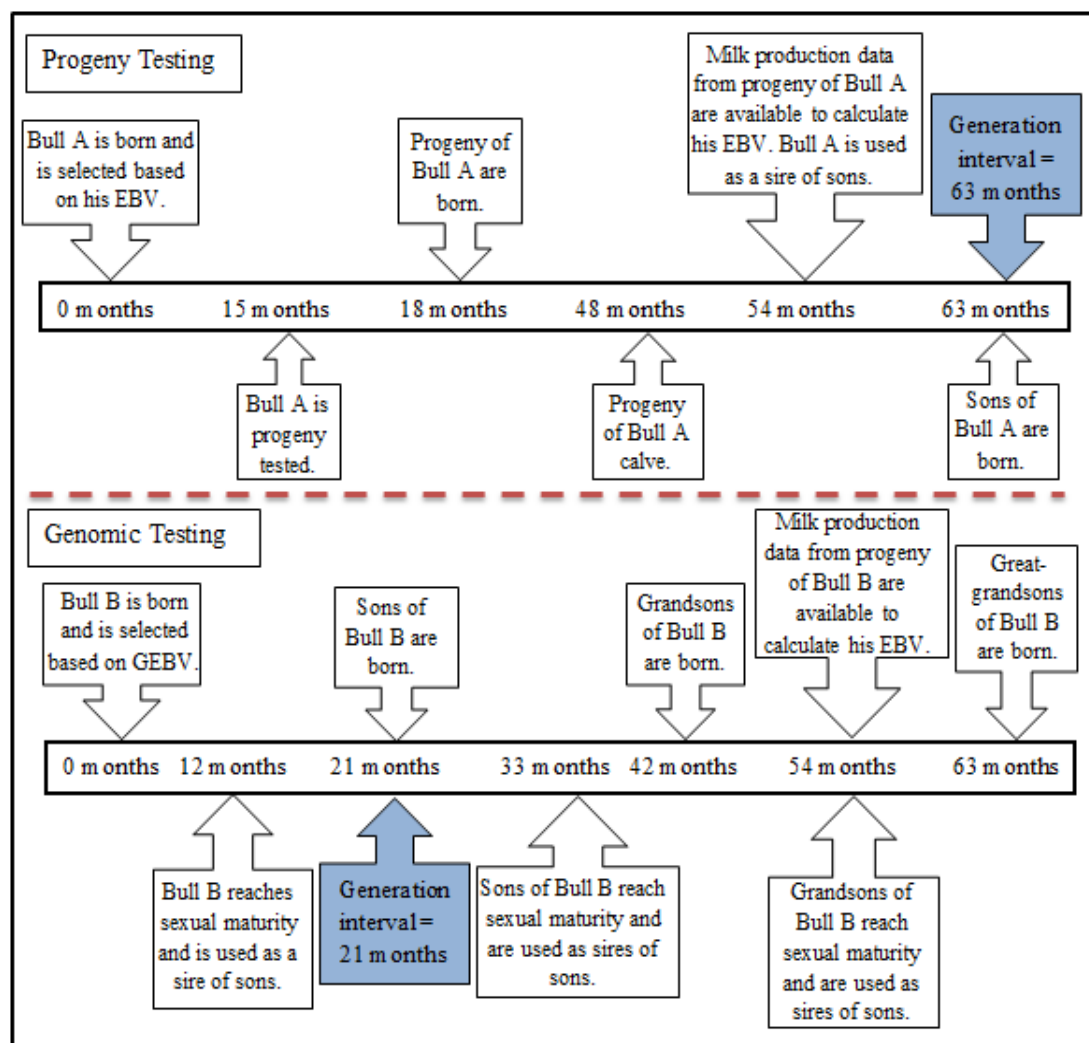


Figure 1.1: Timeline of a traditional artificial insemination breeding program based on progeny testing (upper timeline) versus an aggressive artificial insemination breeding program based on the use of genomics (lower timeline). EBV = estimated breeding value; GEBV = genomic estimated breeding value (Scheffers and Weigel 2012).

Genomic selection allows for the selection of bulls without the requirement for phenotypic data or progeny (Hayes and Goddard 2001). Using genomic selection, the reliability of calving interval breeding values in Ireland for bulls without progeny increased from 20 to 47% (Berry et al. 2014). However, genomics is not without its

limitations as reproductive traits of low heritability, such as calving difficulty, are difficult to predict. Therefore, combining phenotypic and genomic data can aid in improving selection precision (Berry et al. 2014). Nonetheless, the use of genomics is now the method of choice of genetic evaluation in many dairy cattle populations (Spelman et al. 2013) with 88% of the top 50 EBI-ranked bulls in Ireland, as of August 2017, genomically-selected (ICBF, 2017b).

1.3 Factors Affecting Semen Production and Semen Quality

The success of an AI programme depends largely on the genetic merit of the bulls for economically relevant traits as well as the quality and fertility of semen used (Lemma and Shemsu 2015) and, therefore, AI centres need to manage their bulls and semen processing accordingly. Semen quality can be affected by a wide range of factors such as genetic and environmental factors including season, collection interval, collection frequency and bull age, with a number of studies investigating the effect of these factors on semen traits (Fuerst-Waltl et al. 2006, Snoj et al. 2013, Brito et al. 2002a, Fiaz et al. 2010). It is widely acknowledged that peri-pubertal bulls have lower ejaculate volumes than mature bulls, primarily due to differences in testicular and accessory glands size (Perumal 2014, Karabinus et al. 1990, Mathevon et al. 1998). Thus, increasing semen output of bulls, particularly young bulls and those in high demand, by adapting management practices such as early life nutrition, determining appropriate sexual preparation and collection frequency will enable AI centres to extend the genetic progress gained with superior bulls. Consequently, identifying the relationships between factors affecting semen production and quality in both young and mature bulls would be useful for AI centres.

1.3.1 Bull Age

It is widely acknowledged that the age of a bull at collection affects semen characteristics such as ejaculate volume and sperm concentration (Brito et al. 2002a, Mathevon et al. 1998), with older mature bulls having greater semen volume and quality than younger bulls (Fuerst-Waltl et al. 2006, Brito et al. 2002a). This increase in volume with age is primarily due to physiological changes such as an increase in body mass (Balić et al. 2012) and the concurrent development of the testis and accessory glands post-puberty and during sexual maturation, which are believed to continue to develop for up to 5 years post-puberty (Almquist 1978). However, peak ejaculate volumes and total sperm number (TSN) are achieved at different ages in different breeds (Snoj et al. 2013). The pre-pubertal period is generally characterised by rapid increases in both body and testicular weight (Aponte et al. 2005). Following the onset of puberty (as defined by Wolf et al., 1965), at approximately 9-11 months of age in Holstein Friesian bulls (Byrne et al. 2017) the reproductive capacity of a bull increases for several years until he is sexually mature (Amann 1983). For younger bulls in particular, the age at puberty and subsequent maturity is paramount for their success in an AI programme. Due to the rapid advancement in genomics, there is an interest in AI bull production systems which can hasten puberty and sexual maturation such as early life nutrition (Byrne et al. 2017, Brito et al. 2007, Bollwein et al. 2017). The benefits of hastening puberty and thus enabling AI centres to collect semen from bulls at a younger age reduces production costs, shortens the generation interval and increases genetic gain. However, the reproductive performance of young bulls varies greatly mainly due to the large variation in the age of onset of puberty among and within breeds (Barth et al. 2008).

1.3.2 Collection Frequency

Collection frequency and collection interval have been shown to affect bovine semen production and quality (Mathevon et al. 1998, Everett and Bean 1982). The first ejaculate collected is typically of higher volume and sperm concentration compared to subsequent collections on the same day (Everett and Bean 1982, Bhakat et al. 2011). Everett et al. (1978) found that first ejaculates contained 40.8% more sperm per ml than second ejaculates. However, although in general, second ejaculates are of lower volume and sperm concentration, the collection of sequential ejaculates increases productivity per unit time, as more insemination doses can be obtained on a given day. The ability of sperm to maintain their functional status post-thawing in both first and second ejaculates is critical considering that AI in cattle is primarily implemented with the use of cryopreserved semen (Thibier and Wagner 2002). Fuerst-Waltl et al. (2006) reported a higher percentage of motile sperm in first ejaculates; however, Boujenane and Boussaq (2013) reported that the collection of subsequent ejaculates did not affect post-thaw sperm motility.

One possible explanation behind a reduction in semen production and pre-freeze sperm quality associated with second ejaculates may be due to the shortened collection interval of the second ejaculate. Longer collection intervals have been reported to result in greater semen production and quality, however, these collection intervals vary from 3-4 to 10 days (Mathevon et al. 1998, Everett and Bean 1982, Fuerst-Waltl et al. 2006) and so are unrealistic in a commercial environment setting. Amann and Almquist (1962) found that bulls with a high collection frequency (8 times weekly) had 24% fewer sperm reserves in the cauda compared to sexually rested bulls, while those collected twice weekly did not affect caudal reserves.

Furthermore, while Everett et al. (1978) reported 31% greater sperm numbers per mL when collection interval was increased from 2 days to 6 days, daily sperm output was highest when bulls were collected more frequently. Therefore, as overall semen production, such as TSN, increases with the collection of subsequent ejaculates, thus increasing productivity, placing high demand bulls on a collection schedule of twice a day, twice a week would be beneficial to AI centres.

1.3.3 Season

Although cattle are not typically considered seasonal breeders, the effect of season on semen production has been widely assessed in the bull (Bhakat et al. 2014, Brito et al. 2002b, Al-Kanaan et al. 2015, Malama et al. 2017, Snoj et al. 2013); however, data are conflicting, perhaps due to the range of climatic conditions under which these studies have been carried out such as variability in humidity, temperature, day length, etc. (Wildeus and Hammond 1993, Brito et al. 2002b). Sullivan and Elliott (1968) found that semen collections in the US in Winter resulted in higher non-return rates (NRR) than those in Spring. Similarly, Boujenane and Boussaq (2013) and Malama et al. (2017) reported that semen collected in Winter was of higher quality than Summer collections. In contrast, Stålhammar et al. (1989) and Snoj et al. (2013) observed greater sperm concentration and TSN during the Summer months than in any other season in AI centres located in Sweden and Slovenia, respectively. Studies reporting seasonal variations in semen characteristics have mainly attributed these changes to compromised scrotal thermoregulation and heat dissipation mechanisms (Figure 1.2; Menegassi et al. 2015) as well as the endocrine profile and the differential responsiveness of bull testes to gonadotropins (Jiménez-Severiano et al. 2003). Moreover, the adaptability of a bull to local microclimatic conditions has

also been considered to be connected to semen quality (Nichi et al. 2006) and therefore, could account for differences in their reproductive capacity throughout the year.

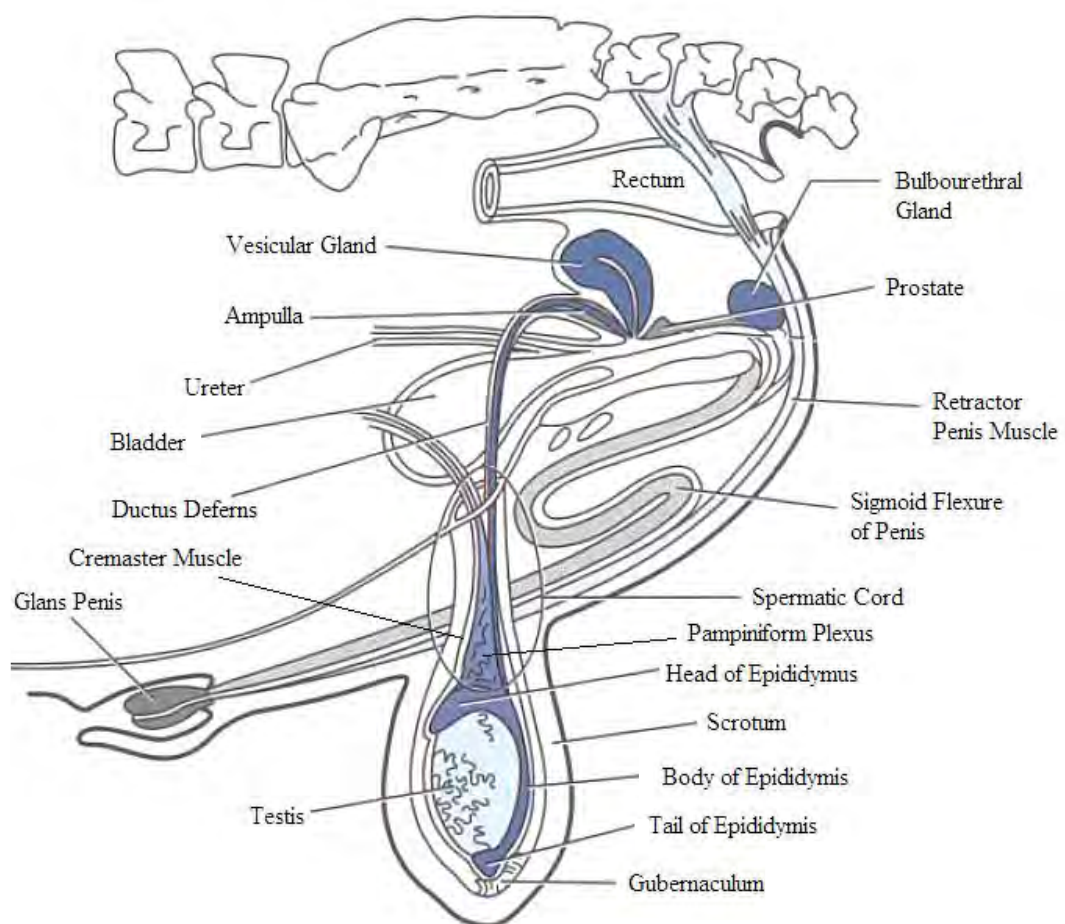


Figure 1.2: Illustration of the bovine reproductive tract including the thermoregulatory structures of the testes such as the pampiniform plexus and the cremaster muscle (Senger 1997).

Spermatogenesis in the bull takes approximately 61 days (Table 1.1; Johnson et al. 2000) and has been shown to be susceptible to temperature variation, as heat stress through scrotal insulation has been reported to result in a decrease in the percentage of progressively motile sperm (Rahman et al. 2011). Thus, the sperm in an ejaculate may reflect conditions to which the bull was exposed 8-9 weeks prior to collection.

Seasonal variation in melatonin levels, responsible for controlling gonadotropin and prolactin secretion, and testosterone secretions have been shown to affect spermatogenesis (Lincoln et al. 1996, Godfrey et al. 1990). Differences in sexual development between Spring- and Autumn-born bull calves has been reported with Spring calves reaching sexual maturity 65 days earlier (Tatman et al. 2004). The delay in acquisition in sexual maturity in Autumn-born bull calves was reported to be a consequence of reduced photoperiod in Winter months resulting in reduced luteinising hormone and testosterone hormone concentrations (Tatman et al. 2004). Additionally, seasonal variation in testosterone concentrations in bulls has also been reported with higher concentrations in Spring and Summer (Godfrey et al. 1990). However, the effect on season on testosterone levels in bulls is not well understood and results are conflicting (Secchiari et al. 1976, Sundby and Tollman 1978, Tatman et al. 2004). Thus, seasonal variations associated with photoperiod, in particular testosterone concentrations, affect the endocrine and spermatogenic functions of the testis with a consequent effect on sperm production and quality, as sperm output, libido, semen volume and fertility are reproductive criteria under direct endocrine control (Convey et al. 1971).

Table 1.1: Duration of spermatocytogenesis, meiosis and spermiogenesis in bull, boar, stallion, ram, buffalo, dog and cat (França et al. 2005).

Species	Spermatocytogenesis (Days)	Meiosis (Days)	Spermiogenesis (Days)	Total (Days)
Bull	21	23	17	61
Boar	14	13	14	41
Stallion	26	19	12	57
Ram	25	3	19	47
Buffalo	25	4	10	39
Dog	23	7	31	61
Cat	21	8	17	46

Previous studies have shown that neither temperature nor humidity affected sperm production or semen quality (Brito et al. 2002b, Taylor et al. 1985). However, the impact of many environmental factors is reduced when bulls are maintained in temperature-controlled barns (Haugan et al. 2005). Thus, semen can be collected from bulls throughout the year, hence, facilitating the build-up of sufficient stocks, consequently, improving efficiency and profitability of AI centres.

1.4 History of Artificial Insemination in Cattle

In 1898, the first attempts to advance AI into a proven method for large-scale genetic improvements of animals were conducted by a group of Russian researchers (Ivanoff 1922). During the early 1940s, prior to the development of cryopreservation techniques, distribution of semen for AI was a challenge due to the relatively short

lifespan. The discovery of egg yolk by Phillips and Lardy (1940) as a buffer medium in extenders to preserve the fertility of bull sperm for prolonged periods enabled the transportation of semen over longer distances. The addition of sodium citrate in combination with egg yolk, permitted the use of semen at 5 °C for up to three days (Salisbury et al. 1941) and the inclusion of antibiotics, which led to a major improvement in fertility in dairy herds, resulted in accelerating the acceptance of AI (Foote and Bratton 1950). The media was further improved with the Cornell University Extender (CUE; Foote et al. 1960) resulting in the highest fertility achieved in AI. Further modifications to this extender included the addition of caproic acid, catalase, 5% egg yolk by volume and nitrogen (N₂) gassing formed the industry gold standard extender for preserving bovine liquid semen at ambient temperatures 'Caprogen' (Shannon 1965, 1964). The concept of reducing sperm numbers per insemination dose lead to the introduction of the term extender by Foote and Bratton (1950) as the yolk-citrate-antibiotic medium enhanced and extended the usefulness of semen.

The development of semen cryopreservation techniques was parallel with the discovery of the protective properties of egg yolk (Phillips and Lardy 1940) and glycerol (Polge et al. 1949) on bovine sperm. Additionally, the use of milk as a medium to extend the life of bovine semen and the importance of thawing rate to sperm survival was also established (Almquist and Wickersham 1962). Tris-buffered egg yolk-glycerol extender was shown to provide excellent protection to sperm and became the most commonly used medium for the cryopreservation of bovine semen (Davis et al. 1963, Iritani 1980). However, several extenders and cryo-protective compounds have been investigated based on their ability to improve the survivability

and post-thaw motility of cryopreserved sperm. A change in frozen storage conditions in the 1950s with the shift from solid carbon dioxide storage at -79°C to liquid N_2 at -196°C was orchestrated when sperm survival at -196°C was demonstrated to be virtually infinite compared to biological changes observed in sperm with storage at -79°C . Thus, the successful cryopreservation of sperm and the development of efficient liquid N_2 storage conditions provided the foundation upon which the entire cryopreservation industry is built (Foote 2002).

1.4.1 Artificial Insemination in Ireland

Reproductive efficiency is important in all production systems; however, it is considered to have a greater importance in seasonal calving systems such as those operated in Ireland and New Zealand (Walsh et al. 2011). In 2016, over 60% of all dairy cows in Ireland were bred using AI, an increase of 20% in the last decade (Cromie et al. 2007). The primary target for Irish dairy farms is for each cow to produce a calf every 365 days with a targeted six-week calving interval of 90%. However, in 2016 the average calving interval in the dairy herd was 389 days, with a six-week calving rate of 58% (ICBF, 2016). As farm profitability increases by €9.26 per cow per year and €3.51 per heifer per year for every 1% increase in six-week calving rate (Shalloo et al. 2014), costs associated with reproductive inefficiency can have significant implications for dairy herds. Due to the continual growth and use of AI within the dairy industry and the importance of genomics in maximising fertility potential, additional challenges on AI centres is evident as the need to maximise semen production from young genomically-selected sires is paramount. This has led for a requirement of AI centres to devise strategies to optimise liquid and frozen-thawed bovine semen protocols, such as evaluating semen diluents, sperm

concentrations, storage temperatures etc. in order to optimise semen production and quality.

1.4.2 Liquid Semen

Currently, in Ireland, the use of liquid (ie: fresh, non-cryopreserved) semen accounts for only approximately 6.3% of total inseminations annually (Table 1.2). However, in order to accommodate the large demand during the peak breeding season, this can increase to approximately 25% during April to June (Al Naib et al. 2011b). Conversely, in New Zealand, the use of liquid semen accounts for approximately 95% of all inseminations (Verberckmoes et al. 2005). The use of liquid semen has many advantages in that it promotes and maximises the utilisation of genetically superior sires, due to the reduced sperm concentration per straw and therefore generates a greater number of straws per ejaculate compared with frozen-thawed semen (approximately 5×10^6 versus 15×10^6 sperm per dose, respectively). This facilitates the acceleration of genetic gain through more intensive sire utilisation as three times more straws are produced. Furthermore, unlike frozen-thawed semen, which must undergo a minimum 30-day quarantine period before being released for insemination, impeding the release of semen from young genomically-selected sires, liquid semen can be released for insemination on the day of collection (Irish Statute Book 2004).

Table 1.2: Total count and percentage of liquid semen on Day 1 and Day 2 of storage and frozen-thawed semen straws used in Ireland in 2016.

Semen Type	Number of Inseminations 2016	Inseminations 2016 (%)
Liquid Day 1	30,825	3.8
Liquid Day 2	20,762	2.5
Frozen-Thawed	765,472	93.7
Total	817,059	100

The use of liquid semen has a distinct advantage for seasonal systems, particularly in the current genomic era, whereby bulls which are of high demand in one season may be surpassed by the next generation of genetically elite bulls the following year (Bailey et al. 2000). The use of liquid semen also has the advantage of inexpensive storage conditions and ease of use on farm (as reviewed by Vishwanath and Shannon 2000). However, despite its advantages, liquid semen has a limited shelf life and is principally used for only 2.5 to 3 days post collection as a reduction in pregnancy rates has been reported thereafter (Vishwanath and Shannon 2000). Although there is a sharp decline in fertility after 4 days, reasons for which have not been fully elucidated, concomitant with this reduction in fertility is a gradual decline in sperm motility for up to 4 weeks post collection (Figure 1.3; Vishwanath and Shannon 1996). The use of liquid semen, however, poses difficulties in long distance transportation (Faigl et al. 2012) and increases the risk of disease transmission as straws are not held for a quarantine period (Bailey et al. 2008).

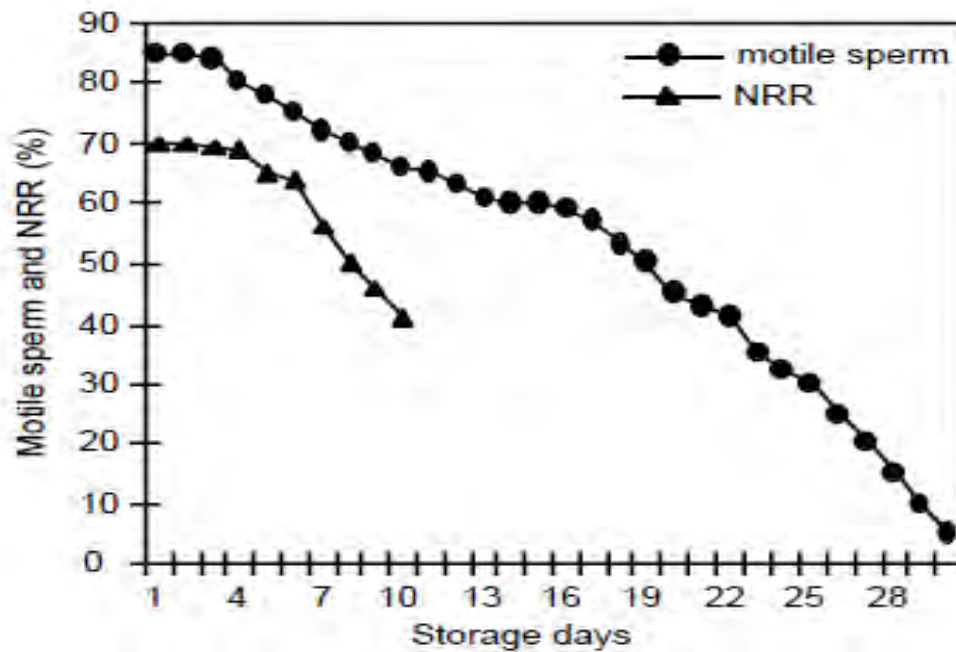


Figure 1.3: Effect of days of storage on bovine semen diluted in Caprogen at ambient temperature (18-21 °C) on the percentage of motile spermatozoa and non-return rate. The spermatozoa concentration was $8-10 \times 10^6$ spermatozoa per mL (Vishwanath and Shannon 1996).

An accepted principle of semen dilution technology is that sperm survival over prolonged periods is inversely related to their metabolic activity (Salisbury and Vandemark 1961). Thus, a number of investigative avenues have been exploited to combat the reduction in fertility associated with increased duration of storage such as an analysis of semen diluents (Shannon 1965), altering storage temperatures (Saha et al. 2014), reducing sperm number (Murphy et al. 2013) as well as N_2 gassing and modifying diluent composition (Shannon 1968).

1.4.2.1 Types of Liquid Semen Extenders

The extensive use of AI in the bovine industry can be partly attributed to the development of suitable diluents for both liquid and frozen-thawed semen with liquid semen extenders ranging from a simple salt solution to a more complex buffered medium (Foote 2002). The main purpose of semen diluents is to maintain the fertilising capacity of sperm. Liquid semen is typically stored in the egg yolk-based diluent Caprogen, which is widely accepted as the gold standard extender for liquid semen dilution (Vishwanath and Shannon 2000). Caprogen diluent is composed of four main constituents; 1) glycerol-antibiotic solution, 2) buffer solution, 3) catalase, caproic acid and citric acid and 4) egg yolk (Shannon 1964, 1965). These components combined with N₂ gassing protects sperm from detrimental effects of seminal plasma, free radicals and pH changes, provides structural stability and an energy source as well as reducing the metabolic activity of sperm (Vishwanath and Shannon 2000). However, apart from Caprogen, several other extenders have been developed for the storage of semen for a variety of domestic species which are either egg yolk-based (BullXcell), milk-based (INRA96) or most recently animal protein free extenders ie: plant-based; BioXcell, AndroMed and OptiXcell, to name but a few. A number of these commercially available soy-lecithin extenders have been evaluated for the preservation of bovine semen (Stradaoli et al. 2007, Aires et al. 2003, Muiño et al. 2007, Batellier et al. 1997) and have maintained better semen quality compared to egg yolk or milk-based extenders (Akhter et al. 2011).

In addition to the dilution of semen, diluents provide protective compounds such as bovine serum albumin, antioxidants and antibiotics to maintain sperm function (van

den Berg et al. 2014). During storage, a family of lipid-binding proteins (bovine seminal plasma proteins; BSP) found in seminal plasma are amongst the principal factors causing damage to sperm cells (Bergeron and Manjunath 2006). Thus, in order to provide sufficient protection to sperm, understanding the mechanism of protection provided by extenders is of paramount importance. It is believed that low density lipoproteins (LDLs) found in egg yolk and casein micelles found in milk interact with BSP proteins to reduce the lipid loss from the sperm membrane, thus, stabilising the membrane and maintaining sperm function during storage (Figure 1.4; (Manjunath 2012, Bergeron et al. 2007). These LDLs are also believed to confer structural stability to sperm membranes during the lipid phase transition, in which alteration of the sperm plasma membrane from a liquid to gel state in conjunction with a reduction in storage temperature occurs (Holt 2000, Drobnis et al. 1993). As the protective action of casein micelles on sperm is thought to be analogous to the protective action of lipoproteins in egg yolk (Bergeron et al. 2007), it is postulated that casein micelles are the effective component in milk-based diluents also protecting cells from the damage arising from the lipid phase transition. The exact mechanism through which plant-based extenders protect sperm is not yet well understood; however, it is believed that exogenous phospholipids and liposomes protect sperm by reversibly binding lipids and phospholipids as well as fusing liposomes with the sperm plasma membrane, thus stabilising the membrane (Ansari et al. 2016).

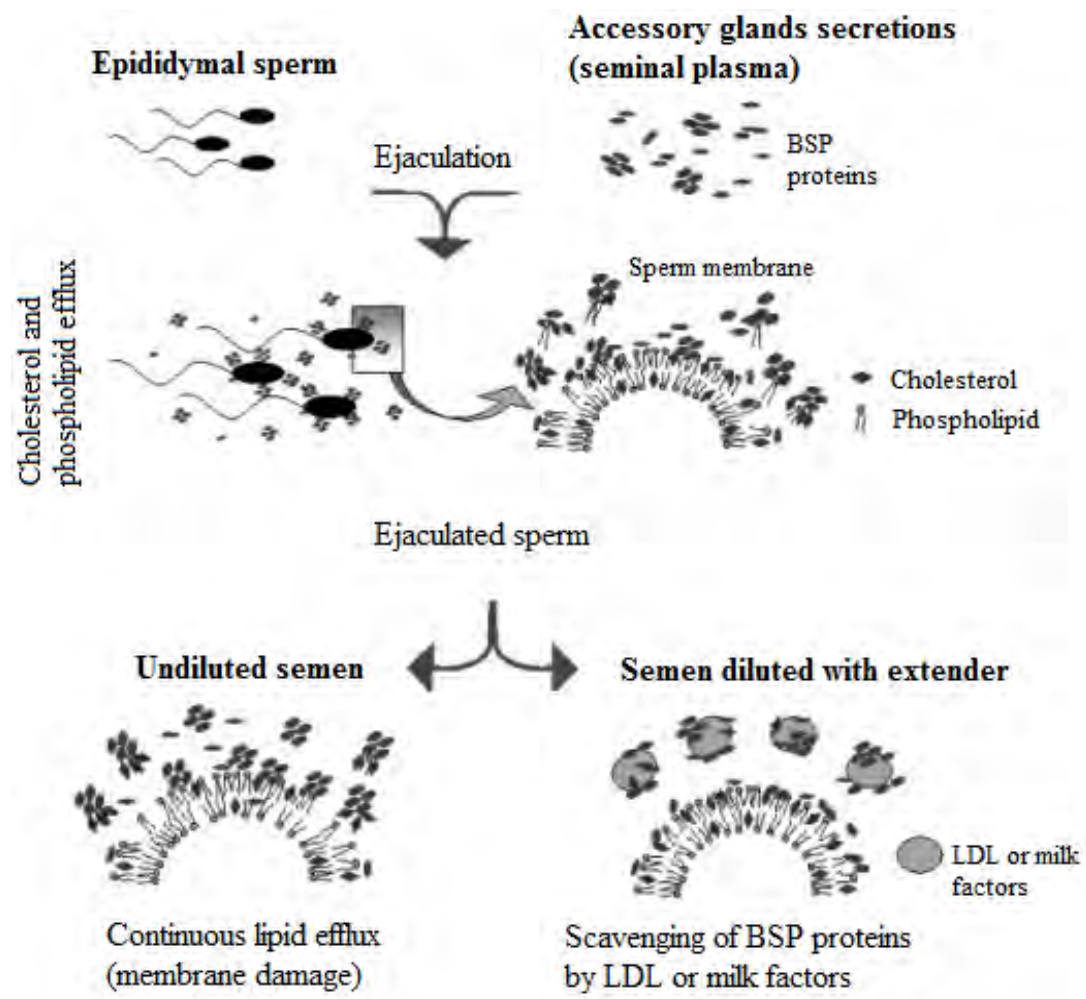


Figure 1.4: Mechanism of sperm protection by egg yolk and milk-based extenders (Bergeron and Manjunath 2006).

1.4.2.2 Effect of Altering Sperm Concentration

In order to increase the rate of genetic gain it is imperative to maximise the number of inseminations doses produced per ejaculate. However, the effect of such a reduction in sperm concentration on semen quality and fertility is unknown. In Ireland, a typical liquid semen dose contains 5×10^6 sperm, irrespective of its usage day (Al Naib et al. 2011a) in comparison to 15×10^6 sperm for a typical frozen-thawed semen dose. In contrast, the AI industry in New Zealand, while also operating a pasture-based system, is capable of achieving NRR of between 65-74%

using liquid semen diluted to 1.25, 1.75 and 2×10^6 sperm per insemination dose on Days 1, 2 and 3 post collection, respectively (Xu 2014). Although the NRR of sires used in Ireland and New Zealand are comparable, the significantly lower sperm number per insemination dose in New Zealand suggests that further advances within liquid semen technology are achievable in Ireland. Furthermore, the optimal fertility of individual bulls is likely to be achieved at differing sperm concentrations (Figure 1.5) with 95% of maximal conception rates obtained within a sperm concentration range of 1 – 11 million viable sperm per dose, however; differences in bull fertility are greater at lower sperm concentrations (Den Daas et al. 1998, Shannon and Vishwanath 1995). AI centres generally have a standard sperm concentration per insemination dose, however, overcompensation of sperm numbers typically occurs, enabling AI centres to minimise differences in pregnancy rates between individual sires (Amann and DeJarnette 2012). Furthermore, increasing sperm numbers per dose enables AI centres to compensate for marginal sperm quality defects. Generally, defects leading to a failure in fertilisation or maintenance of pregnancy are deemed uncompensable traits meaning that increasing sperm numbers per insemination dose will not improve fertility (Saacke 2008), while some defects, which principally impairs sperm motility, are known as compensable traits, because they are capable of being overcome by increasing the sperm concentration per insemination dose (Sullivan and Elliott 1968).

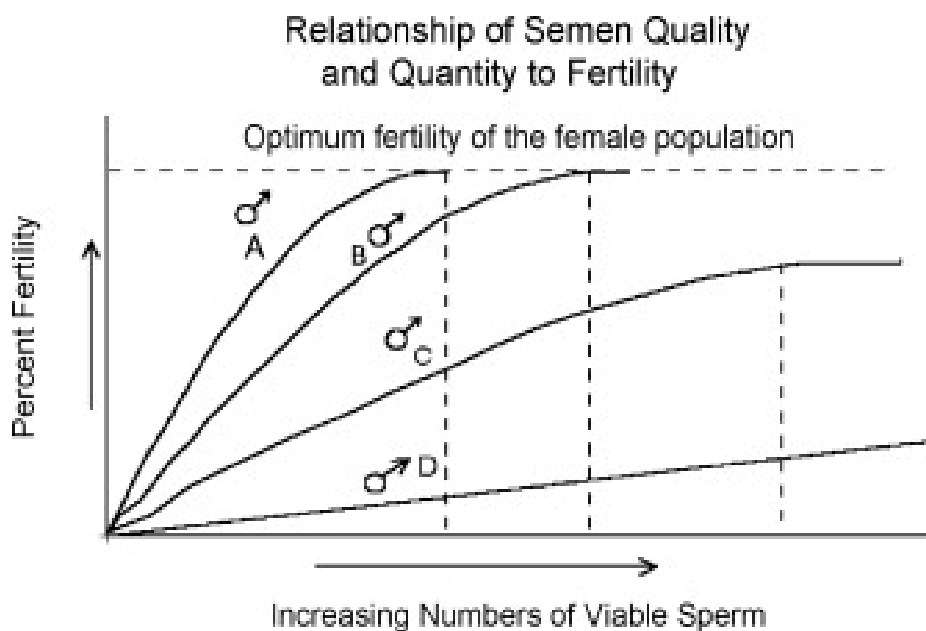


Figure 1.5: Relationship between pregnancy rate and the number of sperm inseminated. The semen of individual bulls varies in the maximum non-return rate and in the rate at which the maximum fertility is achieved with increasing sperm dosage (Saacke 2008).

Previous studies of liquid semen have reported beneficial *in vitro* effects of reducing sperm concentration such as increased sperm viability, reduced oxidative stress (Murphy et al. 2013) as well as a decreased susceptibility to osmotic shock (Prathalingam et al. 2006). Shannon and Curson (1984) reported no significant reduction in fertility of liquid semen when sperm concentration was reduced from 10×10^6 to 1.5×10^6 sperm per insemination dose. In contrast, Murphy et al. (2016) demonstrated that excessive dilution of sperm (5 versus 3 million sperm per dose) had a negative effect on NRR of semen used on Day 2 of storage compared to frozen-thawed semen; however, this did not differ from fertility achieved with liquid semen used on Day 1. Additionally, Shannon and Vishwanath (1995) reported that

liquid semen diluted from 2.5 to 0.5 million sperm per dose resulted in a reduction in NRR of 7% with bull variation ranging between 5.5 to 9.1%. It has previously been reported that increasing the dilution rate, specifically when using an egg yolk-based diluent, was the primary reason for a decline in fertility of liquid semen at lower sperm numbers rather than a direct effect of a lower sperm number being inseminated. This is because higher dilution rates adversely affect viability (Garner et al. 1997, Shannon 1965) as excessive dilution can lead to permanent loss of motility, metabolic activity and fertilising capacity (Maxwell and Johnson 1999). While a larger quantity of egg yolk provides more protection to sperm from the harmful effects of seminal plasma (Bergeron et al. 2004) it also provides a substrate for hydrogen peroxide production from dead sperm (Shannon and Curson 1983), thus, contributing to increasing the generation of reactive oxygen species (ROS). Although are a necessary prerequisite for successful sperm function at basal levels and play a role in the apoptotic pathways of sperm (Koppers et al. 2011), excessive ROS production is detrimental to sperm (de Lamirande et al. 1997).

ROS generation at low concentrations are required to mediate sperm function such as capacitation and the acrosome reaction (O'Flaherty et al. 2006). However, due to the abundance of polyunsaturated fatty acids in sperm plasma membranes, spermatozoa are exceptionally vulnerable to oxidation by ROS (Aitken et al. 2006). Excessive ROS production impairs regular sperm function in terms of sperm motility and reduces membrane fluidity diminishing the sperm's fertilising capability (Guthrie et al. 2011, Kumaresan et al. 2017a). Prathalingam et al. (2006) found that sperm stored at lower sperm concentrations at ambient temperature were found to retain higher viability and were less susceptible to osmotic stress compared with those stored at

higher sperm concentrations. Hence, as osmotic stress is caused due to an imbalance between ROS production and the ability of a system to repair the resulting damage (de Lamirande and Gagnon 1995), sperm concentration may be a crucial component in maintaining sperm quality during liquid storage.

1.4.2.3 Effect of Storage Temperature

It is widely accepted that regardless of storage temperature, sperm motility and thus fertility declines over an extended period of time (Vishwanath and Shannon 2000). Liquid semen straws in Ireland are traditionally stored in an unregulated temperature flask, which is subjected to natural day to night time temperature fluctuations with an average spring temperature of 15 °C and minimum and maximum temperatures reaching 6.4 and 27.9 °C, respectively (Murphy et al. 2016). The optimum storage temperature can depend on the species involved and semen dilution technology implemented with studies suggesting temperatures between 18-24 °C are optimal for bovine semen when semen is purged with N₂ gas (Vishwanath and Shannon 2000), 15-17 °C for porcine semen (Dziekońska et al. 2013), while storage at 5 °C is also recommended for bovine (Black 2006), equine (Ball et al. 2001) and ovine semen in some diluents (O'Hara et al. 2010, Gil et al. 2011).

Various strategies to reduce metabolism have been tested to enhance sperm survival such as reduced storage temperatures, lowering pH (Foote 1964) and N₂ gassing (Shannon 1965). While storage of liquid semen at 5 °C may reduce the metabolic activity of sperm, therefore extending the fertile lifespan (Shannon et al. 1984), one disadvantage is an increase in intracellular sodium concentrations to cytotoxic levels due to a reduction in the activity of the sodium-potassium pump to diffuse ions

across the cell membrane (Sweadner and Goldin 1980). Storing semen at reduced temperatures may also result in the increased incidence of cold shock injuries which are associated with morphological membrane changes consistent with a lipid phase transition (Drobnis et al. 1993). This results in compacting or relaxing the packing of the phospholipid bilayer, causing membrane destabilisation and ultimately cell death (Crowe et al. 1990). Although the mechanisms of cold shock are not yet well understood, it is believed that due to a loss of membrane phospholipids at reduced temperatures, sperm membrane integrity declines resulting in reduced semen quality (Batellier et al. 2001).

In order to avoid the damage sustained by reduced temperatures, protocols to inhibit pathways detrimental to the survival of sperm at ambient temperatures (15-20 °C) were devised (Shannon and Curson 1984). Storing semen at 15 °C may prevent the occurrence of cold shock injuries by regulating the transition phase hence, reducing the damage sustained to sperm (Drobnis et al. 1993). However, it has been postulated that the production of ROS is accelerated at higher storage temperatures (Pino et al. 2013, Vishwanath and Shannon 1996). Furthermore, exposing semen to extreme temperature conditions is detrimental to the survival of sperm (Murphy et al. 2016). Therefore, in countries where a large variation in day to night time fluctuation occurs, consideration should be given to the storage conditions of liquid semen in order to optimise semen quality during storage.

1.4.3 Frozen-Thawed Semen

It is widely acknowledged that cryopreservation results in a loss of sperm cell integrity and functionality with damage to sperm plasma membranes, acrosomal membranes and mitochondrial function resulting in morphological membrane alterations in the sperm membrane (Celeghini et al. 2008, Bollwein et al. 2008). Therefore, successful cryopreservation not only depends on preserving sperm motility but also maintaining their metabolic function (Watson 2000). During cryopreservation, alterations in lipid organisation and composition within the sperm membrane, due primarily, to the thermal and osmotic effects, and increased production of ROS impairs sperm motility, membrane integrity and fertilising potential (Hu et al. 2010, Watson 1995). This ultimately leads to a reduction in sperm viability of approximately 50% during the freeze-thaw process (Thomas et al. 1998). A number of investigative avenues have been followed in order to improve the freeze-thaw process including; modifying freezing rates (Liu et al. 1998), identifying the best thawing procedures (Muiño et al. 2008), optimising sperm concentration (Shannon and Vishwanath 1995), altering equilibration periods (Shah et al. 2016, Fleisch et al. 2017) and comparing semen diluents (Celeghini et al. 2008) as a combination of these key aspects contributes to the generation of suitable species-specific cryopreservation protocols.

Studies report a synergistic relationship exists between freezing and thawing rates on sperm survival (Mazur 1965, Watson 1979); however, other studies report that these factors are independent (Rodriguez et al. 1975, Robbins et al. 1973). Bovine cryopreservation initially involved slow freezing rates of 1-3 °C per min (Polge 1957); however, freezing rates of 15 °C per min from +15 to -100 °C has been found

to be superior in maintaining sperm survival (Chen et al. 1993, Liu et al. 1998). Studies evaluating different thawing rates have concluded that more rapid thawing at 33-35 °C for 30-40 sec results in better sperm survival compared to slow thawing rates such as those at ambient temperature (DeJarnette and Marshall 2005, DeJarnette et al. 2000, Nur et al. 2003). Although, Nur et al. (2003) and Dhimi et al. (1992) reported that temperatures as high as 60-70 °C could further improved post-thaw motility, Muiño et al. (2008) reported that thawing temperatures higher than 35 °C did not improved post-thaw sperm motility, acrosomal integrity or sperm morphology.

Failure of sperm to come into contact with the oocyte has been reported to be the primary reason for fertilisation failure in cattle (Hawk 1987, Dalton et al. 2001) with an absence of adequate sperm numbers at the site of fertilisation being the principal reason in many cases (Shannon and Vishwanath 1995). Provided post-thaw sperm survival equals or is greater than 50%, it is generally accepted that a sperm concentration of 15×10^6 per 0.25 mL dose is sufficient to achieve acceptable fertility (Vishwanath and Shannon 2000). Previous studies have found a significant reduction in NRR when frozen-thawed semen was diluted from optimum (20×10^6 sperm) to suboptimum (5×10^6 sperm) concentrations with a NRR of 67.6 and 59.7%, respectively (Shannon and Vishwanath 1995). DeJarnette et al. (2011) reported that decreasing the concentration of frozen semen from 10 – 2 million sperm per dose resulted in a significant decrease in NRR of 5% at 60 to 55%, respectively. Reducing sperm numbers in frozen semen from the standard 15 million to 2 million sperm per dose has resulted in contradictory results. Ballester et al. (2007) reported no difference in pregnancy rate of 47 versus 43% for 15 and 2

million sperm respectively, while, Andersson et al. (2004), although indicating that the site of sperm deposition did not influence pregnancy rate, reported a decline in pregnancy rate from 44.9 to 31.3%, respectively. Furthermore, Foote and Kaproth (1997) found no effect on NRR when frozen semen concentrations were reduced from 20 to 10 million sperm per dose at 71.5 and 70.5%, respectively. In order to combat damage sustained to sperm during the freeze-thaw process and to minimise differences in pregnancy rates between individual sires, over-compensation of sperm numbers typically occurs in the preparation of frozen-thawed semen, resulting in a sperm concentration which considerably exceeds the number of sperm necessary for maximum fertility. Thus, the 'true fertility' potential of a bull in the field is masked by the greater sperm number per insemination dose (Amann and DeJarnette 2012).

1.4.3.1 Importance of Equilibration Time

Bovine semen used for cryopreservation is typically diluted in an egg yolk-based extender as egg yolk is known to be one of the best cryoprotectant components for the preservation of post-thaw sperm function and subsequent fertility (Vishwanath and Shannon 2000). The components within egg yolk, in particular LDLs, bind to the sperm membrane during the freeze-thaw process, increasing chilling tolerance and preventing loss of membrane phospholipids (Medeiros et al. 2002, Muiño et al. 2007). The addition of cryoprotectants, which are classified as either penetrating (glycerol; 3-6%) or non-penetrating (egg yolk; containing LDLs and cholesterol; (Purdy 2006), minimises the physical and chemical stresses associated with cryopreservation, thus protecting the sperm membrane during freezing (Bathgate et al. 2006). As the name suggest, a non-penetrating cryoprotectant, such as egg yolk, cannot cross the sperm membrane and thus only acts extracellularly to modify the

sperm membrane (Amann 1999). On the other hand, penetrating cryoprotectants, the most common of which is glycerol, are membrane permeable and so act both intra- and extracellularly causing dehydration of the sperm and membrane lipid and protein rearrangement resulting in increased membrane fluidity and a decrease in the freezing point of the cell (Holt 2000).

Semen cryopreservation involves several steps including cooling, equilibration, freezing and subsequent thawing (Ahmad et al. 2015). Typically, freezing protocols for bovine semen generally include cooling to 4 – 5 °C followed by a variable duration of equilibration (0 – 24 h) at this temperature prior to freezing (Leite et al. 2010). Equilibration time was first believed to be important in allowing glycerol sufficient time to penetrate the sperm membrane (Leite et al. 2010). However, Berndtson and Foote (1972) reported that glycerol penetration in bull sperm is rapid, taking no more than 5 min; therefore, it is now suggested that a period of equilibration is necessary to allow sperm membranes sufficient time to adapt to cooler temperatures (Muiño et al. 2007). This facilitates the movement of the cryoprotectant across the cell membrane (in the case of penetrating cryoprotectants) and enables the movement of water out of the cell, thus minimising damage sustained by ice crystal formation during the freeze-thaw process (Vishwanath and Shannon 2000). Studies aimed at identifying the optimum equilibration time have been conducted on semen from a number of species including sheep (Câmara et al. 2011), goats (Deka and Rao 1986) and cattle (Fleisch et al. 2017, Michel et al. 2016). Although the majority of cryopreservation protocols for bovine semen involve an equilibration period of 4 – 6 h, a wide range of equilibration times have been reported: 0 h (Leite et al. 2010), 1 – 4 h (Arifiantini and Yusuf 2010), 18 – 24 h

(Foote and Kaproth 2002) and 24 – 72 h (Fleisch et al. 2017, Crespilho et al. 2014). The implementation of a shorter equilibration time such as 4 h can result in processing difficulties within a commercial AI centre, as semen has to be frozen on the same day of collection. In AI centres involving the collection of a large number of bulls on a daily basis or where semen has to be transported over long distances to a central processing laboratory, allowing an equilibration period of 18 – 24 h may be more convenient for the working schedules, as many studies have reported that allowing a longer equilibration time of greater than 24 hours increases semen quality (Fleisch et al. 2017, Foote and Kaproth 2002).

1.4.3.2 Types of Frozen Semen Extenders

Despite the importance and wide application of frozen-thawed semen in the bovine AI industry, relatively little progress regarding improved sperm cell survival has been made. Typically bovine frozen-thawed semen extenders differ in composition from liquid semen extenders as greater emphasis is placed on stabilising the cell membrane thus, combating the damage sustained by spermatozoa during the cryopreservation and subsequent thawing process. The use of egg yolk within bovine semen extenders is not without its disadvantages as it renders microscopic semen assessment more difficult (Vishwanath and Shannon 2000), particularly when using computer-assisted sperm analysis techniques (CASA; Singh et al. 2012). Furthermore, being a protein of animal origin, egg yolk may introduce the risk of exotic disease transmission, such as avian influenza (Yildiz et al. 2013), or microbial contamination leading to increased widespread health concerns over its use in semen diluents (Aires et al. 2003). Moreover, there is growing demand for full product traceability and increasing emphasis on biosecurity issues in government legislation

regarding animal based products (Layek et al. 2016). In addition, egg yolk is difficult to standardise, with significant potential for variation from batch to batch (Bousseau et al. 1998), posing problems for quality assurance in the laboratory. Therefore, alternatives to components of animal origin in semen extenders such as soya-lecithin in plant-based diluents are now of interest (Akhter et al. 2012, Ansari et al. 2016, Gil et al. 2003), primarily due to the reduced health risk associated with animal protein-free media and would represent a valuable contribution to the AI industry; however, they are still not universally accepted due to concerns of lower fertility (Layek et al. 2016, Leite et al. 2010).

Plant-based extenders contain a natural mixture of phosphatidylcholine and a number of fatty acids such as stearic, oleic and palmitic acid which are known to confer structural stability to spermatozoa (Oke et al. 2010, Chaudhari et al. 2015). Furthermore, Zeron et al. (2002) reported that the fusion of liposomes with sperm membranes decrease the lipid phase transition of bovine sperm resulting in decreased sensitivity of sperm to cryopreservation. Studies using plant-based extenders have reported competitive *in vitro* results in a number of species including bovine (Stradaioli et al. 2007, Aires et al. 2003, Miguel et al. 2008), ovine (Forouzanfar et al. 2010, Gil et al. 2003) and equine (Papa et al. 2010) in comparison to egg yolk extenders. In addition, comparable fertility rates have been reported in buffalo (Akhter et al. 2012). However, a number of other studies have reported a reduction in semen quality when comparing plant-based versus egg yolk-based extenders (Muiño et al. 2007) with some studies also reporting a reduction in fertility (Van Wagendonk-de Leeuw et al. 2000, Crespilho et al. 2012).

1.5 Semen Quality Assessments

Most AI centres worldwide still rely heavily on the routine evaluation of ejaculate volume, sperm concentration, motility (total and progressive) and morphology via standard microscopic techniques (Chenoweth and McPherson 2016). A number of studies have reported a significant relationship between semen traits, specifically sperm motility, and field fertility (Christensen and Lehn-Jensen 1999, Christensen et al. 2005). However, the accuracy of these initial basic laboratory parameters is controversial and are not sufficient to determine the true fertilising capabilities of individual semen samples (Gadea et al. 2004) as correlations between motility and fertility have resulted in contradictory findings (Tardif et al. 1999, Januskauskas et al. 2003). Male fertility is complex and is influenced by a number of factors, therefore, identifying a single laboratory test reliable to predict the potential fertility of semen or a given sire for AI is considered utopian (Rodríguez-Martínez 2003). Thus, over the last decade the ‘tool box’ of semen evaluation techniques has expanded to include more advanced technologies such as CASA and flow cytometry and are now seen in conjunction with traditional parameters, in order to attain detailed *in vitro* cellular assessments of semen samples (Petrunkina et al., 2007). This is primarily because fertilisation is a multi-factorial process and thus, a combination of several assays must be undertaken to accurately predict fertility (Amann 1989). Despite these techniques assessing a multitude of intracellular sperm parameters, which in theory should correlate more strongly *to in vivo* fertility, further improvements in sperm functional assessments is warranted

1.5.1 Microscopic Evaluation

The *in vitro* evaluation of semen, complementary to clinical examinations, is an important component when estimating the fertility potential of a sire as it aids in the swift elimination of males with obvious cases of poor reproductive performance (Rodríguez-Martínez 2006). Motility is one of the most important characteristics associated with fertilising potential of sperm as it is indicative of sperm viability and structural integrity (Nagy et al. 2015). Thus, sperm motility assessment constitutes an integral part of semen quality analysis in semen collection and processing procedures (Rodriguez-Martinez 2006). However, few studies have investigated its predictive value and discrepancies exist between studies among correlations with fertility with some reporting a significant correlation (Kathiravan et al. 2008, Gillan et al. 2008) or no correlation of motility with fertility (Puglisi et al. 2012, Januskauskas et al. 1999). While these basic assessments set a minimum standard for semen quality they have limited value in predicting subsequent fertility due to the highly variable correlations achieved with simple *in vitro* sperm characteristics and *in vivo* fertility (Rodriguez-Martinez 2005). Accurate subjective assessment of sperm characteristics is markedly slow and largely depends on the training level of each technician leading to coefficient variation in assessment of sperm samples between technicians and laboratories of 20 and 37%, respectively (Hoflack et al. 2007). This highlights the necessity for standardisation in semen analysis in order to reduce the high variability upon assessment (Verstegen et al. 2002). Thus, estimating the fertility potential of a semen sample or that of a particular bull is difficult to accurately achieve via standard microscopic assessments alone. Hence, in order to increase the precision of quality control analysis and fertility prognostics in semen

production centres, a multi-parametric approach of semen quality is recommended (Malama et al. 2017).

1.5.2 Computer Assisted Sperm Analysis

The development of sperm analysis technologies such as CASA systems holds many advantages as it has led to the objective accurate assessment of motility and sperm morphological characteristics, which allows for the assessment of subtle changes in spermatozoa which would otherwise be difficult to observe using standard microscopic techniques (Verstegen et al. 2002). The CASA system allows for a rapid, detailed description of each individual sperm for several specific motility parameters such as total motility, progressive motility, linearity (LIN) and numerous velocity parameters such as curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), as well as amplitude of head displacement (ALH) and direction of movement (Mortimer 2000). The quantitative evaluation of semen by CASA is devised based on the kinematic parameters of individual sperm heads and analysis is conducted using sequential digital imaging to reconstruct the trajectory of each individual sperm (Mortimer et al. 1997).

CASA is currently becoming a popular method to evaluate sperm motility (Amann and Waberski 2014). However, a disadvantage to the system is that the results are highly susceptible to external factors such as operator variability, semen handling and system settings (Kathiravan et al. 2011). A number of studies have correlated motility kinematics of bull sperm with field fertility (Nagy et al. 2015, Kathiravan et al. 2008, Oliveira et al. 2013, Farrell et al. 1998, Januskauskas et al. 2003); however, many of these studies are conflicting in terms of the importance of the various

motility characteristics with field fertility. In many studies, the combination of multiple CASA parameters had a higher correlation with bull fertility than a single variable such as straight line velocity (Gillan et al. 2008, Cseh et al. 2003), beat cross frequency (BCF), ALH in conjunction with total and progressive motility (Oliveira et al. 2013). In contrast, Oliveira et al. (2012), Amann and Waberski (2014) and Simonik et al. (2015) found that sperm kinematic characteristics are not an accurate predictor of fertilising potential and so not a tool for the precise prediction of bull fertility. However, they report that CASA could instead be used to provide important information relating to the quality assurance of semen and can serve as parameters for improving and increasing the value of *in vitro* assessments. This is particularly beneficial when assessing the *in vitro* quality of various semen treatments. Therefore, semen parameters assessed by CASA should be correlated with advanced *in vitro* sperm function analysis in order to improve semen quality control within AI centres as Oliveira et al. (2012) reported that sires producing lower semen quality typically results in lower pregnancy rates.

1.5.3 Flow Cytometry Analysis

Flow cytometry is an accurate *in vitro* technique used in assessing sperm function and its use in routine assessments in sperm evaluation has increased over the last decade. Flow cytometry comprises many advantages as it allows for less subjective and statistically more reliable analysis of an increased numbers of sperm over a short time-period (Marchetti et al. 2002). The discovery of a number of fluorochromes and compounds conjugated to fluorescent probes has allowed for a more objective analysis of semen quality at a biochemical, ultra-structural and functional level. These fluorescent allow rapid evaluation of usually ten thousand cells (compared to

100-200 for microscopy based techniques) for a number of characteristics (Gillan et al. 2005). Flow cytometry is especially useful for taking the “negative” biomarker-based approach to andrological evaluation and can detect subtle inadequacies in sperm whether or not they are manifested in a visible morphological phenotype or diminished sperm motility. Fluorescent assessments associated with sperm functional parameters are abundant across a range of species such as viability (Rodriguez-Martinez and Barth 2007), membrane integrity (Christensen et al. 2005, Christensen et al. 2011, Bollwein et al. 2008), acrosomal integrity (Kumaresan et al. 2017b, Murphy et al. 2016), ROS generation (Gürler et al. 2016), chromatin integrity (Januskauskas et al. 2003) and lipid peroxidation status (Singh et al. 2016). However, as most studies assess a limited number of sperm functions, results have been highly variable and do not always correlate with field fertility (Sudano et al. 2011, Martínez-Pastor et al. 2010). An extensive evaluation of *in vitro* quality parameters by Sellem et al. (2015) on a batch by batch basis reported that combinations of microscopic and flow cytometric analysis could still only explain, at best, approximately 40% of the variation in 56-Day NRR (adjusted r^2 ranged between 0.24 and 0.40). Nevertheless, flow cytometric assessments are useful in assessing semen quality and provide interesting data on *in vitro* sperm function; however, a wider range of assessments must be used to capture the physiological aspects of sperm.

1.6 Inter-Bull Variation in Fertility

In AI centres, bull management and quality control programs have two main objectives; (i) ensure production and sale of highly fertile insemination straws containing sufficient sperm numbers in order to maximise conception rate achieved

for each sire and (ii) minimise fertility differences among bulls (DeJarnette et al., 2010). NRR is the predominant phenotype used for assessing male fertility in the field (Clay and McDaniel 2001, Kuhn and Hutchison 2008). However, although NRR is a good indicator of fertility, inflation in the calculation of NRR can occur, particularly in relation to heifers (Foote 2003). As NRR is calculated based on an assumption that a cow has conceived, reporting pregnancy rate, which is strongly correlated with NRR (Berry et al. 2011), or CR gives a more precise and accurate assessment of fertility.

Although few sires are completely sterile, approximately 20-40% of bulls are classified as sub-fertile (Kastelic 2013). Furthermore, despite semen meeting minimum routine quality control standards within a commercial AI laboratory setting, pregnancy rate from AI bulls can vary significantly by 20-28% (Kastelic and Thundathil 2008, Holden et al. 2017, Al Naib et al. 2011a). Additionally, while individual bull fertility can vary over time, the accuracy of results can be affected by other factors such as variability among AI technicians and farms in which the semen was used (Foote 2002). It is widely reported that inter-bull variation in fertility exists (Figure 1.6; Murphy et al. 2017, Ballester et al. 2007, DeJarnette et al. 2011) as the level of deterioration of semen quality during processing is bull specific (Den Daas et al. 1998). This may indicate that sperm numbers should be adjusted on an individual bull basis so as to reduce differences in fertility between low and higher fertility bulls. However, increasing fertility through increasing sperm numbers has a maximum threshold in terms of fertility and the rate at which this maximum value is approached also differs between bulls (Vishwanath and Shannon 2000).

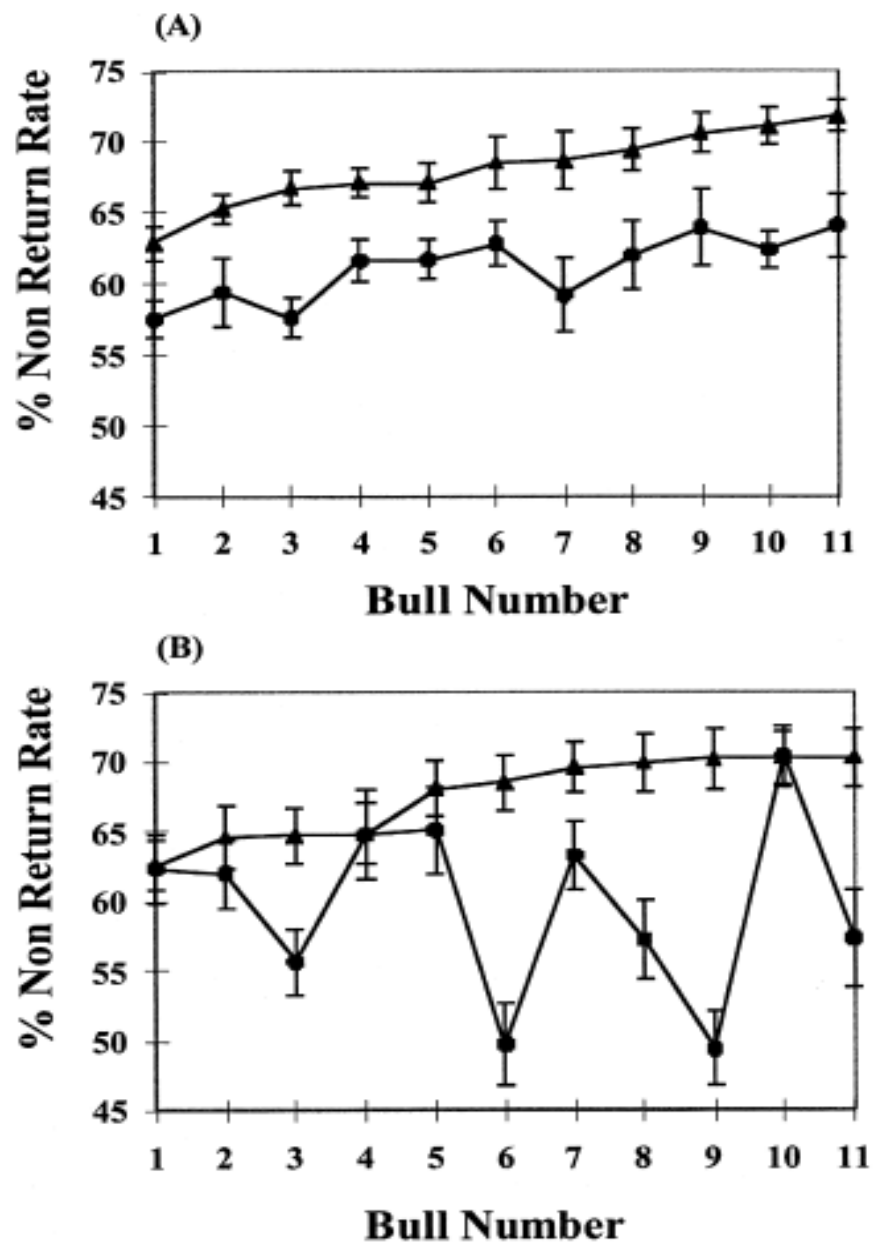


Figure 1.6: Variation in non-return rate at optimum (▲) and suboptimum (●) sperm concentration in liquid (A) and frozen-thawed (B) semen treatments (Shannon and Vishwanath 1995).

Although cryopreserved semen is exposed to various insults compared to liquid semen, thus requiring greater sperm numbers per dose, recent studies have shown no difference of semen type on fertility as indicated by NRR (71.1 versus 71.6%;

Murphy et al. 2016) and pregnancy rate (53 versus 52%; Richardson et al. 2017) for liquid and frozen-thawed semen, respectively. Furthermore, Shannon and Vishwanath (1995) reported no difference in fertility between liquid and frozen semen diluted to 2.5 and 20 x 10⁶ sperm per dose, respectively. However, when semen was diluted from optimum to sub-optimum sperm concentration conditions a bull by dose rate interaction was evident in frozen-thawed semen with a difference in NRR varying between 0.1 and 20.3%; but this interaction was not observed in liquid semen. A similar bull by dose rate interaction for frozen-thawed semen was observed by den Daas (1992). Thus, while the response of bulls to the liquid semen processing is similar, the response varies greatly where the freezing process is employed, indicating that bulls inherently differ in their susceptibility to the effects of freezing.

1.6.1 Use of Power Analysis in Fertility

In order to accurately predict fertility an understanding of statistical procedures, such as the ability to identify dependant and independent variables, as well as biological assessments is required (Utt 2016). Furthermore, a detailed understanding of factors affecting statistical models is required (Amann and DeJarnette 2012). Most AI centres track bull fertility using either NRR or more accurate (and complex) adjusted sire fertility models which account for environmental factors (herd, technician, month of insemination, age of cow, cow genotype, days in milk (DIM), milk production, etc.) and express a bulls fertility relative to the population mean of 0%. A detailed review by DeJarnette and Amann (2010) demonstrated that the fertility of 90% of the bulls marketed is within a 6 percentage unit range. On a population basis, DeJarnette and Amann (2010) concluded that AI companies will never be able to measure "fertility" more precisely than ± 3 percentage units from the population

mean, due to factors which are difficult to control such as binomial variation, herd environment, measurement errors, and bias in semen use. Additionally, in order to confidently rank bulls on their fertility, understanding the importance of insemination numbers per sire is required. DeJarnette and Amann (2010) illustrates that in order to confidently (2 tail test, $P = 0.05$, 80% power) identify detectable differences between high and low fertility bulls a minimum of 1,000 inseminations are required to achieve a detectable difference of $\pm 4\%$ from the average of the bull population (Table 1.3).

Table 1.3: Effect of sample size on minimum detectable difference and range in conception values that cannot be statistically considered different than average conception for a herd with a 30% theoretical average fertility rate (DeJarnette and Amann 2010).

Number of services per sire	Minimum detectable difference from average	Range in conception rate not different from 'average' value
10	$\pm 41\%$	-11% to 71%
50	$\pm 18\%$	12% to 48%
100	$\pm 13\%$	17% to 43%
300	$\pm 7\%$	23% to 37%
500	$\pm 6\%$	24% to 36%
1000	$\pm 4\%$	26% to 34%

This large sample size is required in order to counteract any deviation from the average due to environment and random chance. Furthermore, an understanding of statistical significance and the factors affecting models when predicting fertility is also required (Amann and DeJarnette 2012). Therefore, it is incumbent that studies, which use *in vitro* methods to attempt to predict sire fertility, take into consideration the reliability of the fertility phenotype while using sufficient bulls across the fertility spectrum.

1.7 Objectives

The objectives of this thesis were to

- Identify factors influencing semen production and quality in Holstein Frisian bulls
- Compare commercially available liquid semen diluents against the industry standard, Caprogen, in order to prolong the fertile lifespan of liquid bovine semen
- Define the optimal sperm concentration for liquid semen so as to maximise the use of young genetically superior sires
- Determine the optimum storage temperature for liquid bovine semen
- Ascertain the optimal equilibration time prior to freezing for frozen-thawed bovine semen
- Investigate the efficacy of plant-based versus egg yolk-based diluents for the cryopreservation of frozen-thawed bovine semen.

Chapter Two

Influence of Bull Age, Collection
Frequency and Season of Collection on
Semen Production and Sperm Quality
Characteristics in Holstein Friesian AI
Bulls

Abstract

In the current era of genomic selection, there is an increased demand to collect semen from young genomically-selected elite sires at a young age. The objective of this study was to assess the effect of bull age, collection frequency and season of collection on semen production (ejaculate volume, sperm concentration and TSN and sperm quality (pre-freeze and post-thaw total and gross motility) parameters in Holstein Friesian bulls in a commercial AI centre. The study involved the interrogation of a large dataset collected over a period of four years, involving a total of 8,983 ejaculates from 176 Holstein Friesian bulls aged between 9 months and 8 years. There was a linear increase in ejaculate volume and TSN with bull age ($P < 0.01$). Bulls aged less than one year had the poorest semen production and sperm quality values for all parameters assessed in comparison to older bulls ($P < 0.01$). While there was no significant increase in sperm concentration, there was a linear increase in TSN with increasing ejaculate volume ($P < 0.01$). First ejaculates had a higher volume, sperm concentration, TSN and greater pre-freeze total and gross motility than second ejaculates collected on the same day ($P < 0.01$), but despite this, there was no difference in post-thaw motility between first and second ejaculates. When subsequent ejaculates were collected from bulls aged less than one year, semen production and quality did not differ significantly compared to mature bulls, highlighting the potential to increase productivity from these younger bulls. Semen collected in Winter was poorest in terms of sperm concentration and TSN, but best in terms of post-thaw total and gross motility ($P < 0.01$). In conclusion, second ejaculates can be collected from bulls less than one year of age without a significant decrease in post-thaw semen quality and thus may be a useful strategy to increase semen availability from young genomically selected AI bulls in high demand.

2.1 Introduction

AI is one of the most influential biotechnologies in the dairy breeding industry as it allows for the wide dissemination of superior genetics and promotes faster genetic gain (Oliveira et al. 2013). The success of an AI centre depends largely on the genetic merit of the bulls for economically-relevant traits as well as the quality and fertility of semen used (Lemma and Shemsu 2015). Therefore, AI studs need to manage their bulls and semen processing accordingly. While over the last decade the 'tool box' of semen evaluation techniques has expanded to include more sophisticated technologies such as CASA and flow cytometry, these measures in combination can still only explain, at best, approximately 40% of the variation in bull fertility (Sellem et al. 2015). Therefore, most AI studs worldwide still rely heavily on the routine evaluation of ejaculate volume, sperm concentration, motility (pre-freezing and post-thawing) and morphology via standard microscopic techniques (Chenoweth and McPherson 2016). Indeed, a number of studies have reported a significant relationship between semen traits, specifically sperm motility, and field fertility (Christensen et al. 2005, Christensen and Lehn-Jensen 1999). In recent years, the advent of genomic selection has allowed the dairy industry to reliably select AI bulls at a younger age and has thereby hastened genetic progress by reducing the generation interval (Goddard and Hayes 2007). However, there are major challenges to collecting sufficient high quality semen to meet demand from these elite young bulls. Therefore, identifying the relationships between factors affecting semen production and quality in both young and mature bulls would be useful for AI studs.

Semen quality can be affected by a wide range of genetic and environmental factors including bull age, collection interval, collection frequency and season (Snoj et al. 2013, Fiaz et al. 2010, Fuerst-Waltl et al. 2006, Brito et al. 2002a). It is widely acknowledged that the age of a bull at collection affects semen characteristics such as ejaculate volume and sperm concentration (Brilo et al. 2002a, Mathevon et al. 1998), with older mature bulls having greater semen volume and quality than younger bulls (Fuerst-Waltl et al. 2006, Brito et al. 2002a). This increase is primarily believed to be due to physiological changes such as an increase in body mass (Balić et al. 2012) and the simultaneous development of the testis and accessory glands post-puberty and during sexual maturation which consequently leads to an increase in semen production (Almquist 1978). However, peak ejaculate volumes and total sperm number are achieved at different ages in different breeds (Snoj et al. 2013).

While the first ejaculate of a bull collected on a given day is typically of higher volume and sperm concentration compared to subsequent collections on the same day (Everett and Bean 1982), collection of multiple ejaculates on the same day did not affect post-thaw sperm motility (Boujenane and Boussaq 2013). The reduction in semen production seems to be largely related to the short collection interval between consecutive ejaculates as longer intervals produce higher ejaculate volumes and TSN (Fuerst-Waltl et al. 2006, Mathevon et al. 1998). However, although in general, second ejaculates are of lower volume and sperm concentration, the collection of sequential ejaculates increases productivity per unit time, as more insemination doses can be obtained on a given day.

The effect of season on bovine semen production has been widely assessed (Malama et al. 2017, Brito et al. 2002a, Mathevon et al. 1998, Stålhammar et al. 1989); however, data are conflicting, perhaps due to the range of climatic conditions under which these studies have been carried out (Wildeus and Hammond 1993, Brito et al. 2002b). Studies reporting seasonal variation in semen characteristics have mainly attributed these changes to compromised scrotal thermoregulation and heat dissipation mechanisms (Menegassi et al. 2015) as well as the endocrine profile and the differential response of bull testes to gonadotropins (Jiménez-Severiano et al. 2003). Seasonal variations associated with photoperiod, in particular luteinising hormone, testosterone concentrations and melatonin levels, have been shown to affect spermatogenesis (Tatman et al. 2004, Lincoln et al. 1996, Godfrey et al. 1990) as sperm output, libido, semen volume and fertility are under direct endocrine control (Convey et al. 1971). Moreover, the adaptability of a bull to local microclimatic conditions may have consequences for semen quality (Nichi et al. 2006) and therefore, could account for differences in their reproductive capacity throughout the year.

For younger bulls in particular, age at puberty and subsequent sexual maturity is paramount for their success in an AI programme. Due to the rapid advancement in genomics, there is an interest in AI bull production systems which hasten the onset of maturity (Byrne et al. 2017, Dance et al. 2016), thus enabling AI centres to collect semen from bulls at a younger age, reducing production costs, shortening the generation interval and increasing genetic gain. However, the reproductive performance of young bulls varies greatly mainly due to the large variation in the age of onset of puberty among and within breeds (Barth et al. 2008). Thus, increasing

semen output of bulls, particularly young bulls and those in high demand, by adapting management practices such as determining appropriate sexual preparation and collection frequency would enable AI centres to increase the dissemination of genetic merit of superior bulls.

Given this background, the aim of this study was to assess the effect of bull age, collection frequency and season of collection on semen production and quality characteristics in Holstein Friesian AI bulls.

2.2 Materials and Methods

2.2.1 Animal Management

Semen collection data from Holstein Friesian bulls ($n = 176$ bulls, $n = 8,983$ ejaculates), ranging between 9 months to 8 years of age (Figure 2.1), from Ireland's largest AI centre (National Cattle Breeding Centre, Naas, County Kildare) were used in this study. Bull semen production and quality records over a period of four years (2012 to 2016) were analysed. Data were categorised according to season of collection: Spring (February, March, April), Summer (May, June, July), Autumn (August, September, October) and Winter (November, December, January). Mean temperature over the four years during these periods was 7, 14, 13 and 6 °C, respectively (Met Éireann 2017) with a mean number of collections per day of 33, 25, 23 and 17 for Spring, Summer, Autumn and Winter, respectively. Bulls were individually housed in a barn with ambient (i.e., unregulated) temperature, fed and maintained under similar management and feeding conditions. Bulls were fed a standard ration of 85% dry matter haylage as well as approximately 5 kg of a 14%

protein cereal-based ration daily with *ad libitum* access to water. The mean age of the bulls analysed in Spring, Summer, Autumn and Winter was 24 ± 0.20 , 26 ± 0.25 and 29 ± 0.29 and 28 ± 0.31 months, respectively. Typically, semen was collected from a bull on one to three days per week, depending on demand. In cases where semen from a particular bull was in very high demand, regardless of bull age, a second ejaculate was collected within 1 h of the first collection (as described below).

2.2.2 Semen Collection and Processing

All bulls were sexually stimulated using a teaser bull and allowed to false mount a minimum of three times. The time between each false mount varied between individual bulls but was determined by an experienced barn technician and typically took no longer than 2-3 min. Semen was collected from all bulls using an artificial vagina, once bulls were deemed to be sufficiently stimulated. This method of stimulation and collection was similar for all collections, regardless of the ejaculate number, and remained constant from year to year. Ejaculates were kept separate throughout and were initially partially diluted in 10 mL pre-warmed ($37\text{ }^{\circ}\text{C}$) BullXcell extender (IMV Technologies, L'Aigle, France) and transported in a temperature-regulated box at $18\text{ }^{\circ}\text{C}$ to the laboratory (within 3 h). On arrival, the ejaculate was assessed for weight to determine volume, sperm concentration using a coulter counter (Z Series, Beckman Coulter, Clare, Ireland), total motility (% of the total sperm population both motile and non-motile) and gross motility (5-point scale: 1 = twitching/no forward progressive motility; 5 = excellent forward progressive motile sperm) to ensure all semen samples were of a commercial standard. Initial quality control cut-off values were a total and gross motility of $\geq 70\%$ and a score of ≥ 3 , respectively. Any ejaculates failing to meet these criteria were rejected for

commercial production but the data were still included in this study. Although morphology was not accurately assessed, consideration was given to the percentage of morphological abnormalities within an ejaculate by the experienced laboratory technician and the degree of abnormality was reflected upon when assessing motility.

Following *in vitro* assessments, the ejaculate was fully extended in BullXcell to a final concentration of 15×10^6 sperm per 0.25 mL semen straw (IMV Technologies). Straws were filled, sealed and printed as per routine procedures using the IS4 instrument (IMV Technologies). Straws from each ejaculate were then cooled to 4 °C over 3 h and were frozen to -140 °C as follows: -5 °C per min from +4 °C to -10 °C, -40 °C per min from -10 °C to -100 °C and thereafter -20 °C per min from -100 °C to -140 °C in a programmable freezer (IMV Technologies), followed by submersion and storage in liquid nitrogen at -196 °C until use (Murphy et al. 2017). Four straws from each ejaculate of each bull were assessed immediately post-thaw via standard microscopic techniques for total and gross motility. Post-thaw quality control cut-off values were a total and gross motility of $\geq 50\%$ and a score of ≥ 3 , respectively.

2.2.3 Assessment of Sperm Motility

Sperm motility (total and gross) was assessed pre-freezing and post-thawing using a phase contrast microscope (CX31; Olympus, Centre Valley, PA, USA) at a magnification of 400 X. A droplet (5 μ L) of diluted semen was placed on a pre-warmed glass slide and covered with a pre-warmed coverslip (18 x 18 mm; 37 °C). Total motility was assessed by counting a minimum of 100 sperm over at least five

different fields of view on each collection day, while gross motility was evaluated by assessing the swimming pattern of the entire sperm sample on a scale of 1 to 5 as described above. Total motility was expressed as a percentage of the total sperm population (motile and non-motile).

2.3 Statistical Analysis

Data were checked for normality and homogeneity of variance using histograms, QQ plots, and formal statistical tests in the Univariate procedure (version 9.1.3; SAS Institute, Cary, NC, USA). Data that were not normally distributed were transformed by raising the variable to the power of lambda. The appropriate lambda value was obtained by conducting a Box-Cox transformation analysis using the TRANSREG procedure of SAS. Semen production (ejaculate volume, sperm concentration per mL and TSN) and sperm quality (pre-freeze and post-thaw total and gross motility) parameters were analysed using the MIXED procedure of SAS with a model that included fixed effect of bull age, season and collection frequency. All two- and three-way interactions were tested for among the main factors. Bull was included as a random effect. Differences among means were determined by F-tests using Type III sums of squares. The PDIFF option and the Tukey test were applied to evaluate pairwise comparisons between means. Where appropriate, Spearman partial correlation analysis was carried out between variables using the PROC CORR accounting for year and GLM procedure of SAS was also used to determine the relationships responses between the main factors and semen production and quality variables.

2.4 Results

2.4.1 Effect of Bull Age on Semen Production and Quality

There was an effect of bull age on ejaculate volume, TSN ($P < 0.01$) and sperm concentration (Figure 2.1; $P < 0.05$) as well as pre- and post-thaw total and gross sperm motility (Figure 2.2; $P < 0.01$). Semen quality, as assessed by pre-freeze and post-thaw total and gross motility was lowest for bulls collected at less than one year of age compared to all other age categories; however, the difference in motility scores between bulls aged less than one year and older bulls was small (~2%) and unlikely to be of biological or commercial importance (Figure 2.2; $P < 0.01$). Ejaculate volume was strongly positively correlated with bull age ($r = 0.62$; $P < 0.01$) and increased by approximately 0.5 mL per year. As a result TSN also increased with age, with bulls aged less than one year producing the lowest ejaculate volume and TSN, while bulls aged more than four years produced the largest semen volume and TSN (Figure 2.1; $P < 0.01$). There was a linear increase in TSN with increasing ejaculate volume ($r = 0.71$; $P < 0.01$). Bulls aged between one and two years had a greater sperm concentration per ejaculate than bulls aged less than one or more than two years old (Figure 2.1; $P < 0.05$).

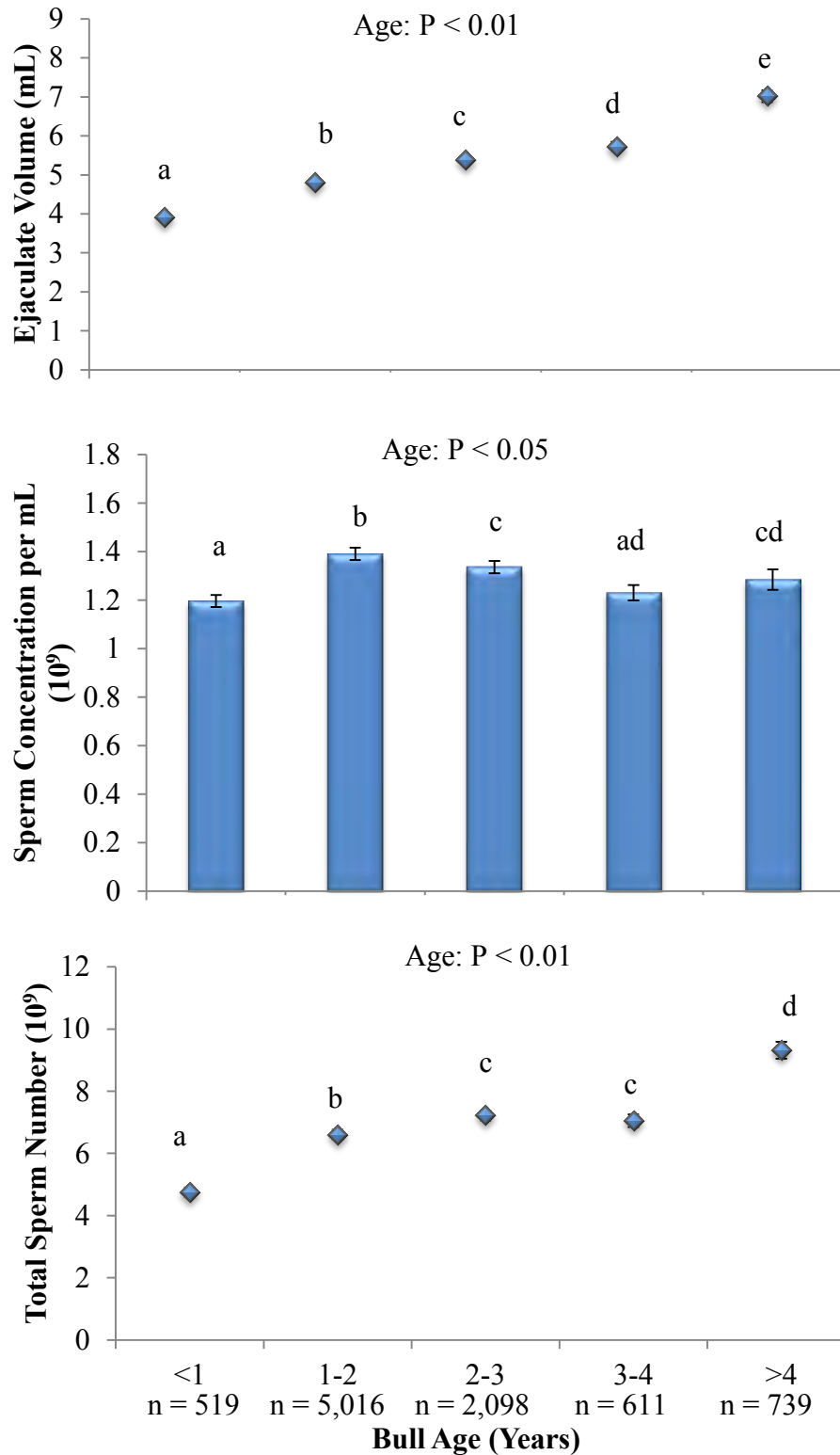


Figure 2.1: The effect of bull age on ejaculate volume (upper panel), sperm concentration (middle panel) and total sperm number (lower panel). Vertical bars represent sem. ^{abcde}Differing superscripts differ between bull ages within each parameter (P < 0.01). n = number of ejaculates.

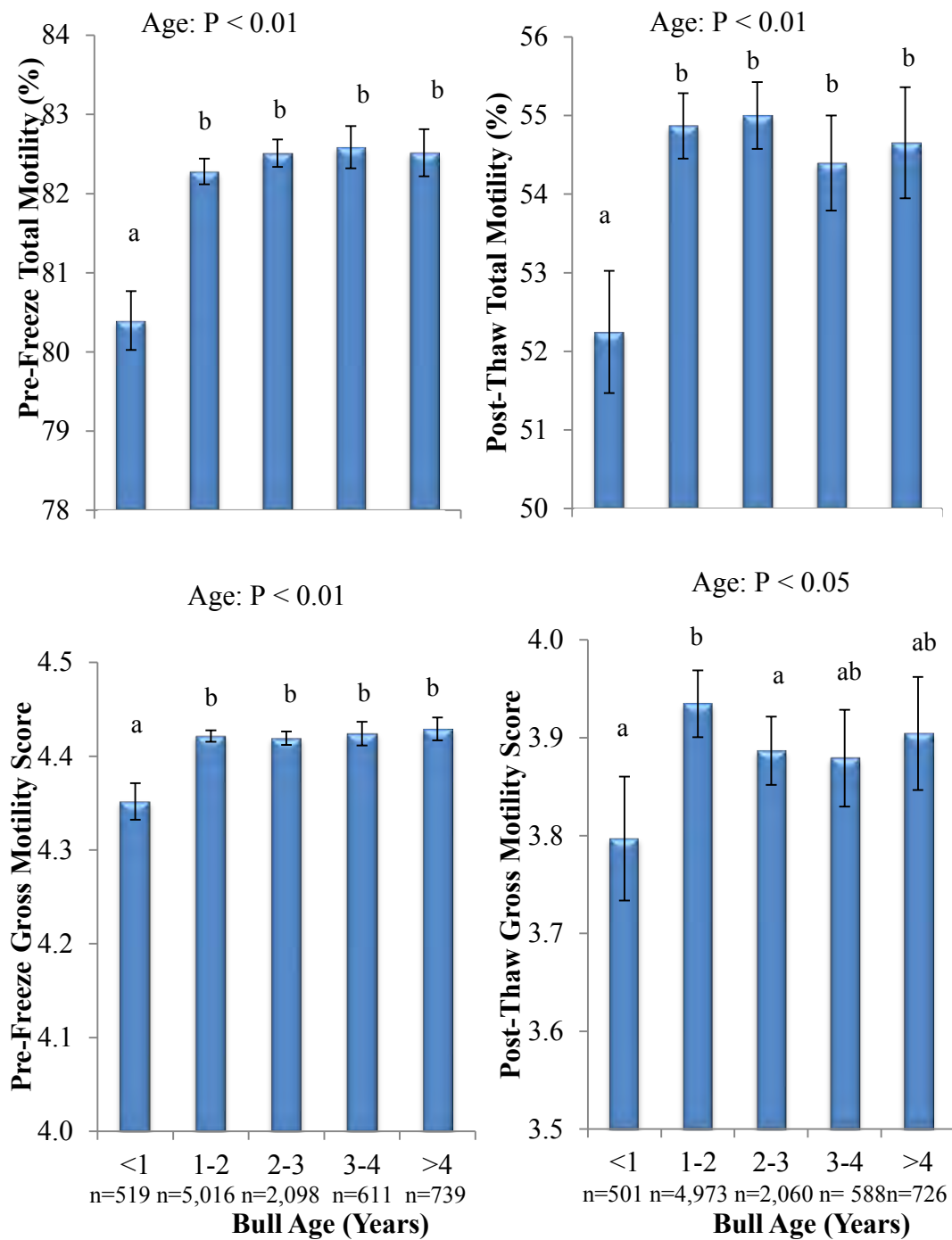


Figure 2.2: The effect of bull age on pre-freeze (upper left panel) and post-thaw total motility (upper right panel) and pre-freeze (lower left panel) and post-thaw gross motility (lower right panel). Vertical bars represent sem. ^{ab}Differing superscripts differ between bull ages within each parameter (P < 0.01). n = number of ejaculates.

2.4.2 Effect of Collection Frequency on Semen Production and Quality

There was an ejaculate number by bull age interaction on volume ($P < 0.05$), sperm concentration and TSN ($P < 0.01$) as bulls aged greater than one year had a reduced ejaculate volume, sperm concentration and TSN in their second ejaculate (Figure 2.3; $P < 0.01$); however, there was no effect of ejaculate number on bulls aged less than one year ($P > 0.05$). There was an effect of collection frequency on ejaculate volume, sperm concentration, TSN (Figure 2.3: $P < 0.01$) as well as pre-freeze total and gross motility (Figure 2.4; $P < 0.01$). First ejaculates exhibited higher pre-freeze total and gross motility scores ($P < 0.01$) and had a higher ejaculate volume and sperm concentration than second ejaculates, by approximately 15 and 40%, respectively ($P < 0.01$). However, ejaculate number did not affect post-thaw total and gross motility (Figure 2.4; $P > 0.05$). Overall, first ejaculates resulted in more than twice the number of total sperm than a second consecutive collection, with a TSN of 5.5 and 2.6×10^9 , respectively, primarily as a result of the large difference in sperm concentration between subsequent ejaculates.

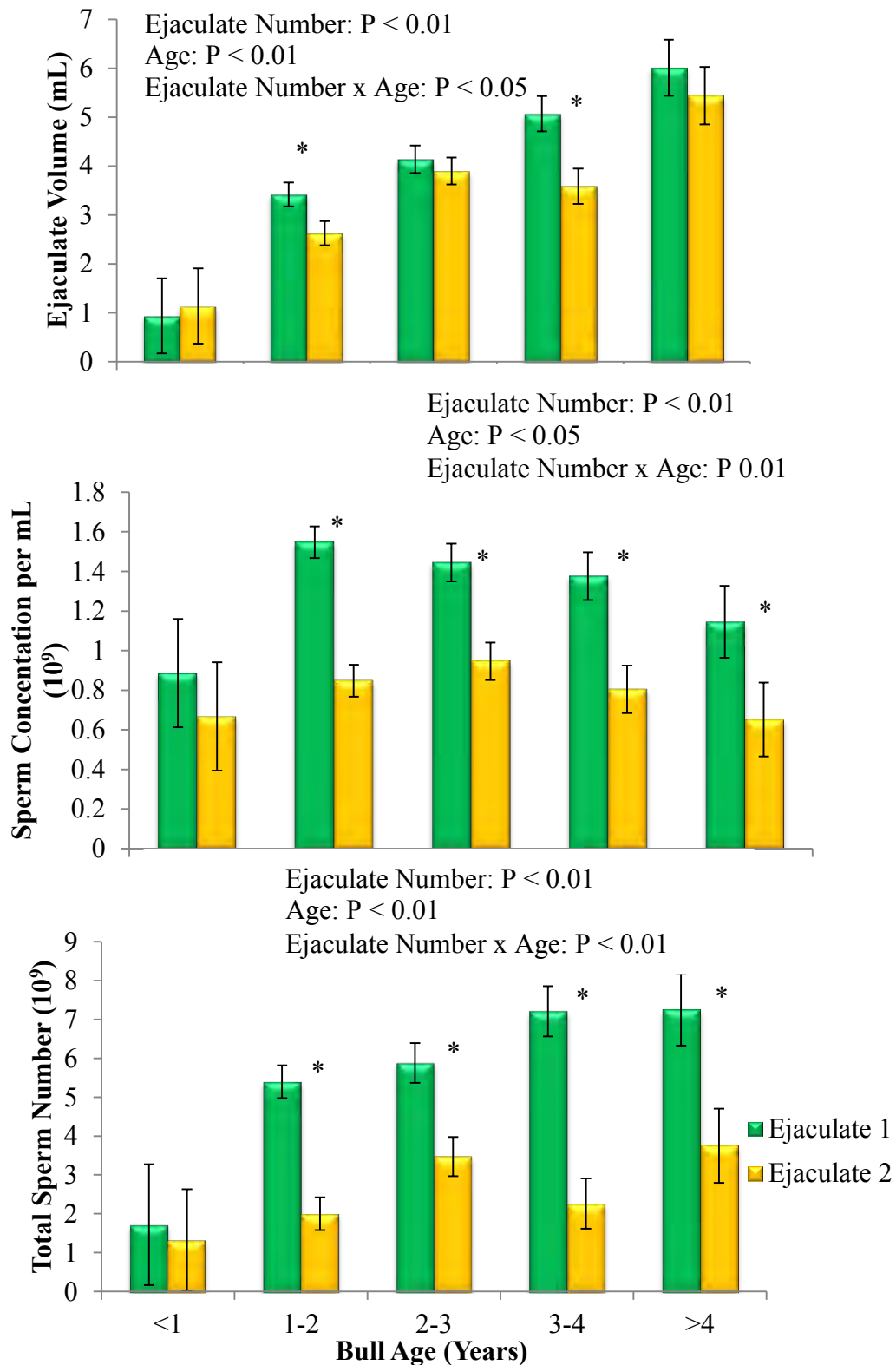


Figure 2.3: The interaction of ejaculate number and bull age on ejaculate volume (upper panel), sperm concentration (middle panel) and total sperm number (lower panel). Vertical bars represent sem. *Asterisk represents differences between ejaculate number within each parameter ($P < 0.01$).

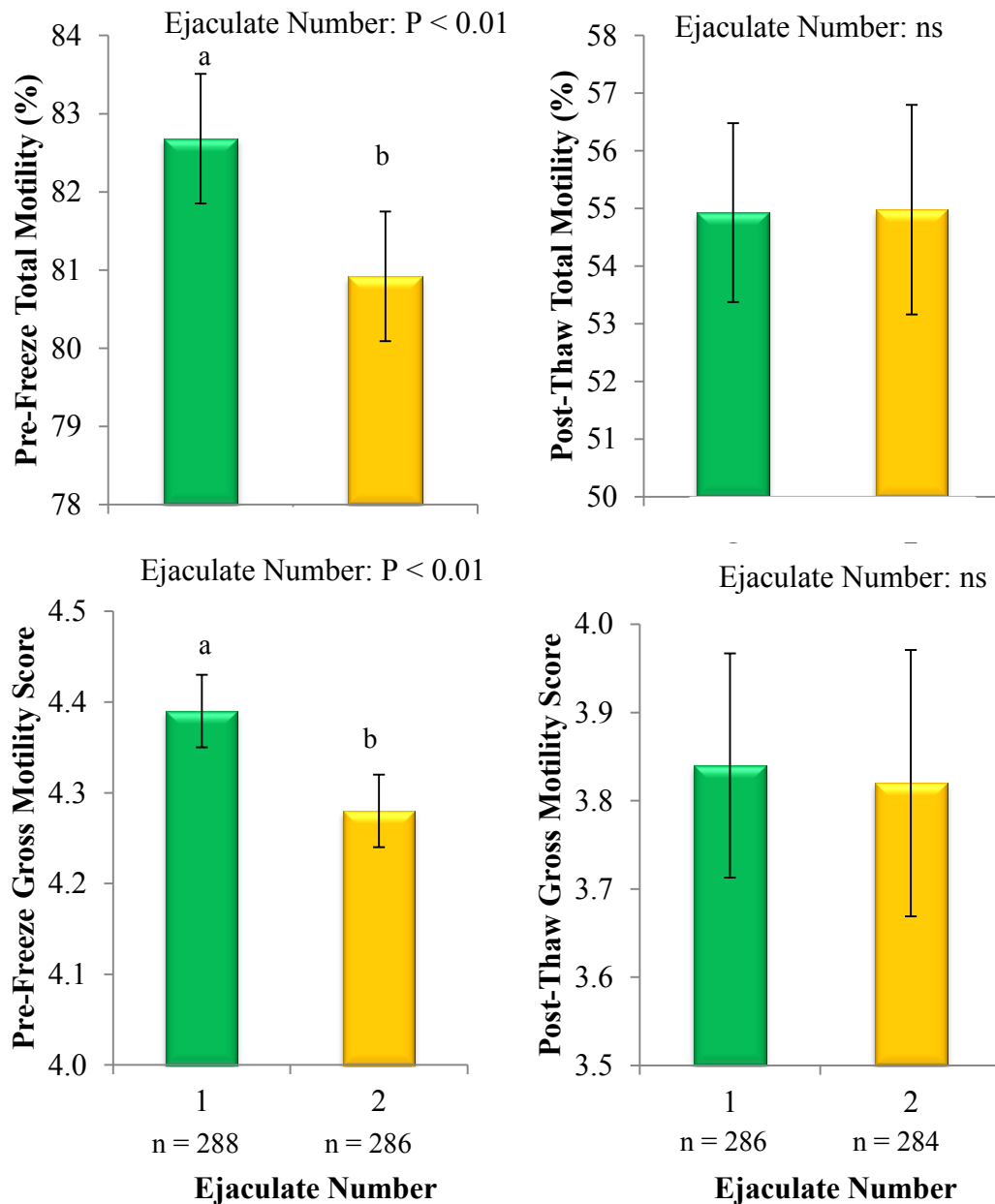


Figure 2.4: The effect of collection frequency on pre-freeze (upper left panel) and post-thaw total motility (upper right panel) and pre-freeze (lower left panel) and post-thaw gross motility (lower right panel). Vertical bars represent sem. ^{ab}Differing superscripts differ between ejaculate numbers within each parameter ($P < 0.01$). ns = non-significant, n = number of ejaculates.

2.4.3 Effect of Season of Collection on Semen Production and Quality

There was a season by bull age interaction for ejaculate volume, sperm concentration, TSN, post-thaw gross motility ($P < 0.01$) and pre-freeze gross and post-thaw total motility ($P < 0.05$). Although bulls aged less than one year had a reduced ejaculate volume, sperm concentration and TSN in Winter than any other season, there was no clear biological pattern for any other age category. There was an effect of season on sperm concentration, TSN (Figure 2.5; $P < 0.01$) as well as post-thaw total and gross motility (Figure 2.6; $P < 0.01$). There was a tendency for season to affect ejaculate volume ($P = 0.065$) with semen collections in Spring having the lowest volume. Semen collections in Winter had the greatest post-thaw total and gross motility score compared to semen collections in Spring (Figure 2.6; $P < 0.01$). Ejaculates collected in Summer and Autumn had higher sperm concentration and TSN in comparison to Spring and Winter (Figure 2.5; $P < 0.01$). Thus, regardless of the parameter assessed, semen collections in Winter resulted in the poorest semen production output, while collections in Summer and Autumn had the best semen production characteristics in terms of sperm concentration and TSN.

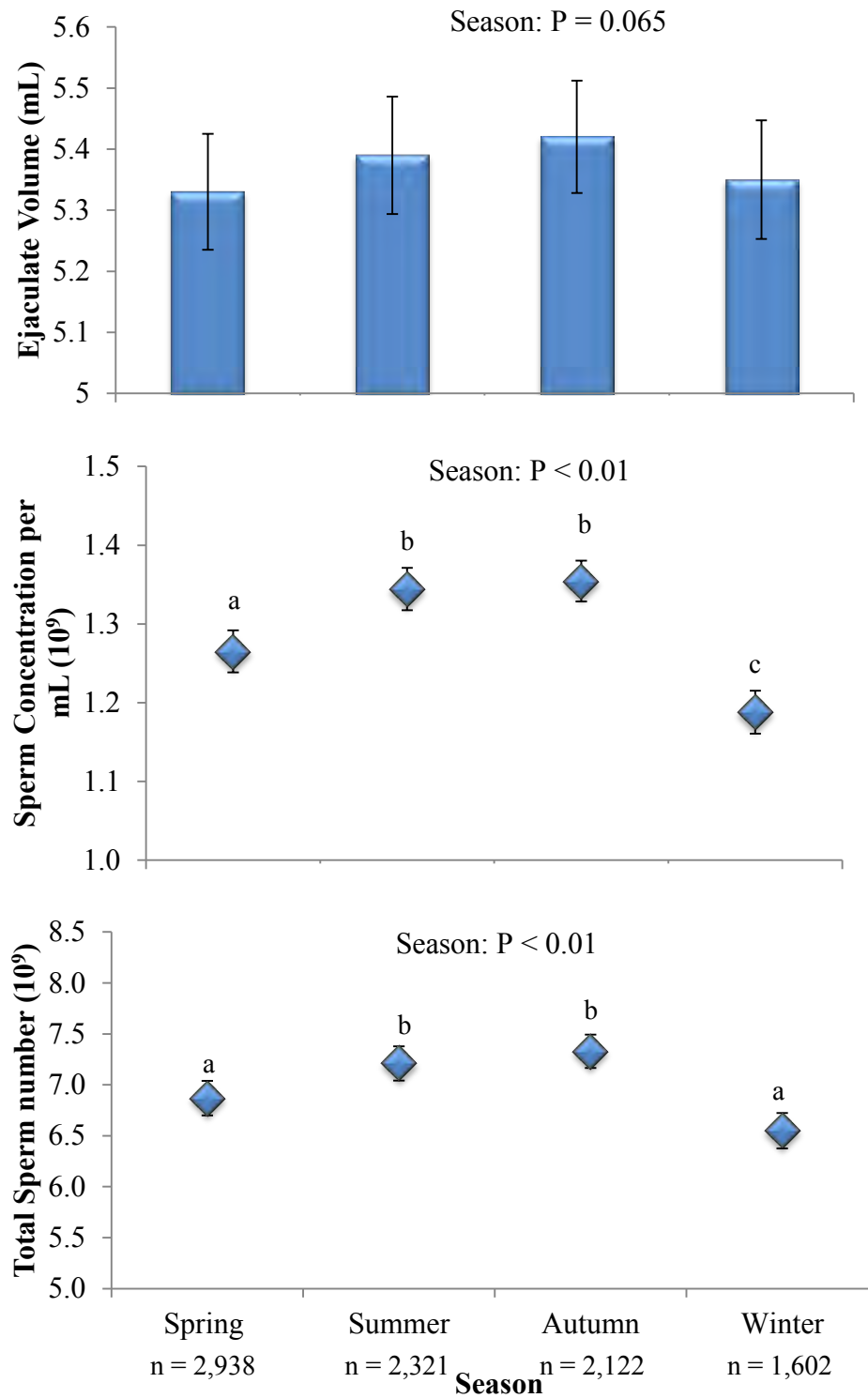


Figure 2.5: The interaction of season of collection and bull age on ejaculate volume (upper panel), sperm concentration (middle panel) and total sperm number (lower panel). Vertical bars represent sem. ^{ab}Differing superscripts differ between ejaculate numbers within each parameter (P < 0.01). ns = non-significant, n = number of ejaculates.

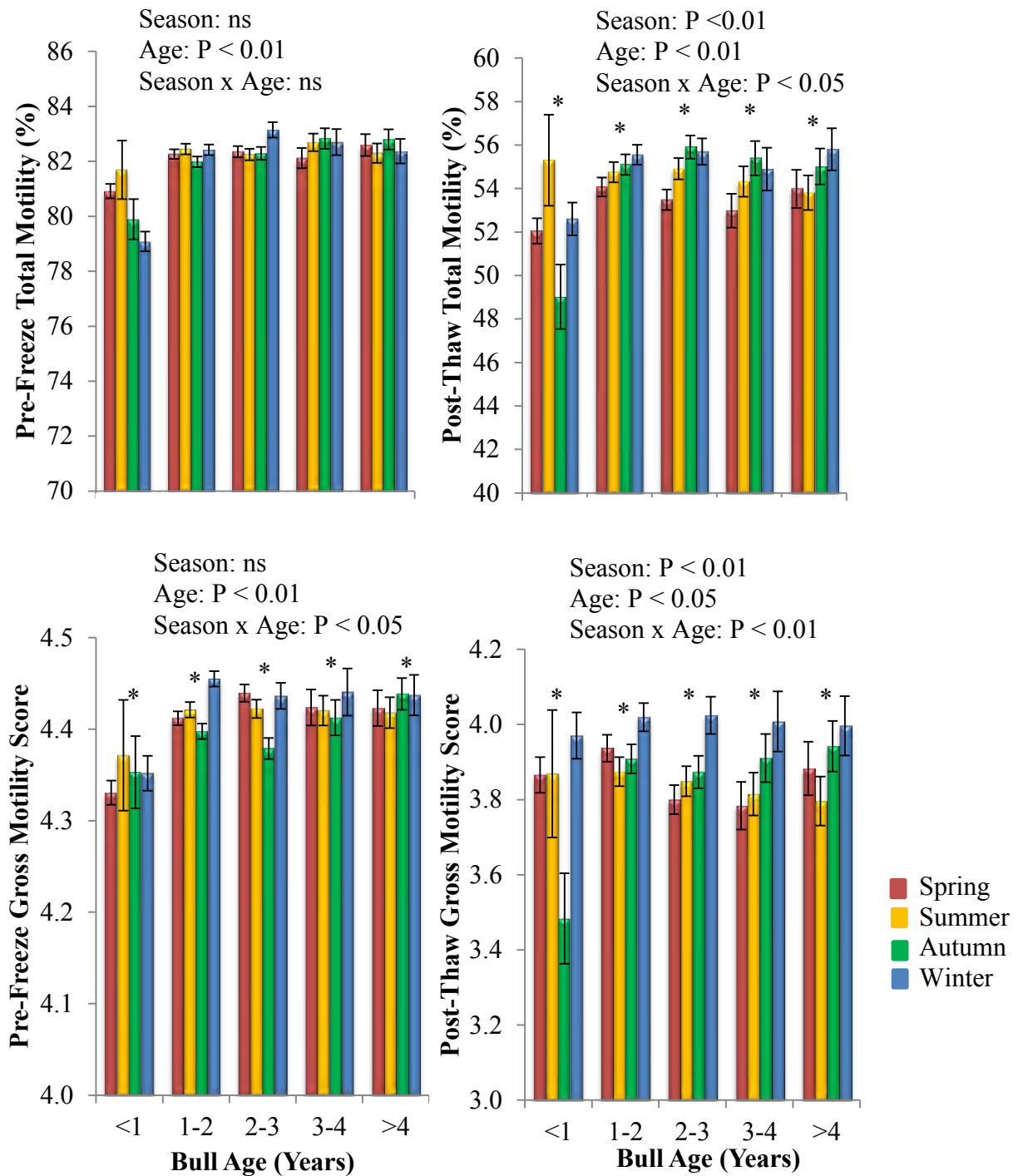


Figure 2.6: The interaction of season of collection and bull age on pre-freeze (upper left panel) and post-thaw total motility (upper right panel) and pre-freeze (lower left panel) and post-thaw gross motility (lower right panel). Vertical bars represent sem. *Asterisk represents differences between season within each parameter ($P < 0.01$). ns = non-significant.

2.5 Discussion

This study involved the interrogation of a large dataset collected over a period of four years, involving a total of 8,983 ejaculates from 176 Holstein Friesian bulls aged between 9 months and 8 years of age, thereby facilitating an in-depth assessment of semen quality of AI bulls in a comprehensive attempt to identify factors affecting semen production and quality in a commercial AI setting. The main novel findings of this study were that second ejaculates can be collected from young bulls without a concomitant significant decrease in post-thaw semen quality and thus may be a useful strategy to increase semen availability from AI bulls in high demand. This study also clearly illustrates the challenges surrounding the collection of ejaculates of sufficient volume and quality from bulls of less than one year of age.

The observed increase in volume and TSN associated with increasing bull age is consistent with a number of other reports (Everett and Bean 1982, Mathevon et al. 1998, Taylor et al. 1985, Brito et al. 2002a). Since sperm concentration remained constant after 1 year of age the increase in TSN with age was being driven by increases in ejaculate volume up to four years of age, consistent with the findings of Everett and Bean (1982). Not surprisingly, bulls aged less than one year had the lowest values (Al-Kanaan et al. 2015). It is widely acknowledged (Perumal 2014, Mathevon et al. 1998, Karabinus et al. 1990) that peri-pubertal bulls have lower ejaculate volumes than mature bulls, which is in agreement with the findings in this study, and that the pre-pubertal period is generally characterised by rapid increases in both body and testicular weight (Aponte et al. 2005). Therefore, the increase in ejaculate volume with age may be related to an increase in activity of the hypothalamic-pituitary-testicular axis and the concurrent development of the testis

and accessory glands with sexual maturity, which are believed to continue to develop for up to 5 years post-puberty (Almquist 1978). Following the onset of puberty (as defined by Wolf et al. 1965), at approximately 9-11 months of age in Holstein Friesian bulls (Byrne et al. 2017; Dance et al. 2016), the reproductive capacity of a bull increases for several years until sexually maturity is reached (Amann 1983).

In the current study, there was an ejaculate number by bull age interaction for ejaculate volume, sperm concentration and TSN for bulls older than one year of age, with the collection of a subsequent ejaculate resulting in lower semen production values. First ejaculates had a greater volume, sperm concentration and TSN for bulls older than one year in comparison to second ejaculates collected on the same day; however there was no effect of ejaculate number on bulls aged less than one year. This finding is similar to that of Fuerst-Waltl et al. (2006) and Bhakat et al. (2011) as first ejacuaes recorded greater semen production values for all age categories; however, these studies did not investigate the effects on bulls aged less that one year with the lowest bull age category for each study of 16-18 months and less than three years, respectively. Conversely, although volume increased with bull age, ejaculate number did not significantly affect ejaculate volume for all ages as bulls aged between 2-3 years and greater than 4 years recorded similar volumes for both collections. Surprisingly, the effect of ejaculate number was not significant in bulls aged less than one year for any semen production parameter. This may be primarily due to the significantly lower semen production values associated with young bulls and the large variation within their analysis compared to more mature bulls. Furthermore, although semen production decreases with the collection of multiple ejaculates on the same day, the overall production of semen increases, resulting in an

increase in the number of semen doses produced per bull per day. To place it in perspective, for an average bull, the first and second consecutive ejaculates typically produce approximately 400 and 200 straws, respectively, with a concentration of 15×10^6 sperm per 0.25 mL straw. Therefore, a bull collected twice a day, twice a week (1,200 straws) compared to once a day, twice a week (800 straws) would result in increasing overall production by 400 straws. Thus, a second collection may be justified for bulls which are in high demand, particularly those less than one year of age, as semen production from these young bulls was not negatively impacted by the collection of a second ejaculate.

Additionally, in the current study first ejaculates recorded a higher pre-freeze total and gross motility score than second ejaculates which is in agreement with Fuerst-Waltl et al. (2006) who reported a higher percentage of motile sperm in first ejaculates. However, similar to the findings of Boujenane and Boussaq (2013), there was no difference observed in post-thaw motility. One possible explanation behind a reduction in semen production and pre-freeze sperm quality associated with second ejaculates may be due to the shortened collection interval of the second ejaculate (although not formally assessed in this study the norm was within 1 h). Longer collection intervals have been reported to result in greater semen production and quality, however, these collection interval vary from 3-4 to 10 days (Mathevon et al. 1998, Everett and Bean 1982, Fuerst-Waltl et al. 2006) but are unrealistic in a commercial environment setting. Due to the high demand, it is impractical for AI centres to allow up to 10 days between collections. In the current study, critically there was no effect of ejaculate number on post-thaw semen quality in which a collection interval of approximately 1 h was implemented. This is important as the

ability of sperm to maintain their functional status post-thaw in both first and second ejaculates is essential considering that AI in cattle is primarily implemented with the use of cryopreserved semen (Thibier and Wagner 2002). Therefore, the results of the current study highlight that semen production and quality can be maintained with a shorter collection interval of approximately 1 h, hence increasing productivity.

The effect of season on semen production has been widely assessed in the bull (Malama et al. 2017, Snoj et al. 2013, Bhakat et al. 2014, Al-Kanaan et al. 2015). Spermatogenesis has been shown to be susceptible to temperature variation (Rahman et al. 2011) and as it takes approximately 61 days in the bull (Johnson et al. 2000), the quality of sperm in an ejaculate may reflect conditions to which the bull was exposed 8-9 weeks prior to collection. The impact of many environmental factors, however, is reduced when bulls are maintained in temperature-controlled barns (Haugan et al. 2005) as other studies have shown that neither temperature nor humidity affected sperm production or semen quality (Brito et al. 2002b, Taylor et al. 1985). Under the temperate climatic conditions of the current study, there was a season by bull age interaction on semen production; ejaculate volume, sperm concentration, TSN and semen quality; post-thaw total motility and pre- and post-thaw gross motility. Bulls aged less than one year recorded poorest semen production values in terms of volume, sperm concentration and TSN in Winter than any other season; however, there was no clear biological pattern for any other age category. Furthermore, Winter collections also recorded significantly higher post-thaw semen quality values; but, while there was a statistical difference in semen quality, the difference between seasons was relatively small and is unlikely to be of biological importance or have a significant impact on quality control in a commercial

environment as all values recorded were sufficient to pass quality control analysis. The results of the current study are consistent with (Sullivan and Elliott 1968) who reported that semen collections in the US in Winter resulted in higher non-return rates than those in Spring, which may be related to better semen quality in line with the current study. Similarly, Boujenane and Boussaq (2013) reported that semen collected in a Moroccan AI centre in Winter was of higher quality than Summer collections. In contrast, Stålhammar et al. (1989) and Snoj et al. (2013) observed greater sperm concentration and TSN during the Summer months than in any other season in AI centres located in Sweden and Slovenia, respectively. This is broadly in agreement with the results of the current study as collections in Summer recorded the highest values for sperm concentration and TSN; however, this did not differ from collections in Autumn.

In conclusion, this study characterised the challenges surrounding the collection of young Holstein Friesian bulls in a commercial AI setting. The low semen ejaculate volume typically associated with young bulls not only reduces sperm numbers but also pre-freeze and post-thaw semen quality. As these young bulls are typically of a higher genomic value compared to older bulls, AI centres require large quantities of their semen in order to meet demand and therefore, need to minimise the amount of inferior quality semen being handled. The collection of a second consecutive ejaculate, although having a significantly lower volume and TSN, with the exception of bulls less than one year, does not affect post-thaw sperm quality and therefore, should be considered, particularly for bulls in high demand.

Chapter Three

A Comparison of Semen Diluents on the
In Vitro and *In Vivo* Fertility of Liquid
Bull Semen

Abstract

The aim of this study was to assess the effect of semen diluent on CR following AI with liquid bull semen stored for up to 3 days post collection. In Experiment 1, the effect of storing liquid semen maintained at a constant ambient temperature in 1 of 7 different diluents (Caprogen, OptiXcell, BioXcell, BullXcell, INRA96, NutriXcell or AndroMed) on total and progressive motility was assessed on Days 0, 1, 2 and 3 post collection. In Experiment 2, the field fertility of liquid semen diluted in Caprogen, BioXcell or INRA96 and inseminated on Days 1, 2 or 3 post collection was assessed in comparison to frozen-thawed semen (total of $n = 19,126$ inseminations). In Experiment 3, the effect of storage temperature fluctuations (4°C and 18°C) on total and progressive motility following dilution in Caprogen, BioXcell and INRA96 was assessed on Days 0, 1, 2 and 3 post collection. In Experiment 1, semen stored in Caprogen, BioXcell and INRA96 resulted in the highest total and progressive motility on Days 1, 2 and 3 of storage compared to OptiXcell, BullXcell, NutriXcell and AndroMed ($P < 0.01$). In Experiment 2, there was an effect of diluent on calving rate ($P < 0.01$) as semen diluted in BioXcell had a lower CR on Days 1, 2 and 3 of storage (46.3, 35.4 and 34.0%, respectively) in comparison with Caprogen (55.8, 52.0 and 51.9%, respectively), INRA96 (55.0, 55.1 and 52.2%, respectively) and frozen-thawed semen (59.7%). There was an effect of parity ($P < 0.01$), cow fertility sub-index ($P < 0.01$) as well as the number of days in milk ($P < 0.01$) on CR. In Experiment 3, when the storage temperature of diluted semen was fluctuated between 4 and 18°C , to mimic what occurs in the field (night-time vs day-time), BioXcell had the lowest total and progressive motility in comparison to Caprogen and INRA96 ($P < 0.01$). In conclusion, diluent significantly affected sperm motility

when stored for up to 3 days. Semen diluted in INRA96 resulted in a similar CR to semen diluted in Caprogen and to frozen-thawed semen, while that diluted in BioXcell resulted in a decreased CR. Consistent with this finding, semen diluted in BioXcell was less tolerant of temperature fluctuations than that stored in Caprogen or INRA96. Given that it can be used directly off-the-shelf, INRA96 may be a suitable alternative to Caprogen for the storage of liquid bull semen.

3.1 Introduction

AI is the single most important technique devised to facilitate the genetic improvement of animals as it facilitates the widespread use of elite males (Oliveira et al. 2013, Black 2006). Dairy animals in Ireland are ranked on an EBI which was established in 2001. The EBI is a profit index aimed at helping farmers identify the most profitable bulls and cows for breeding dairy herd replacements and has contributed significantly to improved fertility of dairy cows in Ireland (Murphy et al. 2016, Berry et al. 2005). The EBI comprises information on seven sub-indexes related to profitable milk production, including: (i) Milk production, (ii) Fertility, (iii) Calving performance, (iv) Beef Carcass, (v) Cow maintenance, (vi) Cow management and (vii) Health. One of the key components of the EBI is the fertility sub-index which accounts for approximately 35% of the total EBI and is based primarily on calving interval and cow survival (Irish Cattle Breeding Federation 2014).

Currently, within the Irish dairy industry, 95% of AI is conducted using frozen-thawed semen, with liquid (i.e., fresh, non-cryopreserved) semen accounting for only 5% of inseminations (Murphy et al. 2016). However, Ireland has a seasonal grass-

based production system and during the peak breeding season, from mid-April to early June, the use of liquid semen can increase to approximately 25% of inseminations in order to accommodate the large demand (Al Naib et al. 2011a). A typical dose of liquid semen contains 5 million sperm (Murphy et al. 2013) in comparison to 15 to 20 million sperm for a typical frozen-thawed semen dose (Vishwanath et al. 1996). Liquid semen processing yields more doses per ejaculate, thereby, facilitating the greater utilisation of genetically superior sires. This is particularly beneficial for young genomically-selected sires as these sires are in high demand but produce lower semen volumes in comparison to more mature bulls (Brito et al. 2002a).

The extensive use of AI in the bovine industry can be partly attributed to the development of suitable diluents for both liquid and frozen-thawed semen (Foote and Kaproth 2002). In Ireland, liquid semen is typically stored at ambient temperature in thermo-insulated containers in order to reduce the effects of the natural day to night time temperature fluctuations. Murphy et al. (2016) reported that although fluctuating storage temperature between 5 and 32 °C had no effect on viability, total progressive motility was greatest for liquid semen stored at 15 °C compared to other temperatures assessed. In addition to dilution of the semen, diluents provide protective compounds such as BSA, antioxidants and antibiotics to maintain sperm function (van den Berg et al. 2014). Despite this, liquid semen has a limited shelf life and semen stored in Caprogen (the gold standard for liquid semen dilution) is principally used for only 2.5 to 3 days post collection as a reduction in pregnancy rates has been reported thereafter (Vishwanath and Shannon 2000). A number of studies have been conducted on liquid bull semen diluents with the aim of combating

the reduction in fertility associated with increased duration of storage (Murphy et al. 2016, Verberckmoes et al. 2004, Vishwanath and Shannon 2000). Many of these studies have focused on reducing the metabolic activity of sperm cells as the survival of sperm for extended periods of time has been shown to be inversely related to their metabolic activity (Vishwanath and Shannon 2000). Approaches taken have included storing semen at 5 °C (Saha et al. 2014), reducing sperm concentration (Murphy et al. 2013), altering the pH (Ferdinand et al. 2014) as well as N₂ gassing and modifying the diluent composition (Shannon 1968). The ability to extend the shelf life of liquid semen is important as: (i) the distribution of liquid semen would be simplified if it could be used for more days and (ii) the work load involved in collecting and processing semen would be greatly reduced as bulls used for liquid semen would have a reduced collection schedule.

Apart from Caprogen, several other diluents have been developed for the storage of semen for a variety of domestic species. BioXcell is an animal protein-free medium (soya lecithin-based extender) which has been routinely used for the cryopreservation and preservation at chilled temperatures of buffalo (Akhter et al. 2011, Akhter et al. 2010) ram (Kulaksiz et al. 2012) and bovine semen (Stradaioli et al. 2007). Although the benefits of using lecithin-based extenders on the cryopreservation of bovine semen have been reported by several authors (Crespilho et al. 2014, Aires et al. 2003, Van Wagtendonk-de Leeuw et al. 2000), many of these studies have focused on *in vitro* analysis only. INRA96 is a milk-based diluent which was primarily developed to maintain the fertility of stallion semen during chilled storage at 4 or at 15 °C for up to 72 h (Batellier et al. 1997) but has also been used for the cryopreservation of equine semen (Pillet et al. 2008, Fayrer-Hosken et

al. 2008). In recent years, INRA96 has also been used as a storage medium for the sperm of other species including rabbit (De Amicis et al. 2004), dog (Sahashi et al. 2011), goat (López-Fernández et al. 2011) and sheep (Olivera-Muzante et al. 2011) and has yielded acceptable fertility *in vivo* in sheep following cervical AI after 24 h storage (O'Hara et al. 2010). Other commercially available extenders include OptiXcell, a protein-free egg yolk-like media for frozen and liquid bovine semen, NutriXcell, a long-term extender primarily used for the preservation of boar semen up to 6 days (Kaeoket et al. 2010), AndroMed, an egg yolk-free, soya lecithin-based medium for freezing of bovine (Maxwell et al. 2007), ram (Fukui et al. 2008), buffalo (Herold et al. 2004) and buck semen (Gacitua and Arav 2005) and BullXcell, an egg-yolk tris extender used for bovine semen cryopreservation. While all of the aforementioned diluents have been used for the preservation of semen from domestic species, many of these studies have solely reported *in vitro* data and there is a dearth of published studies on the *in vivo* fertility of bovine semen stored in the majority of these diluents.

Using a combination of *in vitro* assessments and a large-scale commercial field trial, the objectives of this study were to assess the effect of (i) liquid semen diluent on total and progressive sperm motility (ii) liquid semen diluent on calving rate (CR) (iii) cow characteristics, namely parity, breed, cow fertility sub-index and number of DIM on CR and (iv) temperature fluctuation of liquid semen on total and progressive motility of liquid bovine semen. To our knowledge, this is the first report to examine the effect of a large number of diluents on liquid bull semen during *in vitro* storage, where each treatment was prepared from the same ejaculate. This eliminates any

potential confounding effects arising from the collection of diluent treatments from different bulls or ejaculates and thus, provides clear and reliable data.

3.2 Materials and Methods

3.2.1 Experiment 1: Effect of Storing Liquid Semen Maintained at a Constant Ambient Temperature in 1 of 7 Different Diluents on Total and Progressive Sperm Motility

The aim of this experiment was to assess the ability of 7 liquid semen diluents (1 homemade and 6 commercially available) to sustain total and progressive motility of bull sperm for up to 3 days post collection. Semen was collected from Holstein Friesian bulls (n = 6) at a commercial AI centre on three different occasions (occasion = replicate). The raw ejaculate was placed into a 50 mL tube and transported in a temperature-regulated cooler box at 18 °C to the laboratory (up to 3 h transport). On arrival, the ejaculate was assessed for weight, sperm concentration using a coulter counter (Z Series, Beckman Coulter, Clare, Ireland), total motility (%) and progressive motility on a 5-point scale (1 = twitching/no forward progressive motility; 5 = excellent forward progressive motile sperm) to ensure all semen samples were of a commercial standard. Microscopic assessments were conducted by the same technician and initial quality control cut-off values were a total and progressive motility of $\geq 70\%$ and a score of ≥ 3 , respectively, and any ejaculates failing to meet these criteria were rejected.

The raw ejaculate was split and diluted to achieve a concentration of 5×10^6 sperm per 0.25 mL insemination dose in 1 of 7 different diluents, namely Caprogen

(homemade), OptiXcell (IMV Technologies, Normandy, France), BioXcell (IMV Technologies), BullXcell (IMV Technologies), INRA96 (IMV Technologies), NutriXcell (IMV Technologies) and AndroMed (Minitube, Tiefenbach, Germany). Prior to dilution, the Caprogen diluent was purged in food fresh N₂ gas (BOC, Dublin, Ireland) as per standard preparation procedures to dispel oxygen from the media and create an anaerobic environment, limiting the metabolic activity of sperm during liquid storage (Vishwanath and Shannon 2000). All other diluents were prepared as per the manufacturers' instructions. Semen from each bull was kept separate and ejaculates were split such that each bull was represented in each treatment. Semen straws were filled as per routine procedures and placed in a temperature regulated cooler box at 18 °C. Samples from the different treatments were assessed in a randomised sequence to remove bias as a result of sampling order. Total and progressive motility; n = 3 replicates) were assessed *in vitro* on Days 0, 1, 2 and 3 post-collection (Day 0 = 4 h after collection). Within each replicate, on each assessment day, two straws from each bull (n = 6) for each diluent (n = 7) were assessed.

3.2.1.1 Assessment of Sperm Motility

Sperm motility (total and progressive) in liquid semen was assessed on Days 0, 1, 2 and 3 post semen collection using a phase contrast microscope (CX31; Olympus, Centre Valley, PA, USA) at a magnification of 400 X. A droplet of diluted semen (5 µL) was placed on a pre-warmed glass slide, covered with a pre-warmed coverslip (37 °C) and assessed by counting a minimum of 100 sperm, over at least five different fields of view, for each treatment on each assessment day. Total motility was expressed as a percentage of the total sperm population (motile and non-motile).

A sperm was deemed to display progressive motility if it moved in a linear fashion; progressive motility was expressed as the percentage of live and motile sperm that displayed forward progressive motion.

3.2.2 Experiment 2: Field Fertility of Liquid Semen Diluted in Caprogen, BioXcell and INRA96

The aim of this experiment was to assess the effect of three liquid bovine semen diluents, selected based on the outcome of Experiment 1, on CR following AI. Semen was collected from Holstein Friesian bulls ($n = 8$; denoted A-H) at a commercial AI centre from early May to the end of May 2015. There were 11 collection days in total, with two bulls used per collection day (total of 22 ejaculates). Following assessment for volume, concentration and motility (as described in Experiment 1), each acceptable ejaculate was split into three equal volumes and diluted to 5×10^6 sperm per 0.25 mL insemination dose in one of three different diluents: namely Caprogen, BioXcell or INRA96 (Figure 3.1). All three diluents were prepared as per Experiment 1. Each batch of liquid semen was clearly labelled and distributed for insemination on the day of collection. Liquid semen was used for up to 3 days post collection on both heifers ($n = 192$) and multiparous ($n = 9,611$) dairy cows. Due to logistical constraints, frozen-thawed semen doses were derived from previously collected ejaculates from the same 8 bulls which were processed and frozen using routine procedures ($n = 9,323$ inseminations consisting of 526 heifers and 8,797 multiparous dairy cows). Upon collection of semen samples for cryopreservation, the raw ejaculate was partially diluted in 10 mL of pre-warmed BullXcell (37 °C). Semen samples were assessed for volume, sperm concentration and total and progressive motility as described in Experiment 1. Only ejaculates

achieving a total motility score of $\geq 70\%$ and a progressive motility score of ≥ 3 were used for cryopreservation. Following *in vitro* assessments, the semen was fully extended with pre-warmed BullXcell to achieve a concentration of 15×10^6 sperm per 0.25 mL insemination dose. Straws were frozen to -140°C as follows: -5°C per min from $+4^\circ\text{C}$ to -10°C , -40°C per min from -10°C to -100°C and thereafter -20°C per min from -100°C to -140°C in a programmable freezer (IMV Technologies), followed by submersion and storage in liquid nitrogen at -196°C until use.

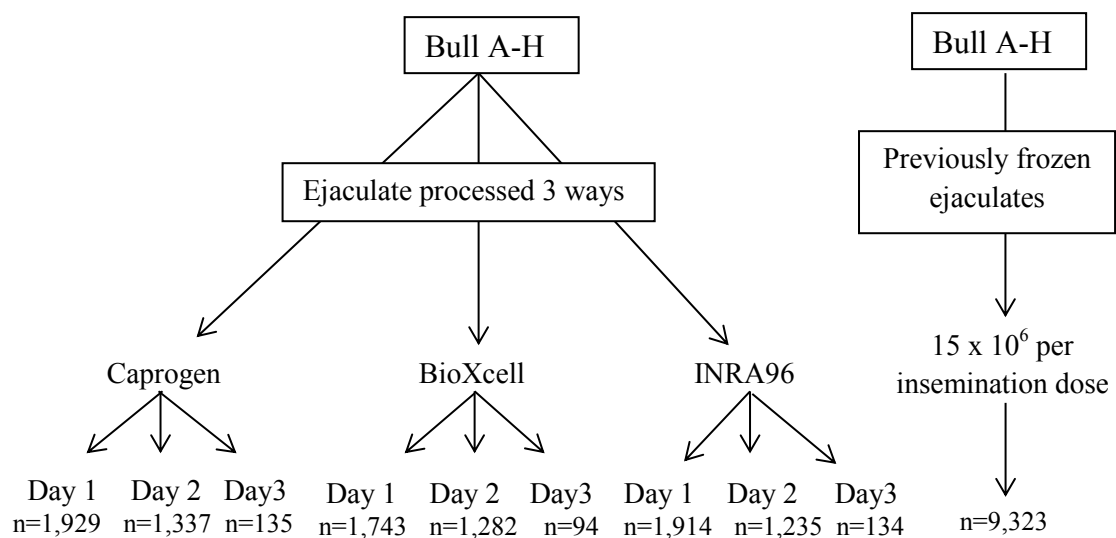


Figure 3.1: Experimental design for Experiment 2. Ejaculates were split for the liquid semen treatments and previously frozen ejaculates from the same bull were sourced for the frozen-thawed treatment (n = the total number of inseminations per diluent per day).

3.2.2.1 Field Inseminations

Inseminations were carried out in May 2015 (coinciding with the peak dairy breeding season) in Irish dairy herds ($n = 2,490$). The majority of inseminations were in Holstein Friesian cows ($n = 18,304$) but small numbers of cows of other breeds were represented: Jersey ($n = 375$), Montbeliarde ($n = 113$), Norwegian Red ($n = 268$), Swedish Red cows ($n = 10$) and other ($n = 56$; includes Normande, Rotbunte, Danish Red and Red Poll). Technicians ($n = 108$) were grouped into geographical areas and treatments were rotated on each collection day to ensure that technicians received different diluent treatments from each of two bulls on each day (Figure 3.2). Technicians were blind to treatments. For each insemination the AI technician recorded the bull code, cow tag number and the straw code on a handheld electronic device. Insemination and calving rate data were captured using the Irish Cattle Breeding Federation (ICBF; Bandon, Co Cork, Ireland) database by cross-referencing the technician name with the bull code and semen type used on each date within the trial period. Obvious errors were extracted from the dataset and data were then interrogated to remove animals based on the following criteria: cows which were not at first AI, cows which received two inseminations from two different bulls or diluent treatments, or cows which were not of a dairy breed. However, if a cow received two inseminations from the same bull with the same diluent treatment within five days of each other, the record was kept and the second date was assumed to be correct. Post editing, a total of 19,126 inseminations remained. Calving rate was measured using a cut-off value of 275 and 290 days from date of insemination to calving date.

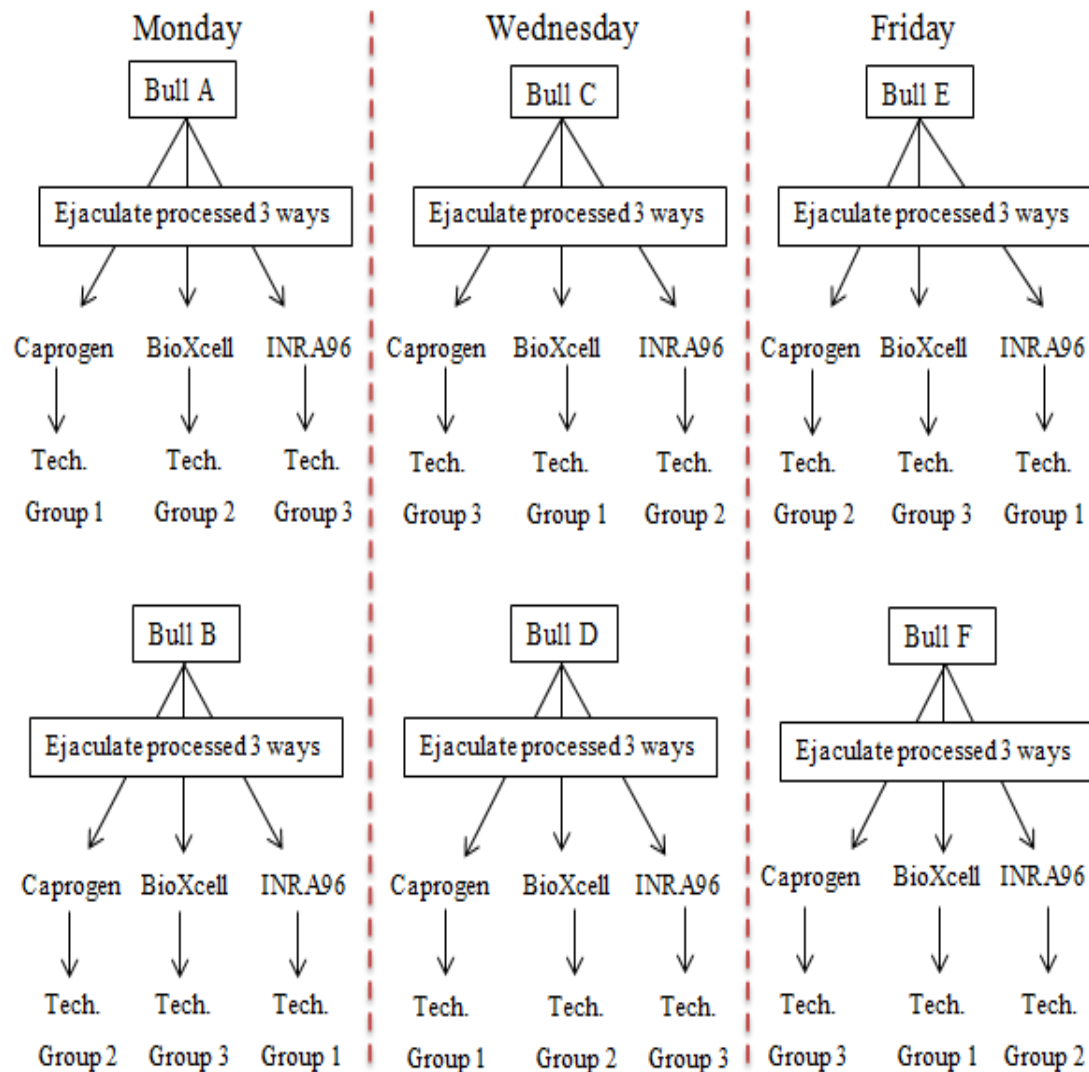


Figure 3.2: Experimental design for Experiment 2, highlighting the distribution of different liquid semen treatments to technicians within a week (Displaying Bulls A-F only). Each technician received two different treatments each day. Frozen-thawed semen was also inseminated throughout the period.

3.2.3 Experiment 3: Effect of Temperature Fluctuations on Semen Diluted in Caprogen, BioXcell and INRA96 on Total and Progressive Sperm Motility

Based on the outcome of Experiment 2, in which BioXcell performed poorly, we hypothesized that the effect of fluctuating temperatures experienced in practice during storage of liquid semen would be different for semen diluted in Caprogen,

BioXcell and INRA96. Semen was collected from Holstein Friesian bulls ($n = 6$) at a commercial AI centre on 3 occasions (occasion = replicate). Semen from each bull was kept separate and ejaculates were assessed and diluted in Caprogen, BioXcell and INRA96 (as per Experiment 2). After packaging, straws were placed in a polystyrene box and stored at 18 °C during the day and gradually brought to 4 °C during the night in order to mimic the unregulated temperature fluctuations to which liquid semen is typically subjected when stored in thermo-insulated containers in practice (Murphy et al. 2015). Samples from the different treatments were assessed on Days 0, 1, 2 and 3 post collection in a randomised sequence to remove bias as a result of sampling order. Total and progressive motility was assessed *in vitro* as described in Experiment 1 using computer assisted sperm analyser (CASA; IVOS II, IMV Technologies).

3.3 Statistical Analysis

Data from Experiments 1 and 3 were examined for normality of distribution, homogeneity of variance and analysed using the general linear model (GLM) repeated-measures procedure with a compound symmetry covariance structure in Statistical Package for Social Science (SPSS, Version 22.0; IBM, Chicago, USA). In Experiment 2, the CR data were compared using Pearson's chi-squared procedure in SPSS. Data were cross checked using an analysis of variance (ANOVA) model. The dependent variable in the analysis was CR (1 = calved, 0 = not calved). Calving rate was evaluated and correlations were investigated with a number of fixed effects, namely bull, parity number, cow breed, cow fertility sub-index and DIM. Each fixed effect was assessed for an interaction with diluent treatment. All post-hoc tests were carried out using the Bonferroni test and results are reported as the mean \pm the

standard error of the mean (sem) in Experiments 1 and 3 and as the estimated marginal mean in Experiment 2, to adjust for imbalance between the number of inseminations in each treatment. Data were considered to differ significantly at $P < 0.05$.

3.4 Results

3.4.1 Experiment 1: Effect of Storing Liquid Semen Maintained at a Constant Ambient Temperature in 1 of 7 Different Diluents on Total and Progressive Sperm Motility

There was an effect of diluent ($P < 0.01$) and day ($P < 0.05$) on both total and progressive motility; from Day 0 to Day 3 across all treatments the percentage of motile sperm declined linearly. There was no diluent by day interaction ($P > 0.05$; Figure 3.3). Caprogen, BioXcell and INRA96 maintained Day 3 sperm total motility (64.0 ± 2.66 , 58.5 ± 2.83 and 58.0 ± 2.35 , respectively) and progressive motility (59.2 ± 3.18 , 47.5 ± 2.36 and $56.8 \pm 1.59\%$, respectively) at the highest levels. Sperm stored in BullXcell and AndroMed had intermediate total and progressive motility scores, while NutriXcell and OptiXcell had the lowest total and progressive motility on Day 3, respectively (Figure 3.3).

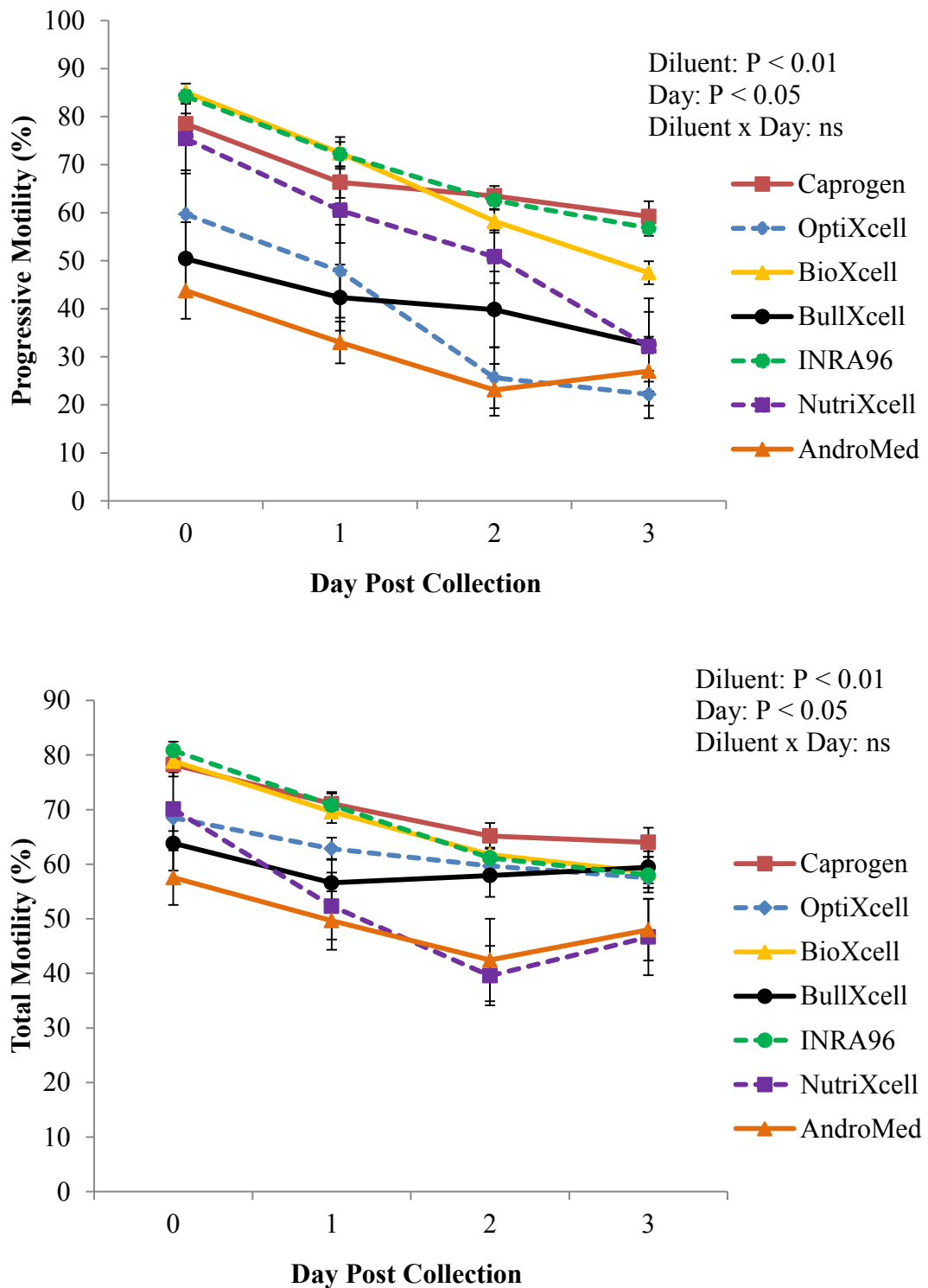


Figure 3.3: The effect of diluent on progressive motility (upper panel) and total motility (lower panel) of liquid bovine semen on Days 0, 1, 2 and 3 post collection (Experiment 1). Vertical bars represent sem. ns = non-significant

3.4.2 Experiment 2: Field Fertility of Liquid Semen Diluted in Caprogen, BioXcell and INRA96

3.4.2.1 Effect of Diluent on Calving Rate

Overall, insemination with liquid semen on Day 1, 2 and 3 post collection resulted in a lower CR (52.7, 47.3 and 47.5%, respectively) in comparison to frozen-thawed semen (59.7%, $P < 0.01$; Table 3.1). However, this was attributed to the poor CR recorded for BioXcell on Day 1, 2 and 3 of storage, as semen diluted in Caprogen and INRA96 and stored for up to 3 days had a similar CR compared to frozen-thawed semen. Semen diluted in BioXcell had a lower CR following storage for 1, 2 or 3 days after collection in comparison with Caprogen, INRA96 and frozen-thawed semen ($P < 0.01$; Figure 3.4).

Table 3.1: The effect of day of storage of liquid semen on calving rate in dairy cows and heifers (Experiment 2). Values with different superscripts differ significantly ($P < 0.01$).

Sperm Treatment	Number of Inseminations	CR (%)
Liquid Day 1	5,575	57.7 ^a
Liquid Day 2	3,846	47.3 ^b
Liquid Day 3	364	47.5 ^{ab}
Frozen-Thawed	9,323	59.7 ^c

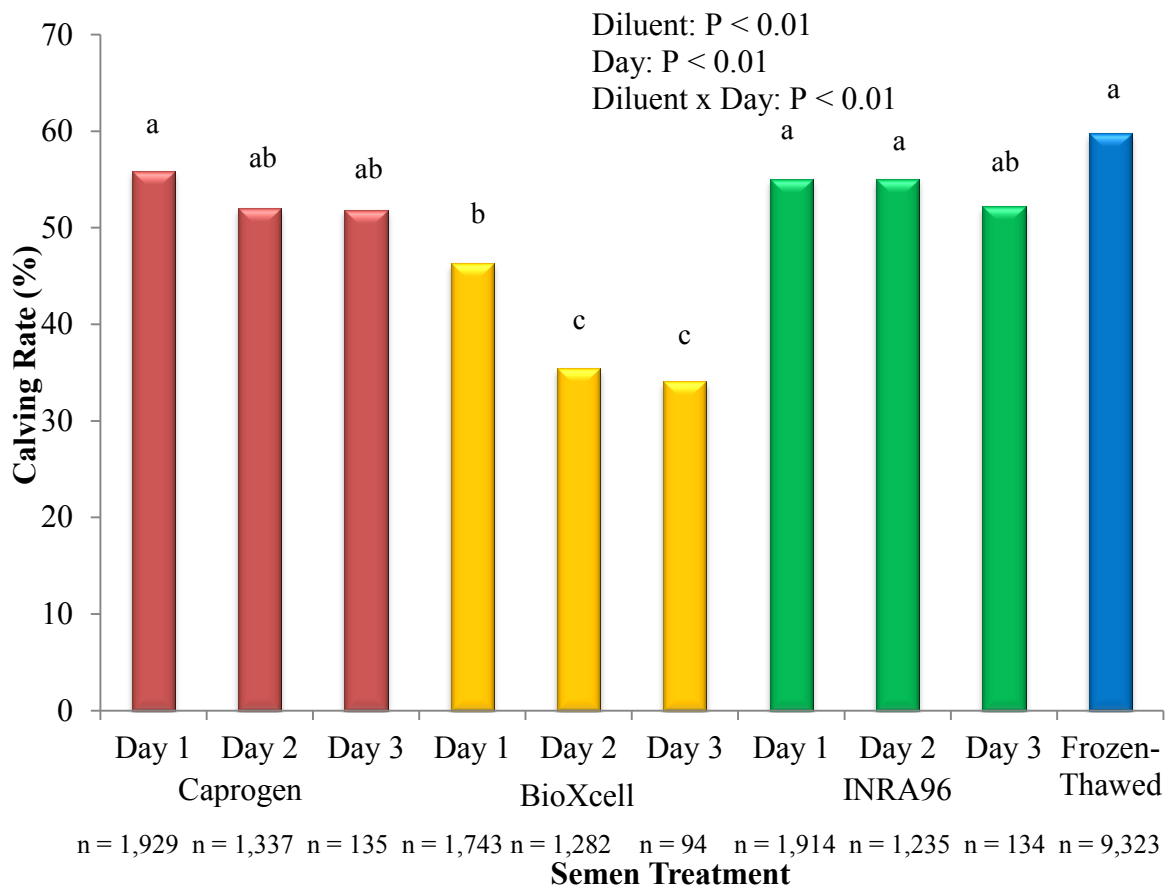


Figure 3.4: The effect of liquid semen diluent and day of storage on calving rate in dairy cows and heifers (Experiment 2). Values with different superscripts differ significantly ($P < 0.01$).

3.4.2.2 Effect of Bull on Calving Rate

There was an effect of bull on CR ($P < 0.01$) with the CR for individual bulls varying from 54.7 to 67.3%. There was a bull by day interaction ($P < 0.01$) as bull G had a lower CR than all other bulls on Days 1 and 2 of storage. Although mean CR following AI with liquid semen on Day 2 was reduced in comparison to Day 1 in all bulls with the exception of bull E, this reduction was only statistically significant in 2 bulls (B and D; Table 3.2). However, bulls B and G had a higher CR when frozen-thawed semen was used in comparison to liquid semen on Days 1 and 2, respectively

($P < 0.01$: Table 3.2) but this result was again confounded by the poor CR recorded for BioXcell.

Table 3.2: The effect of liquid bovine semen inseminated on Day 1, Day 2 or Day 3 post collection and frozen-thawed semen on calving rate in dairy cows and heifers (Experiment 2). Values in the same row with different superscripts differ significantly ($P < 0.01$).

Bull	Liquid Day 1 % Calving Rate (n)	Liquid Day 2 % Calving Rate (n)	Liquid Day 3 % Calving Rate (n)	Frozen-thawed % Calving Rate (n)
A	53.3 (880)	47.2 (697)	46.2 (13)	51.9 (376)
B	53.4 ^a (1,836)	47.4 ^b (1,453)	73.1 ^{abc} (26)	60.0 ^c (1,106)
C	55.1 (234)	52.2 (113)	68.4 (19)	64.4 (877)
D	52.3 ^a (457)	41.5 ^b (415)	54.6 ^{ab} (11)	57.7 ^a (2,593)
E	44.4 ^a (531)	51.1 ^a (276)	43.9 ^a (139)	61.3 ^b (741)
F	54.8 ^a (765)	47.9 ^{ab} (386)	42.0 ^b (150)	59.8 ^a (801)
G	34.2 ^a (79)	36.8 ^a (57)	0 (0)	61.1 ^b (2,272)
H	54.9 ^{ab} (793)	50.1 ^a (467)	83.3 ^{ab} (6)	59.4 ^b (557)
Overall	52.6 ^a (5,575)	47.3 ^b (3,864)	47.5 ^{ab} (364)	59.7 ^c (9,323)

3.4.2.3 Effect of Parity Number and Cow Breed on Calving Rate

There was an effect of parity on CR as primiparous cows had a higher CR in comparison to multiparous dairy cows ($P < 0.01$; Figure 3.5) with the exception of parity 3. Maiden (nulliparous) heifers had a higher CR ($P < 0.05$) when inseminated with frozen-thawed semen (68.8%) in comparison to liquid semen on Day 1 (46.4%) and 2 (46.4%) of storage. There was a parity by diluent interaction ($P < 0.01$); however, there was no clear biological pattern. Cows inseminated with Caprogen had a higher CR for parities 1, 4 and 5, and INRA96 had a higher CR for parities 2 and 3 while cows inseminated with BioXcell recorded the lowest CR for all parities with the exception of those with a parity greater than 5. There was no effect of breed or a breed by diluent interaction on CR ($P > 0.05$).

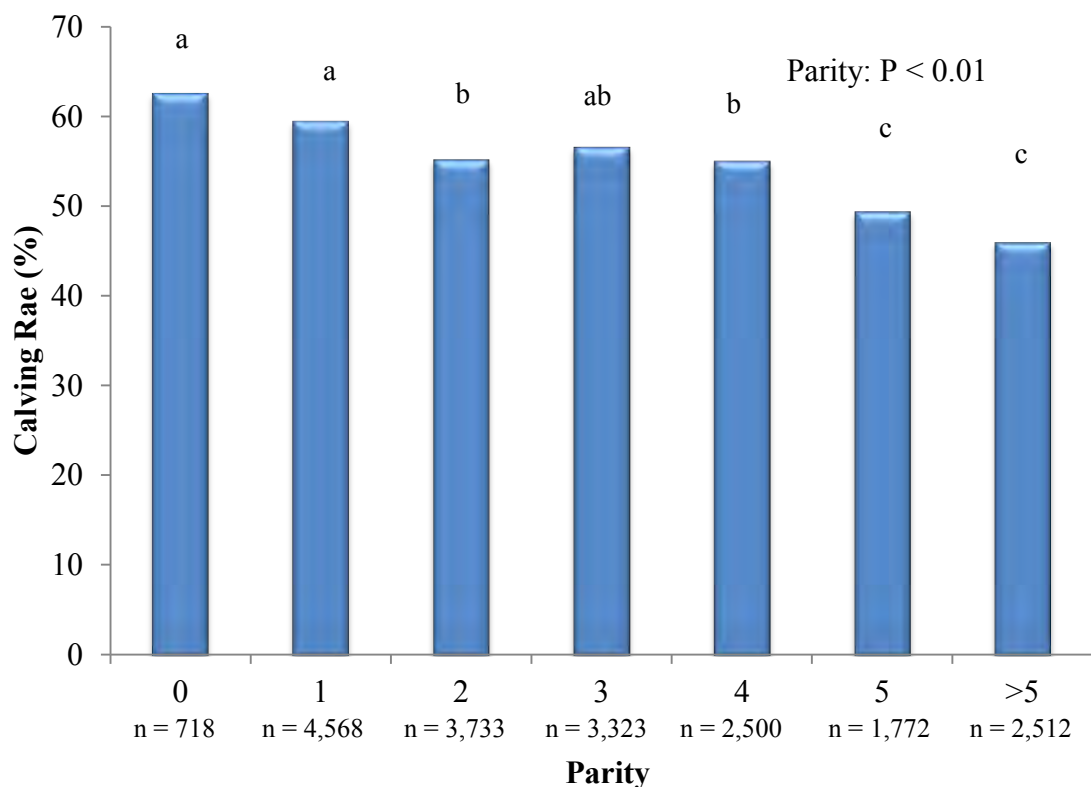


Figure 3.5: The effect of parity number on calving rate in dairy cows and heifers (Experiment 2). Values with different superscripts differ significantly between parity numbers ($P < 0.01$).

3.4.2.4 Effect of Cow Fertility Sub-Index on Calving Rate

Cows with a fertility sub-index greater than €70 had a higher CR in comparison with cows with a sub-index of less than €70 ($P < 0.01$; Figure 3.6). There was a cow fertility sub-index by diluent interaction on CR ($P < 0.01$) as the CR of cows inseminated with BioXcell, INRA96 and frozen-thawed semen increased with increasing cow fertility sub-index.

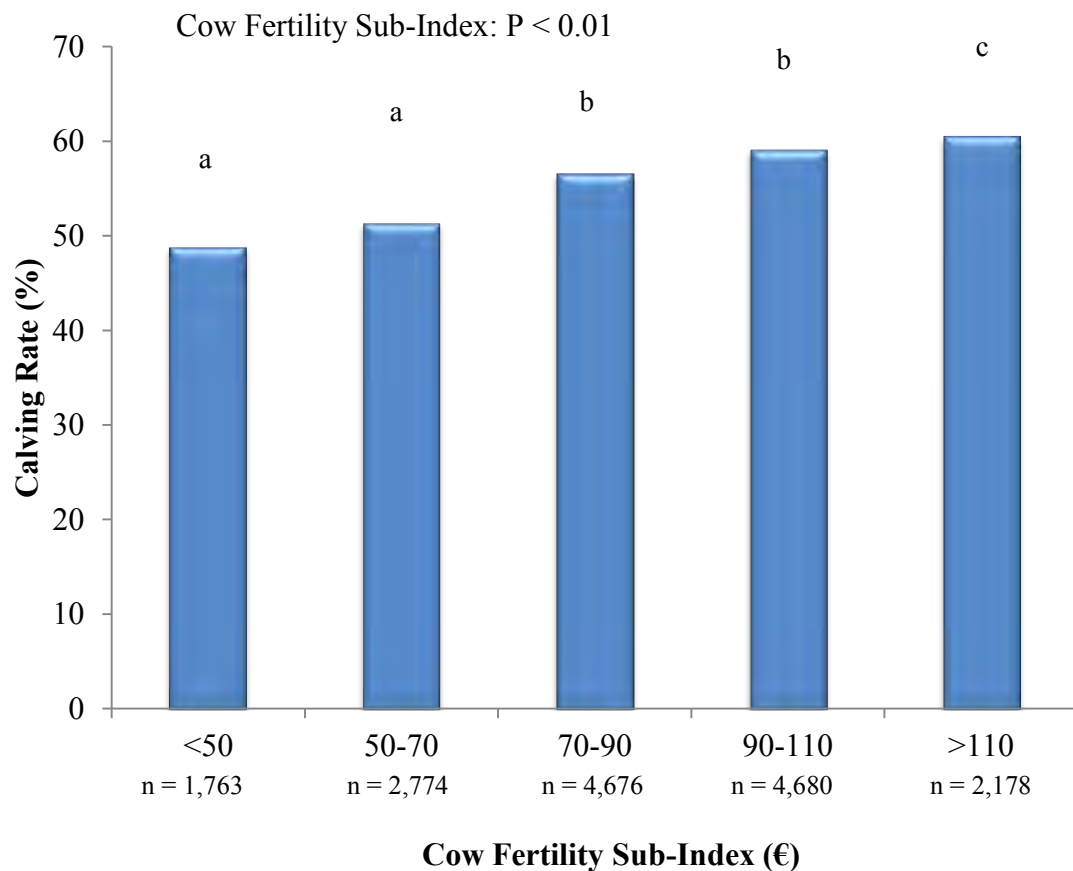


Figure 3.6: The effect of cow fertility sub-index on calving rate in dairy cows and heifers (Experiment 2). Values with different superscripts differ significantly between each sub-index category ($P < 0.01$).

3.4.2.5 Effect of Days in Milk, Herd and Technician on Calving Rate

There was a linear increase in CR to 80 days post calving as the number of DIM increased ($P < 0.01$; Figure 3.7). Cows which were greater than 80 DIM had a higher CR in comparison with cows which were in milk less than 80 days prior to insemination ($P < 0.01$; Figure 3.7). The CR for cows which were less than 20 days calved was 18.4%. There was no DIM by diluent interaction on CR ($P > 0.05$). As expected, CR varied between individual herds ($P < 0.01$) for herds with greater than 20 recorded inseminations but there was no herd by diluent interaction ($P > 0.05$). Similarly, CR was affected by technician ($P < 0.01$) with a range between 32.4 and 79.4% for technicians with greater than 100 recorded inseminations.

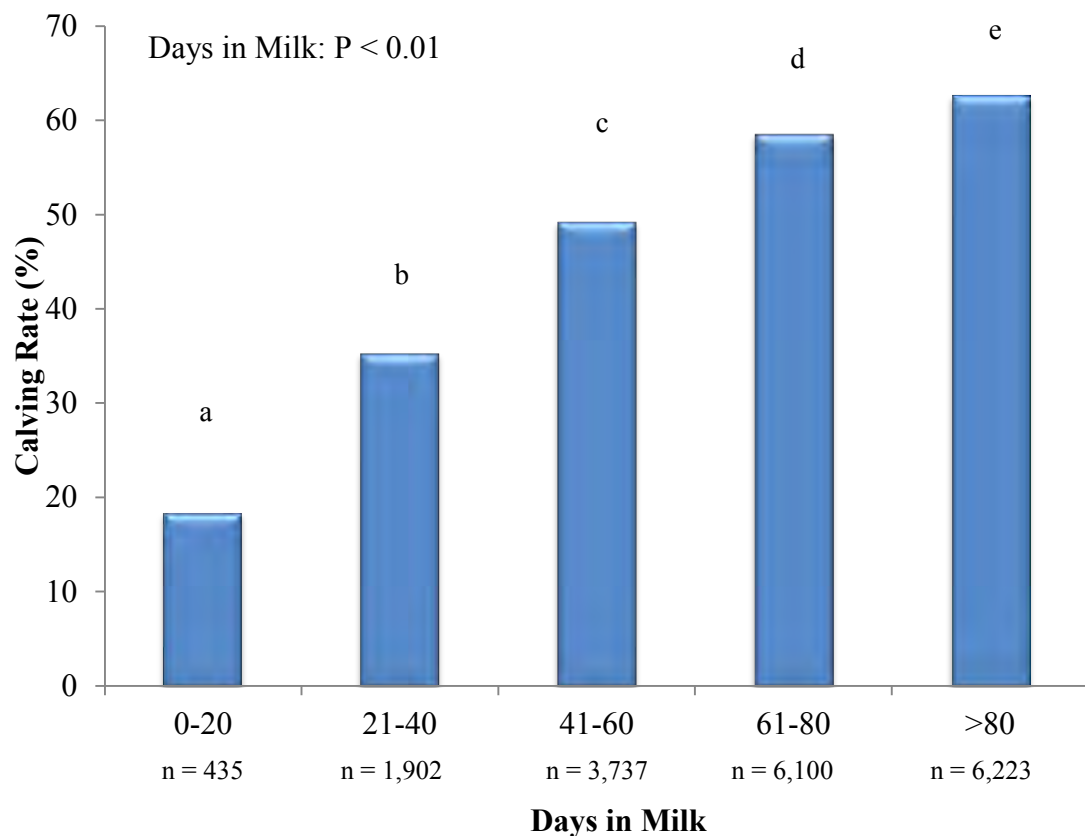


Figure 3.7: The effect of days in milk on calving rate in dairy cows and heifers (Experiment 2). Values with different superscripts differ significantly between number of days in milk categories ($P < 0.01$).

3.4.3 Experiment 3: Effect of Temperature Fluctuations on Semen Diluted in Caprogen, BioXcell and INRA96 on Total and Progressive Sperm Motility

When temperature was fluctuated there was an effect of diluent ($P < 0.01$) and day ($P < 0.01$) on both total and progressive motility. Semen stored in Caprogen and INRA96 recorded a greater total and progressive motility score than BioXcell ($P < 0.01$) but did not differ from each other ($P > 0.05$; Figure 3.8). From Day 0 to Day 3 across all treatments, the percentage of sperm displaying total and progressive motility declined linearly but there was no diluent by day interaction ($P > 0.05$). Fluctuation of temperature between 4 and 18 °C was detrimental to motility of sperm stored in BioXcell as both total and progressive motility declined with increased duration of storage from 62.3 ± 4.61 to $37.4 \pm 10.0\%$ and 54.4 ± 18.7 to $35.4 \pm 10.2\%$, respectively, from Day 0 to Day 3 of storage. Sperm stored in Caprogen and INRA96 maintained total (65.7 ± 1.98 and $65.7 \pm 5.02\%$, respectively) and progressive motility ($52.5 \pm 3.58\%$ and $59.6 \pm 5.04\%$, respectively) up to 3 days of storage (Figure 3.8).

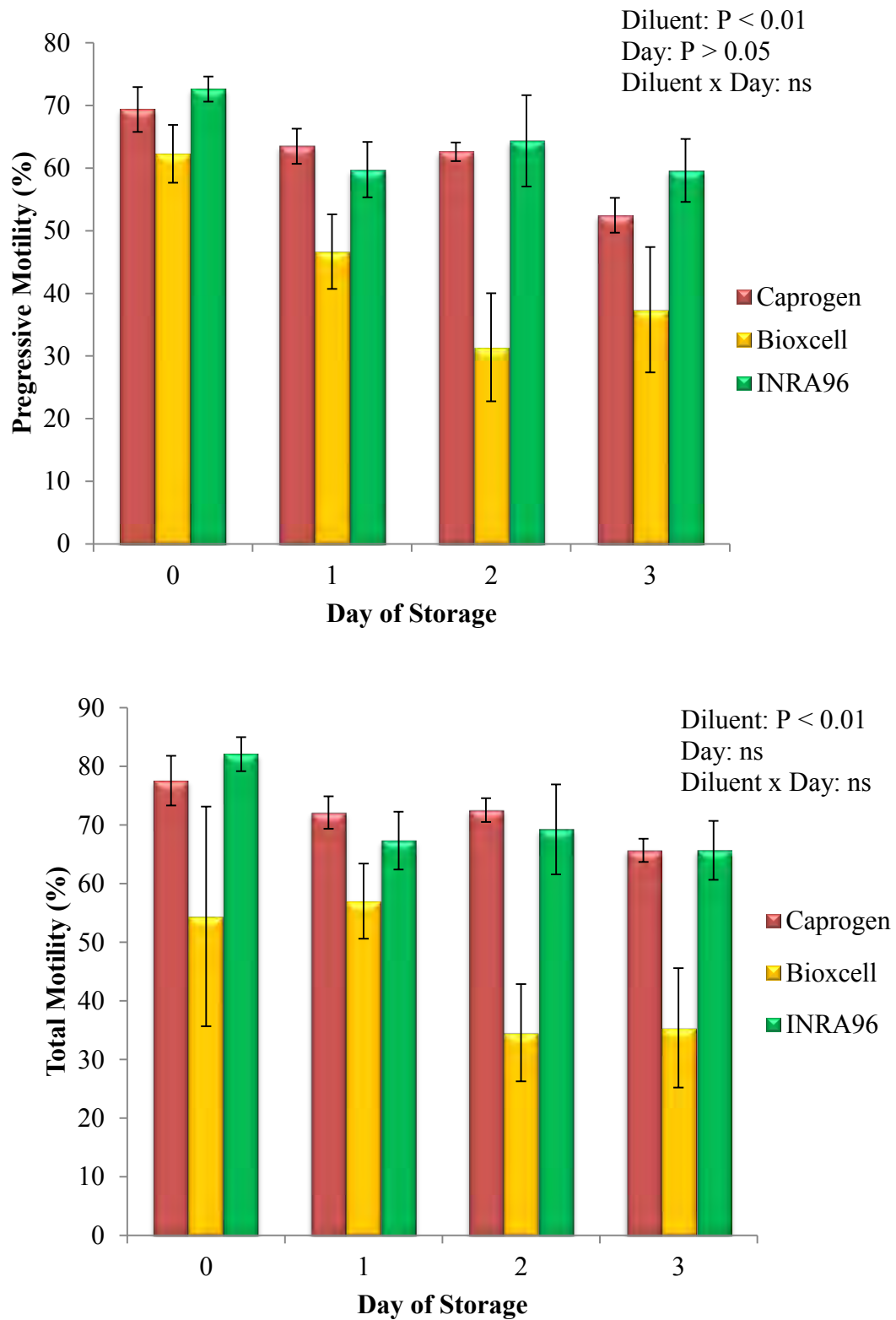


Figure 3.8: The effect of temperature fluctuation on progressive motility (upper panel) and total motility (lower panel) of liquid bovine semen stored in three diluents and assessed on Days 0, 1, 2 and 3 post collection (Experiment 3). Vertical bars represent sem. ns = non-significant.

3.5 Discussion

Despite the importance of AI to the dairy industry, there is a dearth of published studies comparing the field fertility of semen stored in different diluents. We have taken the approach of using split ejaculates and a combination of *in vitro* and *in vivo* assessments in a comprehensive attempt to identify the optimal semen diluent for liquid bull semen. The main findings of this study were that: 1) liquid semen diluted in Caprogen or INRA96 resulted in a similar CR to frozen-thawed semen; 2) liquid semen stored in BioXcell resulted in a reduced CR in comparison to semen diluted in Caprogen or INRA96 and frozen-thawed semen; 3) heifers had a higher CR in comparison with multiparous dairy cows when inseminated with liquid or frozen-thawed semen; 4) cows with a higher fertility sub-index had an increased CR compared to those with a lower fertility sub-index; 5) there was a positive linear relationship between DIM and reproductive performance; and (6) temperature fluctuation between 4 and 18 °C was detrimental to sperm motility when diluted in BioXcell but not in Caprogen or INRA96.

Regardless of storage temperature, sperm motility and thus, fertility declines over an extended period of time (Akhter et al. 2011, Vishwanath and Shannon 2000). In agreement, the results of this study demonstrated that irrespective of semen extender, semen quality, measured in terms of total and progressive motility, declined with increased duration of storage. When liquid semen was stored at a constant temperature Caprogen, BioXcell and INRA96 maintained better total and progressive motility than AndroMed, BullXcell, NutriXcell and OptiXcell. Caprogen, the industry standard liquid bovine semen diluent, although initially developed for the dilution of semen at 5 °C (Shannon 1965), has been shown to

maintain sperm motility for extended periods and result in higher fertility rates when stored at an ambient temperature of 18 °C (Shannon et al. 1984). The results of this study support this finding as Caprogen resulted in the highest total motility score on Day 3 of storage when stored at ambient temperature. INRA96, although not primarily used for the preservation of bovine semen, performed well in comparison to Caprogen and had the highest total and progressive motility score when temperatures were fluctuated between 4 and 18 °C. O'Hara et al. (2010) reported that storing ram semen in INRA96 at a constant 15 °C resulted in significantly reduced sperm motility but when stored at a constant 5 °C INRA96 performed well both in terms of *in vitro* and *in vivo* fertility. In the current study INRA96 recorded the second highest and highest total and progressive motility score, respectively, on Day 3 compared to the other diluents when semen was stored at a constant temperature of 18 °C.

BioXcell (soya lecithin-based extender) has been used routinely as a cryopreservation media for buffalo, ram and bull semen and a number of studies have compared its use on frozen-thawed semen against other commercially available diluents in these species (Sharafi et al. 2009, Stradaioli et al. 2007, Gil et al. 2003). Both Sharafi et al. (2009) and Gil et al. (2003) reported a lack of difference in motility and capacitation status between ram semen diluted in BioXcell and the control extender (L1G7 and milk-egg yolk based extender, respectively). Similarly, Gil et al. (2003) reported no difference in fertility suggesting that although BioXcell did not improve sperm quality *in vitro* or *in vivo* it could offer a safer alternative to preserving ram semen due to the reduced health risk associated with animal protein-free media. Stradaioli et al. (2007) reported BioXcell to be superior in preserving

post-thaw bull sperm motility as well as lowering the proportion of acrosome-reacted and capacitated sperm in comparison to a traditional egg-yolk tris glycerol extender. An *in vitro* study on liquid stored buffalo semen by Akhter et al. (2011) demonstrated that semen quality parameters for BioXcell were comparable to both milk and egg-yolk based extenders up to Day 3 of storage but were higher for BioXcell on Day 5 when semen was stored at a constant temperature of 5 °C (the manufacturer's recommended storage temperature of fresh semen diluted in BioXcell). Kasimanickam et al. (2011) stated that soya-based extenders had similar protection capabilities in liquid ram semen as an egg-yolk extender and were superior to liquid semen diluted in a milk-based diluent. In contrast to these studies, in the current study, although BioXcell performed well up to Day 3 of storage when maintained at a constant ambient temperature, the *in vivo* fertility results were inferior to those recorded by both Caprogen and INRA96.

The reduction in fertility associated with BioXcell observed in this study would indicate that BioXcell is less tolerant of temperature fluctuations and thus would not be a suitable substitute for an egg-yolk or milk-based extender if liquid semen was subjected to temperature variations. One possible explanation for this reduction is that as semen temperature fluctuates, morphological membrane changes consistent with a lipid phase transition occurs (Drobnis et al. 1993). This results in compacting or relaxing the packing of the phospholipid bilayer, causing membrane destabilisation and ultimately cell death (Crowe et al. 1990). Thus, maintaining semen at a constant ambient temperature results in regulating this transition phase and reduces the damage sustained to sperm cells (Crowe et al. 1989). As well as this, a family of lipid-binding proteins (bovine seminal plasma proteins; BSP) found in

seminal plasma are amongst the principal causes of damage to sperm cells during storage (Bergeron and Manjunath 2006). It is believed that low density lipoproteins found in egg-yolk and casein micelles found in milk interact with BSP proteins to reduce the lipid loss from the sperm membrane, thus, stabilising the membrane and maintaining sperm function during storage (Manjunath 2012, Bergeron et al. 2007). These low density lipoproteins are also believed to be important in protecting sperm cells during the lipid phase transition (Holt 2000) and as the protective action of casein micelles on sperm is thought to be analogous to the protective action of lipoproteins in egg-yolk (Bergeron et al. 2007), it could be postulated that casein micelles are the effective component in milk-based diluents also protecting cells from the damage arising from the lipid phase transition.

Caprogen and INRA96 resulted in similar CR suggesting that both diluents are capable of efficiently protecting the sperm membrane from excessive BSP protein binding, which results in the loss of cholesterol and phospholipids, resulting in a deleterious effect on sperm function (Bergeron and Manjunath 2006). The results also highlight that both Caprogen and INRA96 are efficient at maintaining sperm quality when semen is stored at either a constant or fluctuating temperature, suggesting that they are both capable of stabilising the sperm membrane during the lipid phase transition. BioXcell displayed an ability to protect sperm cells when semen was stored at a constant temperature; however, during temperature fluctuations its protection was inferior to both Caprogen and INRA96, as evidenced by a reduction of approximately 30 and 20% in total and progressive motility on Day 3 of storage, respectively, in comparison to Caprogen and INRA96. The reason for this is unclear but as soy lecithin (a plant protein source) is used in animal-protein

free media to substitute for the phospholipids in egg-yolk and casein micelles in milk-based diluents, plant protein sources may be less efficient in protecting sperm cells from temperature variations. Further research is required to understand the exact mechanisms of protection offered to sperm cells by BioXcell.

AndroMed, also a soya-lecithin based extender, has been reported to result in better post-thaw sperm quality than milk-based extenders in the buck (Jiménez-Rabadán et al. 2012), yielded similar pregnancy rates in frozen-thawed semen compared to an egg-yolk extender in the ram (Fukui et al. 2008) and resulted in higher NRRs in frozen-thawed semen when compared to a tris-egg yolk based extender in the bull (Aires et al. 2003). De Paz et al. (2010) reported that liquid ram semen diluted in a soybean lecithin extender maintained higher sperm motility and viability at 5 and 15 °C of storage in comparison to a control egg-yolk extender. These studies suggest that a soya lecithin-based extender can be just as effective in preserving semen, if not better, than egg-yolk and milk-based diluents. In the current study, however, semen diluted in AndroMed performed poorly in comparison to the egg yolk (Caprogen) and milk-based (INRA96) diluent used for liquid semen in this study. BullXcell is used routinely in the cryopreservation of bovine semen and performed adequately when storing liquid semen at a constant temperature. However, Caprogen resulted in superior semen quality in terms of total and progressive motility in comparison to BullXcell. NutriXcell, which is primarily used for the preservation of boar semen, and OptiXcell, a protein-free egg yolk-like diluent, were the least effective extenders in maintaining total and progressive motility of bull semen, respectively, when stored at a constant ambient temperature. Although these extenders have been used

for the preservation of sperm from a number of species, there is limited published data investigating their use against other diluents within the bovine industry.

Previous studies have reported that the production of ROS may be linked to an aging effect on sperm as ROS production ultimately leads to an apoptotic cascade in which sperm lose their motility, DNA integrity and vitality (Aitken et al. 2012). Murphy et al. (2016) reported a lower NRR on Day 2 of storage; however, this was only evident in 50% of the bulls tested, suggesting that individual bulls may be more susceptible to the aging process. The current study supports the notion of a sperm aging effect as a similar reduction in CR was recorded on Day 2 of storage in 2 bulls used in this study. However, there was no significant decline in CR in the other 6 bulls from Day 1 to Day 2 of storage.

It is well documented that the physiological status of nulliparous heifers differs to that of lactating cows (Forde et al. 2015). In addition, cow fertility declines with age as stress associated with calving, lactation and an increased vulnerability to clinical pathologies have a negative effect on fertility traits (Andersen-Ranberg et al. 2005, Miller et al. 2001, Fonseca et al. 1983). A number of studies have found that NRR was higher in primiparous cows compared to multiparous dairy cows (Murphy et al. 2016, Fouz et al. 2011), supporting the results of this study. Pryce et al. (2002) reported that conception rate to first service of maiden heifers of average genetic merit was 71% in comparison to lactating cows at 45% (Pryce et al. 2004). The results of the current study would support this finding as heifers recorded a higher CR in comparison to multiparous dairy cows, with frozen-thawed semen recording a higher CR than liquid semen on Days 1, 2 and 3 of storage; however, this was confounded by the poor CR results of BioXcell.

The number of DIM at breeding has been shown to significantly affect fertility with cows with a greater number of DIM more likely to conceive (Hillers et al. 1984). As the Irish dairy industry is a seasonal grass-based production system, the number of DIM is particularly important for spring-calving herds because the breeding season begins on a fixed date (typically in mid-April). Thus, once the breeding season begins, irrespective of the number of DIM, it is common practice to serve every cow detected in oestrus (Buckley et al. 2003). In this study the number of DIM affected CR, irrespective of diluent, with maximum CR achieved by cows which were greater than 80 DIM. Haan (2012) reported that rapid uterine involution occurs within the first 5-15 days post-partum in which the size and length of the previously gravid horn is halved. In 30-80% of cows, the detection of the first postpartum dominant follicle occurs at approximately 7 to 10 days with the first ovulation occurring at 28.6 ± 1.54 days postpartum (Crowe et al. 1998). This is consistent with the results of this study as cows inseminated <40 DIM had a CR of 32.1%, compared with 57.9% for those calved >40 days.

A high level of fertility in seasonal dairy production systems is critical as it ensures that a high proportion of cows will calve at the start of the grass growing season, thus maximising the amount of milk produced from cheap grazed grass in their diet (Veerkamp et al. 2002). We observed a positive linear relationship between fertility sub-index and CR and interestingly, the CR did not plateau as the fertility sub-index increased. This is clear evidence that the fertility sub-index is improving reproductive performance in the Irish dairy herd and will reduce the costs associated with reproductive inefficiency within dairy herds such as increased calving intervals,

involuntary culling (Esslemont et al. 2001), labour costs as well as increased costs associated with repeated AI (Shalloo et al. 2014).

In conclusion, Caprogen, BioXcell and INRA96 maintained total and progressive sperm motility for a longer period of storage in comparison to OptiXcell, BullXcell, NutriXcell and AndroMed when diluted semen was stored at a constant temperature. Storing semen at fluctuating temperatures between 4 °C and 18 °C had no impact on motility when semen was stored in Caprogen and INRA96, but compromised the motility of liquid bull semen stored in BioXcell indicating that BioXcell has a reduced ability to protect sperm during temperature fluctuations. The dilution and storage of liquid bovine semen in INRA96 resulted in a similar CR to semen diluted in the industry standard, Caprogen. Given that INRA96 can be used directly off-the-shelf, it may be a suitable alternative to Caprogen for the storage of liquid bull semen.

Chapter Four

Effect of Storage Temperature, Nitrogen
Gassing and Sperm Concentration on the
In Vitro Semen Quality and *In Vivo*
Fertility of Liquid Bull Semen Stored in
INRA96

Abstract

The aim of this study was to assess the effect of storage temperature, N₂ gassing and sperm concentration on *in vitro* characteristics and CR following AI of liquid bull semen stored in INRA96. In Experiment 1 the effect of liquid bull semen diluted in either N₂ bubbled or non-bubbled INRA96 at a concentration of 5 x 10⁶ sperm per 0.25 mL insemination dose and stored at 5 or 15 °C was assessed subjectively for total and progressive motility on Days 0, 1, 2, 3 and 4 post collection. In Experiment 2a, the effect of stored liquid semen at three sperm concentrations (3, 4 or 5 x 10⁶ sperm per 0.25 mL insemination dose) on total and progressive motility was assessed subjectively on Days 0, 1 and 2 post collection. In Experiment 2b, the field fertility of liquid semen stored at ambient temperature at a concentration of 3, 4 or 5 x 10⁶ sperm per 0.25 mL dose and inseminated on Days 1 or 2 post collection was assessed in comparison to frozen-thawed semen (total of n = 5,742). In Experiment 1, total and progressive motility decreased with increased duration of storage (P < 0.01); however, there was no effect of N₂ bubbling on motility on Days 0, 1, 2, 3 and 4 of storage (P > 0.05). There was an effect of temperature on total and progressive motility, regardless of treatment, as semen stored at 15 °C recorded higher motility values than semen stored at 5 °C (P < 0.01). In Experiment 2a, there was no effect of sperm concentration on total or progressive motility on Days 0, 1 or 2 of storage (P > 0.05). There was a linear decrease in motility with increased duration of storage (P < 0.01); however, there was no sperm concentration by day interaction (P > 0.05). In Experiment 2b, there was an effect of sperm concentration on CR (P < 0.01); semen diluted to 3 and 4 x 10⁶ sperm per dose resulted in a lower CR after 2 days of storage (41.1 and 44.7%, respectively) in comparison to frozen-thawed semen (55.2%) but did not differ to CR of semen diluted to 5 x 10⁶ sperm per dose on Day 2 of storage

($P > 0.05$). There was an effect of parity, fertility sub-index and DIM at AI on CR ($P < 0.01$). In conclusion, N_2 bubbling and sperm concentration had no effect on *in vitro* sperm motility of liquid semen, but this study demonstrated a reduction in CR on Day 2 of storage at lower sperm concentrations in comparison to frozen-thawed semen.

4.1 Introduction

Seasonal grass-based dairy production systems depend on compact breeding during mid-April to early June in the Northern Hemisphere, in order to coincide milk production with grass growth (Shalloo et al. 2007). AI is the single most important technique devised to facilitate the genetic improvement of animals (Oliveira et al. 2013, Black 2006) and currently, within the Irish dairy industry, 95% of AI is conducted using frozen-thawed semen, with liquid (i.e., fresh, non-cryopreserved) semen accounting for only 5% of annual inseminations (Murphy et al. 2016). However, the use of liquid semen increases to approximately 25% during the peak breeding season in order to accommodate the large demand (Al Naib et al. 2011a). Although liquid bull semen has traditionally been stored at ambient temperature in the egg-yolk based diluent, Caprogen, Murphy et al. (2017) indicated that bull semen stored in INRA96 (a commercially available milk-based diluent) had a comparable CR to Caprogen but was more convenient for the busy working schedule of an AI centre as it could be used directly off the shelf.

In Ireland a typical liquid semen dose contains 5×10^6 sperm, irrespective of its usage day (Murphy et al. 2013), in comparison to 15×10^6 sperm for a typical frozen-thawed semen dose (Vishwanath et al. 1996). Thus, liquid semen processing

yields more doses per ejaculate, thereby facilitating the greater utilisation of genetically superior sires. This is particularly beneficial for young genomically-selected sires as these sires are in high demand but produce lower semen volumes in comparison to more mature bulls (Brito et al. 2002a). An added advantage is that unlike frozen-thawed semen, which must undergo a 30 day quarantine period in the European Union, liquid semen can be released for insemination on the day of collection. Despite its advantages, liquid semen has a limited shelf life and is normally used for only 2.5 to 3 days post collection as a reduction in pregnancy rates has been reported thereafter (Vishwanath and Shannon 2000). In order to combat this reduction in fertility a number of investigative avenues have been exploited with many studies focusing on reducing the metabolic activity of sperm, as sperm survival for extended periods of time has been shown to be inversely related to their metabolic activity (Vishwanath and Shannon 2000). Approaches taken include; altering storage temperatures (Saha et al. 2014), reducing sperm number in semen diluted in Caprogen (Murphy et al. 2016) as well as N₂ gassing and modifying diluent composition (Shannon and Curson 1984), to name but a few.

It is widely acknowledged that reduced storage temperatures and N₂ gassing of media are two primary methods of reducing metabolic activity of sperm. However, storing semen at lower temperatures (ie: 5 °C) can cause membrane damage and has been reported to cause a rise in intracellular levels of sodium to cytotoxic levels as the activity of the sodium-potassium pump declines (Sweadner and Goldin 1980). Storage at ambient temperatures (ie: 15 – 20 °C) avoids the damage sustained by reduced temperatures but it has been postulated that the production of reactive oxygen species is accelerated at higher compared to lower storage temperatures

(Pino et al. 2013, Vishwanath and Shannon 1996). Protocols to reduce sperm metabolic activity at ambient temperature were devised such as N₂ gassing (Shannon and Curson 1984) to displace oxygen in the media creating an anaerobic environment. INRA96, while marketed for use at 4 °C in an aerobic environment, has demonstrated sufficient protection capabilities to bull sperm when stored at ambient temperature (Murphy et al. 2017), however, whether the use of N₂ gassing of INRA96 is beneficial remains to be elucidated.

It has previously been reported that increasing the dilution rate, specifically when using an egg-yolk based diluent, was the primary reason for a decline in fertility of liquid semen at lower sperm numbers rather than a direct effect of a lower sperm concentration, as higher dilution rates adversely affect viability (Garner et al. 1997, Shannon 1965). Excessive dilution can lead to permanent loss of motility, metabolic activity and fertilising capacity (Maxwell and Johnson 1999). While a larger quantity of egg yolk provides more protection to sperm from the harmful effects of seminal plasma (Bergeron et al. 2004) it also provides a substrate for hydrogen peroxide production from dead sperm (Shannon and Curson 1983), thus contributing to increasing the generation of reactive oxygen species (ROS) which is detrimental to sperm (de Lamirande et al. 1997). Previous studies of liquid semen have reported beneficial *in vitro* effects of reducing sperm concentration such as increased sperm viability, reduced oxidative stress (Murphy et al. 2013) as well as a decreased susceptibility to osmotic shock (Prathalingam et al. 2006). Shannon et al. (1984) reported no significant reduction in fertility of liquid semen when sperm concentration was reduced from 10 to 1.5 x 10⁶ sperm per insemination dose. In contrast, Murphy et al. (2016) demonstrated that excessive dilution of sperm had a

negative effect on NRR of semen used on Day 2 of storage compared to frozen-thawed semen; however, this did not differ from fertility achieved with liquid semen used on Day 1.

Therefore, using a combination of *in vitro* assessments and a large-scale commercial field trial, the objectives of this study were to assess the effect of N₂ gassing, storage temperature and reducing the sperm concentration in liquid bull semen diluted in INRA96.

4.2 Materials and Methods

4.2.1 Experiment 1: Effect of Diluting Liquid Semen in INRA96 with/without Nitrogen at 5 or 15 °C on Total and Progressive Sperm Motility

The aim of this experiment was to assess the effect of N₂ bubbling on total and progressive motility of liquid semen stored for up to 4 days post collection. Semen was collected from Holstein Friesian bulls (n = 5) on three occasions (each occasion/collection was one replicate) at a commercial AI centre in Ireland. The raw ejaculate was partially diluted in 10 mL pre-warmed INRA96 (IMV Technologies, L'Aigle, France) at 37 °C and transported in a temperature-regulated cooler box at 18 °C to the laboratory (1 h transportation). On arrival, the ejaculate was assessed for sperm concentration using a coulter counter (Z Series, Beckman Coulter, Co Clare, Ireland) as well as an initial score of total motility (%) and gross motility on a subjective 5-point scale (1 = twitching/no forward progressive motility; 5 = excellent forward progressive motile sperm) to ensure all semen samples were of a commercial standard (results not shown). Microscopic assessments were conducted

by the same technician and initial quality control cut-off values were a total and gross motility of $\geq 70\%$ and a score of ≥ 3 , respectively, and any ejaculates failing to meet these criteria were rejected.

The ejaculate was then split into two equal parts and diluted using either N₂ bubbled or non-bubbled INRA96 to achieve a concentration of 5×10^6 sperm per 0.25 mL insemination dose. Prior to dilution, N₂ bubbled INRA96 (200 ml) was purged in food fresh N₂ gas (BOC, Dublin, Ireland) for 30 min (Krzyzosiak et al. 2001) to dispel oxygen from the media and create an anaerobic environment, limiting the metabolic activity of sperm during liquid storage. Semen from each bull was kept separate and ejaculates were split such that each bull was represented equally in each treatment. Semen straws were filled as per routine procedures, placed in an insulated plastic container (to slow temperature shifts) and stored at one of two temperatures, 5 °C (placed in fridge) and 15 °C (placed in a temperature regulated box) (Murphy et al. 2016, Vishwanath and Shannon 2000, Shannon and Curson 1984). Samples from the different treatments were assessed in a randomised sequence to remove bias as a result of sampling order. Total and progressive motility (%); (n = 3 replicates) were subjectively assessed *in vitro* on Days 0, 1, 2, 3 and 4 post-collection (Day 0 = 4 h after collection).

4.2.1.1 Assessment of Sperm Motility

Sperm motility (total and progressive) in liquid semen was assessed subjectively on Days 0, 1, 2, 3 and 4 post semen collection using a phase contrast microscope (CX31; Olympus, Centre Valley, PA, USA) at a magnification of 400 X. A droplet of diluted semen (5 μ L) was placed on a pre-warmed glass slide, covered with a pre-

warmed coverslip (18 x 18 mm; 37 °C) and assessed by counting a minimum of 100 sperm, over at least five different fields of view, for each treatment on each assessment day. Total motility was expressed as a percentage of the total sperm population (motile and non-motile). A sperm was deemed to display progressive motility if it moved in a linear fashion; progressive motility was expressed as the percentage of motile sperm.

4.2.2 Experiment 2a: Effect of Storing Liquid Semen in INRA96 at varying Sperm Concentrations on Total and Progressive Sperm Motility

The aim of this experiment was to assess the effect of bull sperm concentration on total and progressive motility on Day 0, 1 and 2 of storage. Semen was collected from Holstein Friesian bulls (n = 6; 7 collection days; 3 bulls used per collection day resulting in 3 to 4 ejaculates/replicates per bull) at a commercial AI centre. The raw ejaculate was collected, partially diluted (with 10 mL of INRA96), transported, assessed and processed as described above. The ejaculate was diluted via a two-step dilution, firstly to achieve a concentration of 60×10^6 sperm per mL and then to achieve the final concentrations of 3, 4 and 5×10^6 sperm per 0.25 mL insemination dose. Semen from each bull was kept separate and ejaculates were split such that each bull was represented equally in each treatment. Semen straws were filled as per routine procedures and stored in a temperature regulated cooler box at 15 °C. Samples from the different treatments were assessed in a randomised sequence to remove bias as a result of sampling order. Total and progressive motility; (n = 3 replicates) were assessed *in vitro* as per Experiment 1 on Days 0, 1, 2 and 3 post-collection (Day 0 = 4 h after collection).

4.2.3 Experiment 2b: The Field Fertility of Liquid Semen Diluted in INRA96 at 3, 4 or 5 x 10⁶ Sperm per Insemination Dose

The aim of this experiment was to assess the effect of bull sperm concentration on CR following AI with liquid semen. Liquid semen (3, 4 and 5 x 10⁶ sperm per 0.25 mL dose) on Days 1 and 2 post collection and frozen-thawed semen (15 x 10⁶ sperm per 0.25 mL dose) were used for insemination (Figure 4.1). Semen was collected from Holstein Friesian bulls (n = 6; denoted A-F) at a commercial AI centre during May 2016. There were 7 collection days in total, with 3 bulls used per collection day (total of 21 ejaculates). The raw ejaculate was collected, partially diluted, transported, assessed and processed as described above. Each batch of liquid semen was clearly labelled and distributed for insemination on the day of collection. Liquid semen was stored at ambient temperature (Murphy et al. 2016) and used for up to 2 days post collection on both heifers (n = 391) and multiparous (n = 1,884) dairy cows. Due to logistical constraints, frozen-thawed semen doses were derived from previously collected ejaculates from the same 6 bulls which were processed and frozen using routine procedures (n = 3,467 inseminations consisting of 1,084 heifers and n = 2,383 multiparous dairy cows). Briefly, upon collection of semen samples for cryopreservation, the raw ejaculate was partially diluted in 10 mL of pre-warmed BullXcell (IMV Technologies) at 37 °C and transported to the laboratory. Semen samples were assessed for volume, sperm concentration and total and progressive motility as described above. Only ejaculates achieving a total and gross motility score of $\geq 70\%$ and ≥ 3 were used for cryopreservation. Following *in vitro* assessments, the semen was fully extended with pre-warmed BullXcell to achieve a concentration of 15 x 10⁶ sperm per 0.25 mL insemination dose. Straws were frozen to -140 °C as follows: -5 °C per min from +4 °C to -10 °C, -40 °C per min from -10

°C to -100 °C and thereafter -20 °C per min from -100 °C to -140 °C in a programmable freezer (Digitcool; IMV Technologies), followed by submersion and storage in liquid nitrogen at -196 °C until use.

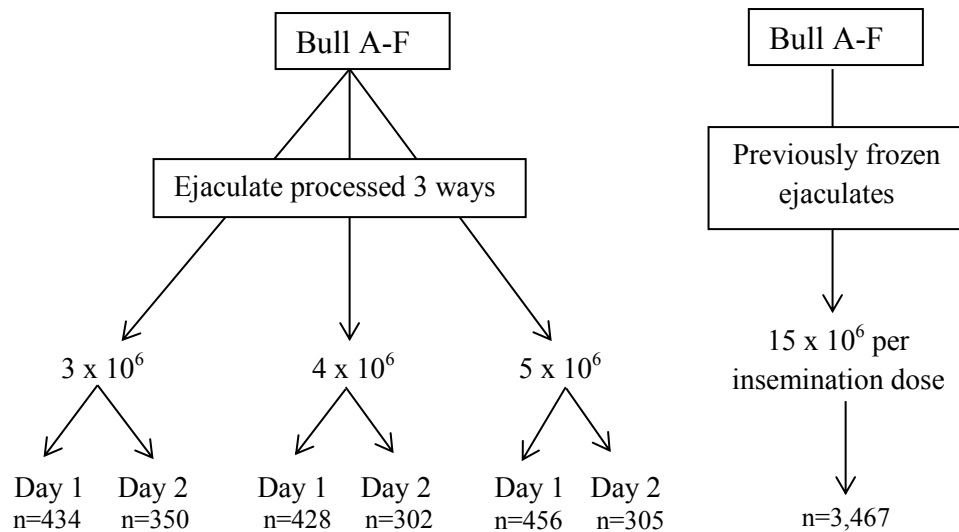


Figure 4.1: Experimental design for Experiment 2b. Ejaculates were split for the liquid semen treatments and previously frozen ejaculates from the same bulls were sourced for the frozen-thawed treatment. (n = the total number of inseminations per sperm concentration per day).

Inseminations (liquid and frozen-thawed semen) were carried out in May 2016 (coinciding with the peak dairy breeding season) in Irish dairy herds (n = 750). The majority of inseminations were in Holstein Friesian cows (n = 5,476) but small numbers of cows of other breeds were represented: Jersey (n = 158), Montbeliarde (n = 40), Norwegian Red (n = 34) and other (n = 34; includes Normande, Rotbunte, Swedish Red, Danish Red and Red Poll). Technicians (n = 61) were grouped into geographical areas and treatments were rotated on each collection day to ensure that technicians, who were blinded to treatments, received different diluent treatments

from each of three bulls on each day (Figure 4.2). For each insemination the AI technician recorded the bull code, cow tag number and the straw code on a handheld electronic device.

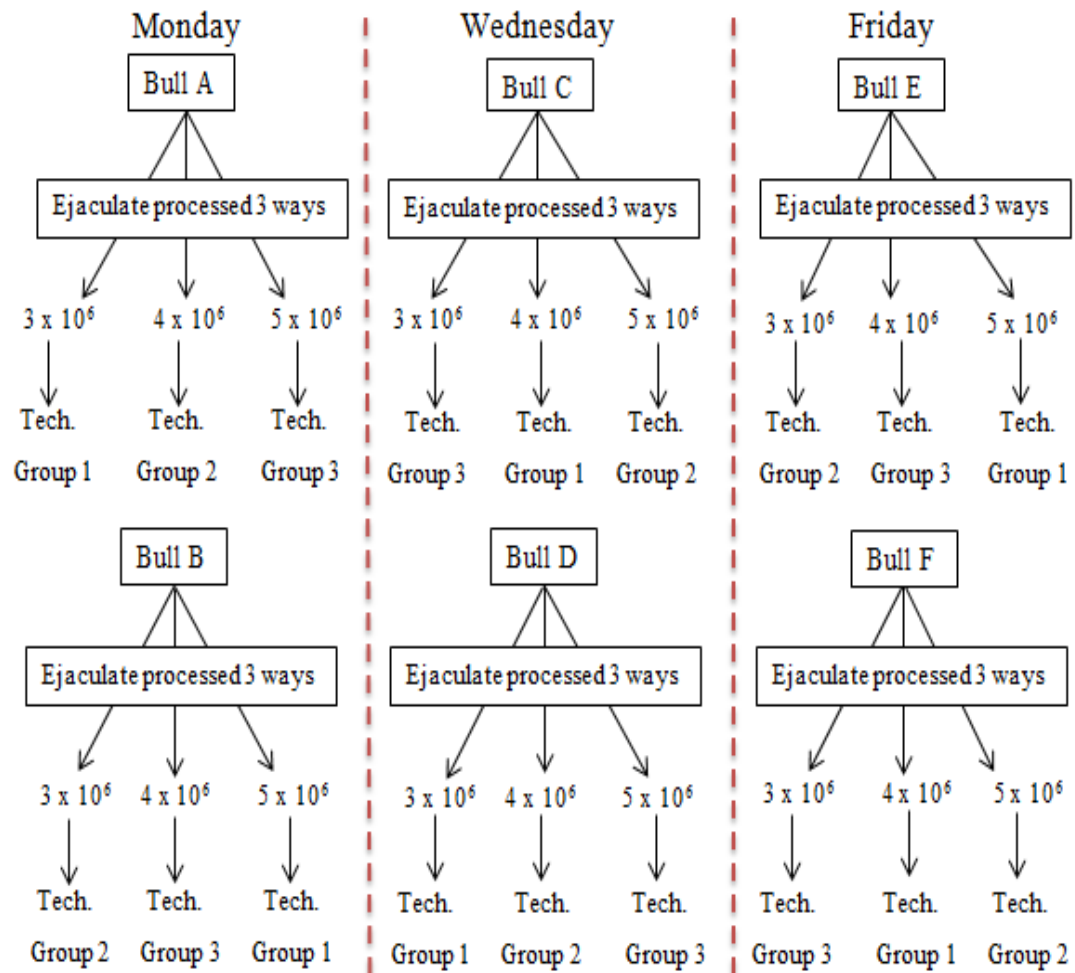


Figure 4.2: Experimental design for Experiment 2b highlighting the distribution of different liquid semen treatments to technicians within a week (displaying bulls A-F). Each technician (tech) received three different treatments each day. Frozen-thawed semen was also inseminated throughout the period.

Cow characteristics such as parity, DIM and fertility sub-index were also assessed. Fertility sub index, a key component of the EBI comprises ~35% of the total EBI and is based on calving interval and cow survival (Teagasc 2014). It was set up to

combat a decline in reproductive performance by providing farmers with a profit index enabling the selection of elite sires to breed replacement heifers with increased milk yield, reproductive performance and improved health traits (Berry et al. 2005).

4.2.3.1 Capturing of Calving Rate Data

Calving rate data were captured using the Irish Cattle Breeding Federation (ICBF; Bandon, Co Cork) database by cross-referencing the technician name with the bull code and semen type used on each date within the trial period. Obvious errors were extracted from the dataset and data were then interrogated to remove animals based on the following criteria: cows which were not at first AI, cows which received two inseminations from two different bulls or sperm concentration treatments, or cows which were not of a dairy breed. However, if a cow received two inseminations from the same bull with the same sperm concentration treatment within five days of each other, the record was kept and the second date was assumed to be correct. Post editing, a total of 5,742 inseminations remained. Calving rate was measured using a cut-off value of 275 and 290 days from date of insemination to calving date.

4.3 Statistical Analysis

Data from Experiments 1 and 2a were examined for normality of distribution, homogeneity of variance and analysed using the GLM repeated-measures procedure with a compound symmetry covariance structure in SPSS. The final model for Experiment 1 included the main effects of N₂ bubbling, day, temperature and their interaction. The final model for Experiment 2a included the main effects of sperm concentration treatment, day and their interaction. In Experiment 2b, CR data were compared using Pearson's chi-squared procedures in SPSS. Data were cross checked

using an ANOVA model. The dependent variable in the analysis was CR (1 = calved, 0 = not calved). In addition, using a general linear model for binomial data, CR data and correlations were investigated with a number of fixed effects, namely sperm concentration, bull, parity number, cow breed, cow fertility sub-index, DIM, herd and technician. Each fixed effect was assessed for an interaction with sperm concentration. All post-hoc tests were carried out using Bonferroni test and results are reported as the mean \pm sem in Experiments 1 and 2a and the estimated marginal means in Experiment 2b, to adjust for the imbalance between the number of inseminations in each comparison/treatment. Data were considered to differ significantly at $P < 0.05$.

4.4 Results

4.4.1 Experiment 1: Effect of Diluting Liquid Semen in INRA96 with/without Nitrogen at 5 or 15 °C on Total and Progressive Sperm Motility

There was no effect of N₂ bubbling on total and progressive motility of liquid semen ($P > 0.05$; Figure 4.3). However, there was an effect of temperature, day and a day by treatment interaction on total and progressive motility ($P < 0.01$). As expected, total and progressive motility declined with increased duration of storage ($P < 0.01$). Semen held at 15 °C had a higher total and progressive motility score throughout the duration of storage compared to semen held at 5 °C regardless of N₂ treatment (N₂ bubbled or non-bubbled INRA96; $P < 0.01$). There was no effect of bull, bull by day and or bull by temperature interaction on total and progressive motility ($P > 0.05$).

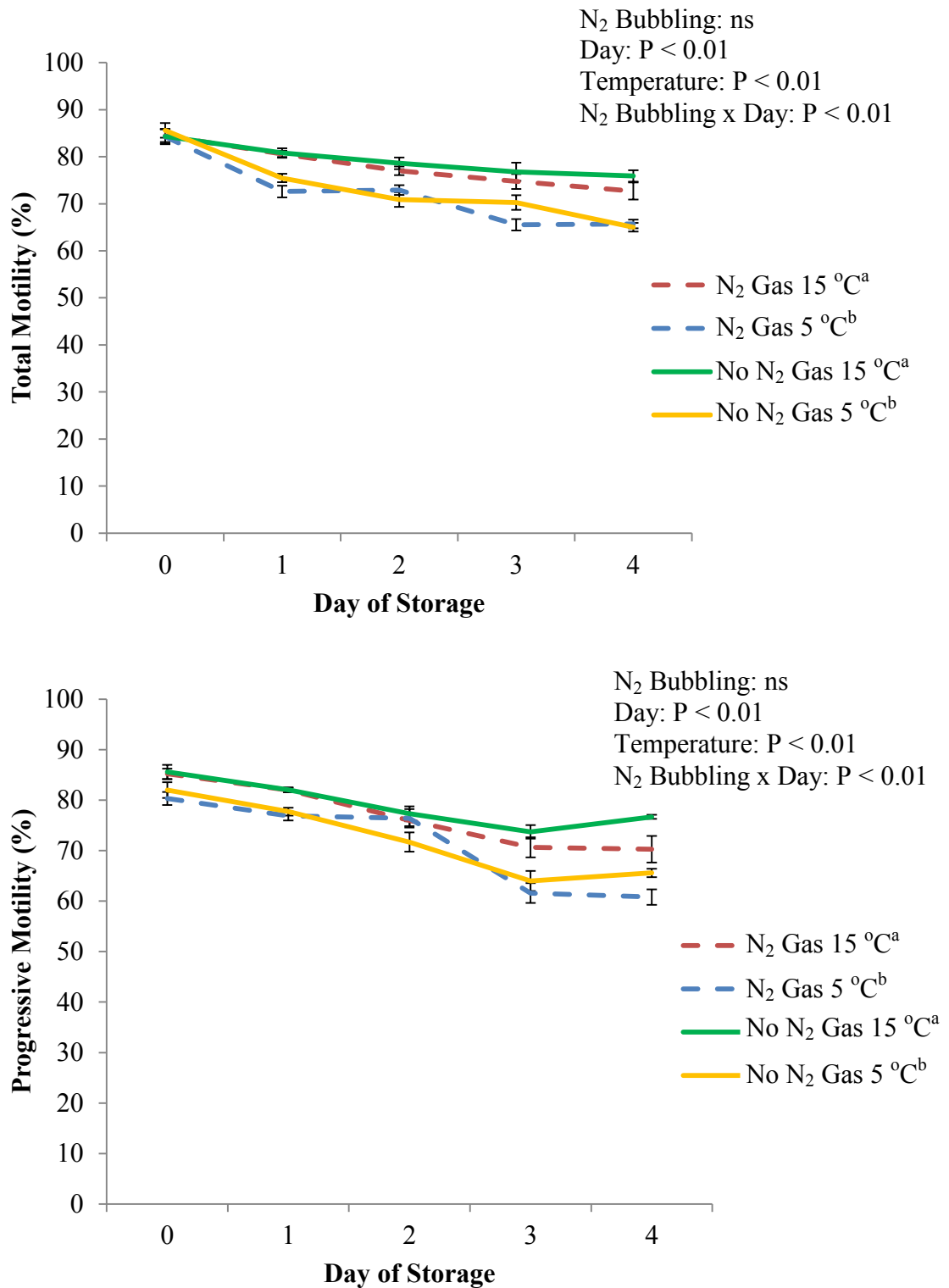


Figure 4.3: The effect of nitrogen bubbling on total (upper panel) and progressive (lower panel) motility of liquid semen on Days 0, 1, 2, 3 and 4 post collection (Experiment 1). Vertical bars represent \pm sem. ^{ab}Treatments with different superscripts differ significantly ($P < 0.01$). ns = non-significant.

4.4.2 Experiment 2a: Effect of Storing Liquid Semen in INRA96 at varying Sperm Concentrations on Total and Progressive Sperm Motility

Although total and progressive motility declined linearly with increased duration of storage ($P < 0.01$), there was no effect of sperm concentration on both total and progressive motility ($P > 0.05$). Semen diluted to a concentration of 3, 4 and 5 x 10⁶ sperm per dose maintained acceptable total and progressive motility scores throughout the duration of storage with a decline in total (81.8 to 74.2, 81.6 to 73.3 and 81.9 to 75.3%, respectively) and progressive motility (83.3 to 74.4, 84.3 to 74.4 and 84.2 to 75.6%, respectively) on Days 0, 1 and 2 of storage. There was also no sperm concentration by day interaction ($P > 0.05$).

4.4.3 Experiment 2b: The Field Fertility of Liquid Semen Diluted in INRA96 at 3, 4 or 5 x 10⁶ Sperm per Insemination Dose

Overall, inseminations with liquid semen on Day 1 post collection resulted in similar CR (52.1%) in comparison to frozen-thawed semen (55.2%; $P > 0.05$). Insemination with liquid semen on Day 2 of storage resulted in a lower CR (45.6%; $P < 0.01$) compared to semen used on Day 1 or frozen-thawed semen (52.1 and 55.2%, respectively; $P < 0.01$). There was an effect of sperm concentration on CR following AI as liquid semen diluted to 3 or 4 x 10⁶ sperm per dose resulted in a lower CR on Day 2 of storage (41.1 and 44.7%, respectively; $P < 0.05$) in comparison to frozen-thawed semen ($P < 0.05$) but did not differ to semen diluted to 5 x 10⁶ on Day 2 of storage ($P > 0.05$; Figure 4.4). There was no effect of bull on CR ($P > 0.05$) with the average CR for bulls used in the trial varying between 50.7 and 54.9 %. There was a bull by sperm concentration interaction ($P < 0.05$), as Bulls D and E in the 3 x 10⁶ treatment had a lower CR (47.6 and 43.7%, respectively) in comparison to frozen-

thawed semen (58.3 and 61.7%, respectively). There was a bull by day interaction as Bulls B and E had a lower CR on Days 1 and 2 (Bull B; 42.2 and 42.3% vs Bull E; 48.8 and 40.2% for Day 1 and 2, respectively) in comparison to frozen-thawed semen (58.7 and 61.7%, respectively; $P < 0.05$) while Bulls C and D had a reduced CR on Day 2 (44.0 and 47.5%, respectively), in comparison to frozen-thawed semen (54.8 and 58.3%, respectively; $P < 0.05$).

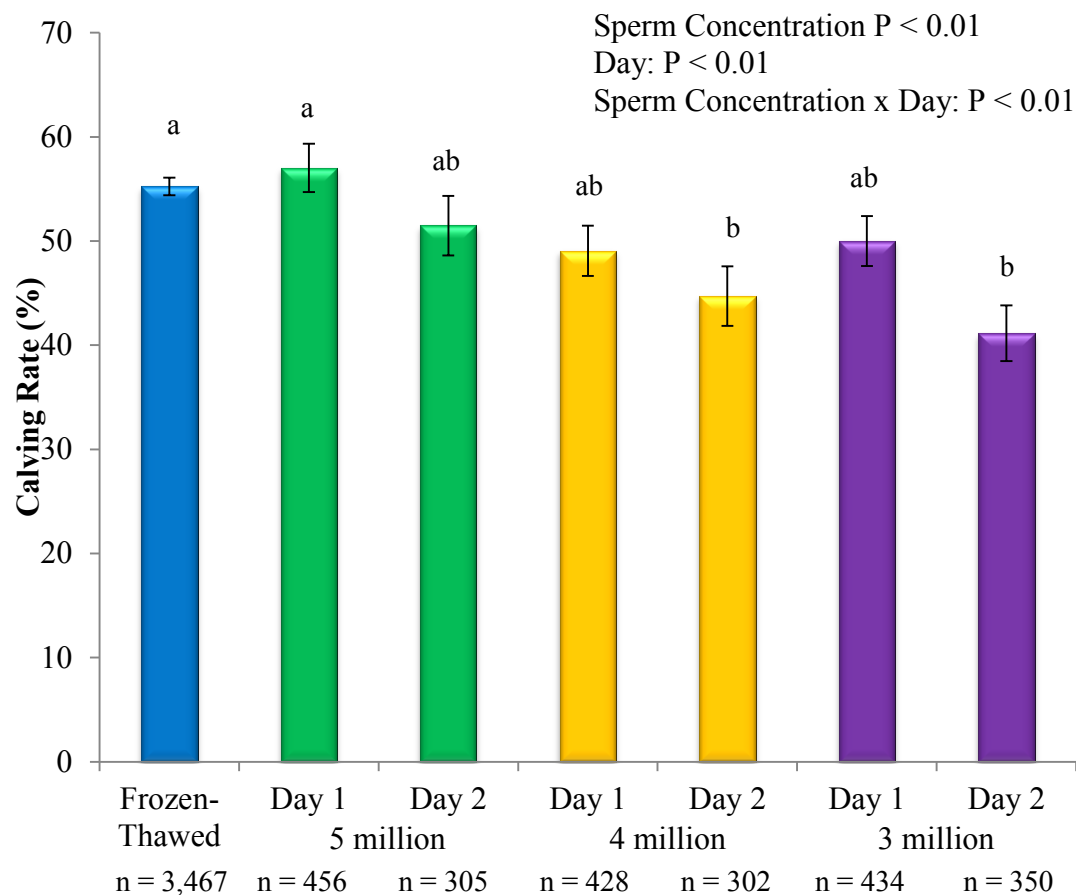


Figure 4.4: The effect of sperm concentration and day of storage on calving rate in dairy cows and heifers (Experiment 2b). Values with different superscripts differ significantly ($P < 0.01$). Vertical bars represent 95% confidence intervals. n = the total number of inseminations per treatment per day.

4.4.3.1 Effect of Cow Characteristics on Calving Rate

There was an effect of parity, cow fertility sub-index and DIM on CR ($P < 0.01$). Maiden heifers had a higher CR (57.9%) than primiparous (52.7%; $P < 0.05$) and multiparous dairy cows (50.6%; $P < 0.01$). Cows with a fertility sub-index of greater than €110 recorded a higher CR (61.0%) in comparison to cows with a fertility sub-index of <€50, €50-70, €70-90 and €90-110 at 45.6, 51.0, 53.8 and 55.4%, respectively. Cows greater than 60 DIM at the time of AI recorded a higher CR (55.4%) than cows with a DIM of <20, 20-40 and 40-60 (CR of 22.7, 36.3 and 41.2%, respectively). As expected, CR varied between individual herds and technicians, for herds and technicians with greater than 40 and 45 recorded inseminations, respectively ($P < 0.01$). There was no effect of breed, or a breed, cow fertility sub-index, DIM, herd or technician by sperm concentration interaction ($P > 0.05$).

4.5 Discussion

The main findings of this study were (i) storage of bull semen in INRA96 at 15 °C is superior to semen stored at 5 °C as assessed *in vitro* (ii) N₂ bubbling of INRA96 or reduced sperm concentration (within the range 10⁶ sperm per dose) had no effect on total and progressive motility *in vitro*, and (iii) insemination with liquid semen stored at 3 and 4 x 10⁶ sperm per dose resulted in a reduced CR following 2 days of storage in comparison with frozen-thawed semen but did not differ from semen diluted to 5 x 10⁶ sperm per dose on Day 2 of storage. As each treatment was prepared from the same ejaculate, any potential confounding effects of day of collection were removed, thus providing clear and reliable *in vitro* and *in vivo* data on the use of liquid bull semen.

It has previously been reported that sperm are quite versatile in relation to sperm quality between storage temperatures of 5 – 22 °C as they were found to retained acceptable *in vitro* standards (Murphy et al. 2016), with semen stored at 15 °C having greater motility compared to semen stored at 5, 22, 32 °C or fluctuating temperatures between 5-15, 5-22 and 5-32 °C. This is in agreement with the results of the current study as although semen held at both a constant 15 or 5 °C recorded acceptable total and progressive motility values up to 4 days of storage, storing semen at 15 °C resulted in better total and progressive motility throughout the duration of storage compared to semen stored at 5 °C. In the current study N₂ bubbling had no effect on sperm motility regardless of storage temperature. This is in agreement with Krzyzosiak et al. (2001) who reported no significant difference in the percentage of motile bull sperm up to Day 3 of storage or on the *in vitro* fertility of sperm stored under aerobic, N₂ gassed and anaerobic conditions (N₂ gassed and placed in an anaerobic chamber overnight with 5% Hydrogen and 95% N₂). Although not assessed in the current study, plasma membrane integrity deteriorated quicker when stored in aerobic conditions compared to anaerobic or N₂ gassed conditions (Krzyzosiak et al. 2001), however, motility on Day 7 was significantly lower after storage under anaerobic conditions compared to aerobic or N₂ gassed storage conditions (Krzyzosiak et al. 2001). Therefore, it may be possible that diluents provides sufficient support for sperm motility over prolonged periods (up to 3 days of storage) regardless of the oxygenated state of the media and that the benefits of N₂ gassing are not observed by assessing motility alone.

As expected, in the current study, total and progressive motility declined linearly in all treatments with increased duration of storage regardless of storage temperature,

N₂ gassing or sperm concentration; however, although all sperm concentration treatments maintained acceptable total and progressive motility on Day 2 of storage with scores above 74% respectively, semen diluted to 3 and 4 x 10⁶ sperm per dose resulted in a reduced CR on Day 2 of storage (41.1 and 44.7%, respectively) compared to frozen-thawed semen (55.2%). A study conducted by Vishwanath and Shannon (1996) reported that bull sperm have gradual decreasing motility scores for up to 4 weeks when stored in Caprogen diluent; however, there was a sharp decline in NRR after 3-5 days (69.9 ± 1.2%) compared to after 10 days (41.5 ± 3.7%). This suggests that additional factors relating to fertility other than sperm motility are essential in achieving high pregnancy rates.

Murphy et al. (2016) previously reported that some bulls are more susceptible to sperm aging resulting in a decrease in their fertility due to the cumulative generation of reactive oxygen species with increased duration of storage. However, the results of the current study would suggest that some bulls may be more suitable for use in frozen-thawed semen rather than liquid semen programs regardless of the duration of storage. Three bulls in particular performed relatively poorly, in relation to CR, following AI on Day 1 and 2 of storage of liquid semen compared to frozen-thawed semen with these bulls combined having an average CR of 45.9% and 42.4% on Day 1 and 2, respectively, compared to 58.4% for frozen-thawed semen. Notwithstanding the limitations of the relatively modest number of inseminations per bull (Foote 2003) a possible explanation for poor fertility performance of liquid semen may be due to the inability of the sperm to adapt to temperature variation associated with storage of liquid semen at ambient temperature. A previous study by our group found that semen stored at ambient temperatures in unregulated temperature control

boxes in the trunk of a car (similar storage conditions to liquid semen in Ireland) where subjected to day to night time temperature variations with minimum and maximum temperature values of 6.4 and 27.9 °C, respectively, (Murphy et al. 2016). Although Murphy et al. (2017) reported that storing diluted semen at constant or fluctuating temperatures between 4 – 18 °C had no impact on motility when stored in INRA96, unpublished data by our group shows that while sperm are quite versatile in terms of storage temperature, fluctuating temperatures between 5 – 28 °C (night-time to daytime over 4 days) resulted in a significant loss of motility (unpublished). Exposure to such daytime/night-time temperature fluctuations may result in a decline in membrane integrity as a consequence of morphological membrane changes which are consistent with the lipid phase transition (Drobnis et al. 1993), thus resulting in a reduction in sperm quality and fertility (Batellier et al. 2001). Therefore, further investigation should be undertaken with a strict temperature regulation regime in place so that liquid semen is maintained at a constant temperature.

In the current study, CR of liquid semen stored at ambient temperature and diluted in INRA96 to 3 and 4 x 10⁶ sperm per dose on Day 2 of storage decreased significantly compared to frozen-thawed semen but did not differ from semen diluted to 5 x 10⁶ sperm per dose after 2 days of storage. Additionally, CR of semen diluted to 3, 4 or 5 x 10⁶ sperm per dose on Day 1 of storage did not differ from frozen-thawed semen. This supports the findings of Murphy et al. (Murphy et al. 2016) as semen diluted in Caprogen had a reduced NRR on Day 2 of storage at lower concentrations compared to frozen-thawed semen. However, in the current study, the overall CR of liquid semen following AI on Day 1 of storage was comparable to the previous study reported by Murphy et al. (2017) who compared semen diluents at the higher

concentration of 5×10^6 sperm per dose. Consequently, it could be recommended to increase sperm numbers for targeted use on subsequent days after collection; namely 4 and 5×10^6 sperm per dose for insemination on Day 1 and 2 post collection, respectively. This would ensure that the number of insemination doses per ejaculate is maximised, thus resulting in an increase in the use of individual sires. In contrast to the results of this study, previous studies have shown that similar conception rates can be achieved with liquid and frozen-thawed semen (Buckley et al. 2003) and that NRRs of liquid and frozen-thawed semen diluted to 2.5×10^6 and 20×10^6 sperm, respectively, do not differ up to Day 2 of storage (Shannon and Vishwanath 1995). However, over-compensation of sperm numbers typically occurs in the preparation of frozen-thawed semen, resulting in a sperm concentration which considerably exceeds the number of sperm necessary for maximum fertility, thus, masking the 'true fertility' potential of a bull (Amann and DeJarnette 2012).

It is well established that cow characteristics such as parity, fertility sub-index and DIM play a role in fertility (Murphy et al. 2016, Murphy et al. 2017). Cow fertility declines with increased age as stresses associated with calving, lactation and clinical abnormalities at parturition or postpartum have a negative effect on fertility (Andersen-Ranberg et al. 2005, Miller et al. 2001). Previous studies have found that heifers had a higher NRR compared to multiparous dairy cows (Gabriel et al. 2011, Pursley et al. 1997). The results of the current study show that, while numerically greater, CR of heifers did not differ to primiparous dairy cows but was ~13% higher than multiparous dairy cows with a parity of greater than 5. Furthermore, there was no treatment by parity interaction highlighting that no particular semen type has an advantage in negating the effects of parity. In the current study, a positive linear

relationship between fertility sub-index and CR was observed with animals in the highest fertility sub-index ($>€110$) having a higher CR than any other sub-index. Therefore, the results clearly highlight the importance of the EBI and its contribution to herd fertility. In this study, the number of DIM affected CR with cows of greater than 60 DIM having a higher CR than those of less than 60 DIM. Therefore, this study demonstrates that increasing the number of DIM increases fertility; consequently, allowing a DIM of 60 days can be beneficial to the overall production of a dairy herd. However, in the case of late calving cows, some confidence can also be taken from inseminating these animals at a shortened DIM interval, with reasonable success, in order to bring forward the calving date of this cohort of cows.

In conclusion, storing semen at 15 °C resulted in superior total and progressive motility values compared to semen stored at 5 °C, while N₂ gassing and sperm concentration (3, 4 or 5 x 10⁶ sperm per dose) had no effect on sperm motility. On Day 1 of storage there was no difference in CR between liquid and frozen-thawed semen; however, on Day 2 of storage insemination with 3 or 4 x 10⁶ sperm per dose resulted in a lower CR in comparison to frozen-thawed semen but did not differ from 5 x 10⁶ sperm per dose. Thus, given that reducing sperm concentration per dose results in an increased number of doses per ejaculate, therefore increasing the utilisation of superior sires, it may be justifiable to increase sperm numbers for targeted use on subsequent days after collection. Furthermore, additional consideration should be given to the storage temperature of liquid semen with an aim to storing liquid semen at a constant temperature.

Chapter Five

**Optimising Storage Temperature of
Liquid Bovine Semen Diluted in INRA96**

Abstract

Temperature regulation of liquid bovine semen can be difficult in field situations. Four experiments were carried out to assess the effect of storage temperature and straw source on *in vitro* sperm characteristics and 60-day NRR following AI of liquid bovine semen. In Experiment 1a, the effect of storage of liquid bovine semen in INRA96 (IMV Technologies) at one of five storage temperatures (5, 15, 28, fluctuated between 5 and 15 or fluctuated between 5 and 28 °C) on total and progressive motility and kinematic parameters was assessed objectively via computer assisted sperm analyser on Days 0, 1, 2, 3 and 4 post collection. Fluctuating temperatures were designed to mimic day to night time variations. In Experiment 1b, the field fertility of liquid semen stored at a constant 5 or 15 °C or in an unregulated manner was assessed and compared to frozen-thawed semen (total of n = 106,738 inseminations). In Experiment 2a, the effect of straw source of liquid bovine semen in INRA96 in either IMV (clear) or Minitube (coloured) liquid semen straws and stored at 5 or 15 °C (based on outcome of Experiment 1a) on total and progressive motility was assessed on Days 0, 1, 2, 3 and 4 post collection. In Experiment 2b, the field fertility of liquid semen stored at ambient temperature filled into IMV or Minitube liquid semen straws was assessed in comparison to frozen-thawed semen (total of n = 19,477 inseminations). In Experiment 1a, there was a linear decrease in motility with increased duration of storage ($P < 0.01$). Semen stored at a constant 15 °C or fluctuated between 5 and 15 °C had greater total motility than semen held at 5 °C ($P < 0.05$), 28 and fluctuated between 5 and 28 °C ($P < 0.01$); however, 15 °C or fluctuated between 5 and 15 °C did not differ from each other. Semen held at 5, 15 or fluctuated 5 and 15 °C, although not differing from each other, had higher progressive motility scores than storage at 28 and or

fluctuated between 5 and 28 °C ($P < 0.01$). Semen stored at a constant 28 °C exhibited poor motility and velocity values but recorded high progressive motion values compared to all other storage temperatures ($P < 0.01$); however all other storage temperatures did not differ from each other in relation to motility kinematics. In Experiment 1b, semen stored at a constant 5 °C resulted in a lower 60-day NRR (62.5%) than storage at constant 15 °C, unregulated or frozen-thawed semen (73.6, 74.6 and 74.4%, respectively; $P < 0.01$). In Experiment 2a, total and progressive motility decreased with increased duration of storage ($P < 0.01$). Sperm stored in IMV straws had a higher progressive motility than those stored in Minitube straws; however there was no effect of straw source on total motility ($P > 0.05$). In Experiment 2b, there was no effect of straw source on 60-day NRR (72.6 versus 72.7% for IMV and Minitube, respectively, $P > 0.05$). In conclusion, sperm stored in IRNA96 are quite tolerant in terms of storage temperature, retaining acceptable motility between temperatures of 5 and 15 °C. Storing semen at a constant 15 °C resulted in greater *in vitro* sperm motility and higher NRR rates than storage at 5 °C and did not differ in NRR from frozen-thawed semen or semen stored at an unregulated temperature; however lower storage temperatures were shown to be more detrimental to sperm *in vivo* than unregulated storage conditions. Furthermore, straw source did not have a commercially significant effect on sperm motility or on field fertility.

5.1 Introduction

Liquid semen has traditionally been confined to countries such as Ireland and New Zealand with seasonal grass based systems where inseminations are confined to a short breeding season (Verberckmoes et al. 2005). Liquid semen is principally used

for only 2.5 – 3 days post-collection as a reduction in fertility has been reported thereafter (Vishwanath and Shannon, 2000). Liquid semen has a distinct advantage over frozen-thawed semen as the reduced sperm concentration per straw (approximately 3-5 million vs 15-20 million sperm, respectively (Murphy et al. 2013)), allows for approximately three times greater production of semen straws. Hence, the use of liquid semen maximises the number of insemination straws produced per ejaculate compared to frozen-thawed semen. Currently, liquid bovine semen straws in Ireland are stored in an unregulated temperature flask, which is subjected to natural day to night time temperature fluctuations with an average spring temperature recorded within the flask of 15 °C while the minimum and maximum temperatures recorded in one study were 6.4 and 27.9 °C, respectively (Murphy et al. 2016). The optimum storage temperature of semen can depend on the species involved and semen dilution technology implemented, with studies suggesting temperatures between 18-24 °C are optimal for bovine semen when semen is purged with nitrogen gas (N₂; Vishwanath and Shannon 2000), while storage at 5 °C has also been recommended for bovine (Black 2006), equine (Ball et al. 2001) and ovine semen (O'Hara et al. 2010, Gil et al. 2011).

An accepted principle of semen dilution technology is that sperm survival over prolonged periods is inversely related to their metabolic activity (Salisbury and Vandemark 1961). Various strategies to reduce metabolism have been assessed to enhance sperm survival such as reducing storage temperature, lowering pH (Foote 1964) and N₂ gassing (Shannon 1965). While storage of liquid semen at 5 °C may reduce the metabolic activity of sperm, therefore extending their fertile lifespan (Shannon et al. 1984), one disadvantage is an increase in intracellular sodium

concentration to cytotoxic levels due to a reduction in the activity of the sodium-potassium pump to diffuse ions across the cell membrane (Vishwanath and Shannon 2000). Storing semen at reduced temperatures may also result in an increased incidence of cold shock injuries which are associated with morphological membrane changes consistent with a lipid phase transition (Drobnis et al. 1993). Although the mechanisms underlying cold shock are not fully understood, it is believed that, due to a loss of membrane phospholipids at reduced temperatures, sperm membrane integrity declines resulting in reduced semen quality (Batellier et al. 2001). In order to avoid the damage sustained by reduced temperatures, protocols to inhibit pathways detrimental to the survival of sperm at ambient temperatures (15-20 °C) were devised (Shannon and Curson 1984). Although storing semen at 15-20 °C may prevent the occurrence of cold shock injuries and thus improve fertility; it has been postulated that the production of reactive oxygen species, as a by-product of metabolism, is accelerated at higher storage temperatures (Pino et al. 2013, Vishwanath and Shannon 1996).

Murphy et al. (2016) reported that semen stored at 15 °C in an egg-yolk based diluent (Caprogen) had greater total and progressive motility than semen stored at 5, 22, 32 °C or fluctuating (day to night-time) between these temperatures. That study also reported that sperm were tolerant to temperatures between 5 and 22 °C. Milk-based extenders, such as INRA96, are widely used in the dilution and storage of equine semen cooled to between 4-8 °C (Batellier et al. 2001). However, Batellier et al. (1997) demonstrated that the survival of equine sperm stored in INRA96 at 15 °C was better than that stored in milk-based extenders at 4 °C and others have demonstrated improved pregnancy rates when equine semen was stored at 15 °C

(Cuervo-Arango et al. 2014, Batellier et al. 2001). Although liquid bull semen has traditionally been stored at ambient temperature in the egg-yolk based diluent, Caprogen, we have recently demonstrated that INRA96 is effective for the preservation of bovine semen stored at ambient temperature, resulting in similar calving rates to semen diluted in Caprogen (Murphy et al. 2017). Furthermore, as the preparation of Caprogen is complex and time consuming, the use of INRA96 is a suitable alternative for the dilution and storage of bovine semen and is more convenient for the busy working schedule of an AI centre as it can be used directly off the shelf, thus, reducing time constraints within the laboratory.

Additionally, the quality of material used to produce liquid semen straws is important in order to ensure semen quality is maintained throughout the duration of storage, regardless of storage temperature. The option of using coloured liquid semen straws for each individual bull is becoming increasingly popular, particularly for older AI technicians, as it is an easier method of identifying bulls. Currently, in Ireland liquid semen is packaged in commercially available clear straws (IMV Technologies, L'Aigle, France) with only a coloured plug at one end used to identify different bulls, creating difficulty in bull identification. Thus, the use of commercially available coloured liquid semen straws (Minitube, Tiefenbach, Germany) may be a suitable alternative; however, it is unclear if the quality of these coloured liquid semen straws is comparable to that of the standard IMV liquid semen straw. Therefore, the objectives of this study were to investigate if temperature regulation or straw source could improve the *in vitro* sperm motility and kinematic parameters and *in vivo* fertility of liquid bovine semen stored in INRA96.

5.2 Materials and Methods

5.2.1 Experiment 1a: Effect of Storing Liquid Semen Stored at a Constant or Fluctuating Storage Temperatures on Sperm Motility and Kinematic Parameters

The aim of this experiment was to establish the optimum storage temperature range for liquid bovine semen stored in INRA96. The effect of five different storage temperature conditions (5, 15, 28, fluctuating 5-15 or fluctuating 5-28 °C) on total and progressive motility and kinematic parameters of liquid bovine semen for up to four days post collection was assessed. Semen was collected from Holstein Friesian bulls (n = 7) at a commercial AI centre on three different occasions (occasion = replicate). The raw ejaculate was partially diluted in 10 mL pre-warmed INRA96 (IMV Technologies, Normandy, France) at 37 °C and transported in a temperature-regulated cooler box at 18 °C to the laboratory (up to 3 h transport). On arrival, the ejaculate was assessed for sperm concentration using a coulter counter (Z Series, Beckman Coulter, Clare, Ireland) and scored for total motility (%) and gross motility on a subjective 5-point scale (1 = twitching/no forward progressive motility; 5 = excellent forward progressive motility) to ensure all samples were of a commercial standard (results not presented). Microscopic assessments were conducted by the same technician and initial quality control cut-off values were a total and gross motility of $\geq 70\%$ and a score of ≥ 3 , respectively. Any ejaculates failing to meet these criteria are rejected and would not be included in the study; however no ejaculates were rejected upon initial quality control checks.

Ejaculates were fully diluted in INRA96 to achieve a concentration of 5×10^6 sperm per 0.25 mL insemination dose. Semen from each bull was kept separate and

ejaculates were split such that each bull was represented equally in each treatment. Straws were filled as per routine procedures and stored at one of five storage temperatures: 5, 15, 28, fluctuated between 5 and 15 or fluctuated between 5 and 28 °C. Fluctuating temperatures were designed to mimic day to night time fluctuations; semen was held at 5 °C at night (in a fridge) and at either 15 (in temperature-regulated box) or 28 °C (in an incubator) during the day. In order to allow a gradual temperature fluctuation, straws were placed in an insulated plastic container and stored at their respective temperatures for a minimum of 12 hours daily. Temperatures were fluctuated between 28 to 5 °C and vice versa over a minimum period of 3.5 h. Samples (n = 4 straws) from each treatment were assessed in a randomised sequence to remove bias as a result of sampling order.

5.2.1.1 Computer-Assisted Sperm Analysis

Total and progressive motility and kinematic parameters were objectively assessed on Days 0, 1, 2, 3 and 4 post semen collection (Day 0 = 3 h after collection) using the IVOS-II Computer Assisted Sperm Analyser (CASA; IMV Technologies) system driven by software version 14 (Hamilton Thorne Inc, Beverly, USA). Straws (n = 4 per ejaculate) were warmed to 37 °C for 30 sec, dried fully, to remove any excess water, cut at the sealed end and separately placed into a pre-warmed eppendorf (35 °C). The plug end of each straw was then cut to expel the contents of the straw into the eppendorf and the semen sample was mixed thoroughly to ensure homogeneity. The samples were incubated for approx. 10 min and a drop (3 µL) of diluted semen was placed in a pre-warmed chamber (37 °C; Leja counting chambers, depth 20 µm; Microptics, Barcelona, Spain) and analysed for sperm motility and kinematic parameters. A minimum of 1000 sperm were analysed in at least eight microscopic

fields with 30 frames acquired per field at a frame rate of 60 Hz. Objects incorrectly identified as sperm were edited out using the playback function. The CASA-derived motility and kinematic characteristics (Mortimer 2000) assessed were total motility (%), progressive motility (%), proximal and distal droplets (%), as well VAP above 10 $\mu\text{m/s}$, VSL, VCL, LIN, straightness (STR), ALH and BCF). Regarding analysis settings, the CASA was set to standard factory settings for bull semen; sperm with straightness of $>80\%$ and VAP $>50 \mu\text{m/s}$ were considered progressively motile.

5.2.2 Experiment 2a: Effect of Straw Source on Total and Progressive Motility of Liquid Semen Diluted in INRA96

The aim of this experiment was to assess the effect of straw source (IMV or Minitube) and storage temperature (based on outcome of Experiment 1a; 5 and 15 $^{\circ}\text{C}$) on total and progressive motility of liquid bovine semen for up to four days post collection. Semen was collected from Holstein Friesian bulls ($n = 5$) on three different occasions (occasion = replicate) and the raw ejaculate was partially diluted, transported and assessed as described in Experiment 1a. Semen from each bull was kept separate and ejaculates were split such that each bull was represented equally in each treatment. Straws were filled as per routine procedures in either IMV or Minitube fresh semen straws and stored at 5 $^{\circ}\text{C}$ (in a fridge) or 15 $^{\circ}\text{C}$ (in temperature-regulated box). Samples were objectively assessed for total and progressive motility as described above Experiment 1a.

5.2.3 Experiment 1b: Effect of Storage Temperature on Field Fertility of Liquid Semen Diluted in INRA96

The aim of this experiment was to assess the effect of three storage temperatures on 60-day NRR following AI; two temperatures were selected based on the outcome of Experiment 1 (5 and 15 °C) and the third was the industry standard which involves storage of straws in an unregulated temperature flask (Figure 5.1). Semen was collected from Holstein Friesian bulls (n = 16; denoted A-P) at a commercial AI centre from mid-April to the early June 2017. There were 20 collection days in total, with three bulls used per collection day (total of 60 ejaculates; approx. 3-4 ejaculates per bull). Following assessment for volume, concentration and motility (as described in Experiment 1), each acceptable ejaculate was diluted to 5×10^6 sperm per 0.25 mL insemination dose in INRA96 and processed and filled as per routine procedure. Each batch of liquid semen was clearly labelled and distributed for insemination on the day of collection. Liquid semen was transported to the distribution centre at a constant 15 °C and distributed to technicians where it was then stored at either a constant 5 °C, 15 °C or in an unregulated flask; mean low and high daily atmospheric temperature values recorded during the trial period were 6.8 and 15.9 °C with minimum and maximum temperatures of 0.3 and 21.6 °C, respectively (Met Éireann 2017). Liquid semen was used for up to 2 days post collection on both heifers (n = 3,644) and multiparous (n = 44,561) dairy cows. Due to quarantine restrictions on frozen-thawed semen, frozen-thawed semen doses (15×10^6 sperm per dose) were derived from previously collected ejaculates from the same 16 bulls and processed and frozen as described above (n = 58,533 inseminations consisting of 10,440 heifers and 48,093 multiparous dairy cows) as described by Murphy et al. (2017).

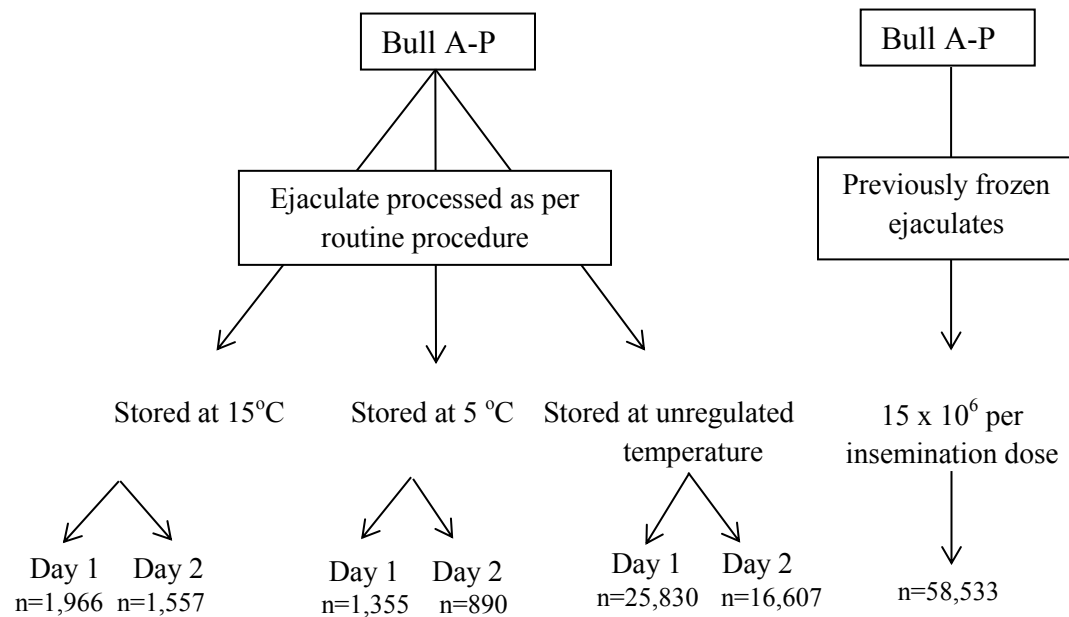


Figure 5.1: Experimental design for Experiment 1b. Ejaculates were split for the liquid semen treatments and previously frozen ejaculates from the same bulls were sourced for the frozen-thawed treatment. (n = the total number of inseminations per storage temperature per day).

5.2.3.1 Field Inseminations

Inseminations were carried out in mid-April to early June 2017 (coinciding with the peak dairy breeding season) in Irish dairy herds (n = 449). The majority (95.7%) of inseminations were in Holstein Friesian cows (n = 102,158) but small numbers of cows of other breeds were represented: Jersey (n = 3,129), Montbeliarde (n = 246), Norwegian Red (n = 969), Swedish Red cows (n = 42) and other (n = 194; includes Ayrshire, Rotbunte, MRI and Brown Swiss). Technicians (n = 243) were grouped by geographical area and each technician was assigned a storage temperature for the duration of the trial: 5, 15 °C or unregulated. For each insemination, the AI technician recorded the bull code, cow tag number and the straw code on an electronic handheld device. Insemination and NRR data were captured using the

ICBF database by cross-referencing the technician name with the bull code and semen type used on each date within the trial period.

5.2.4 Experiment 2b: Effect of Straw Source on Field Fertility of Liquid Semen Diluted in INRA96

The aim of this experiment was to assess the effect of straw source (IMV or Minitube) on 60-day NRR following AI with liquid semen (Figure 5.2). Semen was collected from Holstein Friesian bulls ($n = 6$) at a commercial AI centre during May 2017. There were 9 collection days in total, with 2 bulls used per collection day (total of 18 ejaculates). The raw ejaculate was collected, partially diluted, transported, assessed and processed as described above (Experiment 2a). Semen was stored at ambient temperature and used for up to 2 days post collection on both heifers ($n = 1,237$) and multiparous ($n = 7,389$) dairy cows. Due to logistical constraints, frozen-thawed semen doses were derived from previously collected ejaculates from the same 6 bulls which were processed and frozen using routine procedures as described above (Experiment 1b; $n = 10,851$ inseminations consisting of 2,082 heifers and $n = 8,769$ multiparous dairy cows).

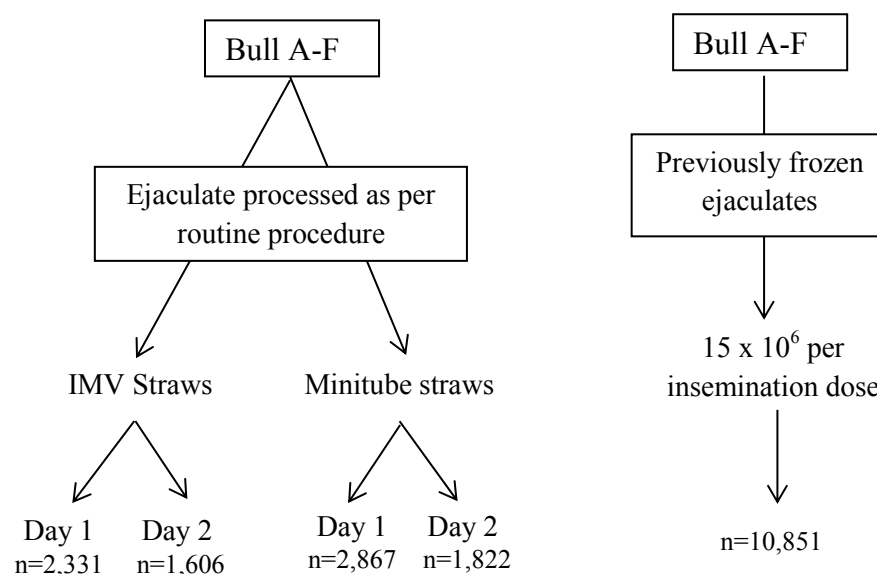


Figure 5.2: Experimental design for Experiment 2b. Ejaculates were split for the liquid semen treatments and previously frozen ejaculates from the same bulls were sourced for the frozen-thawed treatment. (n = the total number of inseminations per straw source per day).

5.2.4.1 Field Inseminations

Inseminations were carried out in May 2017 (coinciding with the peak dairy breeding season) in Irish dairy herds (n = 365). The majority of inseminations were in Holstein Friesian cows (n = 19,328) but small numbers of other breeds were represented: Jersey (n = 526), Montbeliarde (n = 36), Norwegian Red (n = 99) and other (n = 58; includes Ayrshire, Rotbunte, Swedish Red, Danish Red and MRI and Brown Swiss). Technicians (n = 53) were blind to treatments, grouped into geographical areas and treatments were rotated on each collection day to ensure that technicians received different straw type treatments from each bull on each day. For each insemination, the AI technician recorded the bull code, cow tag number and the

straw code on a handheld electronic device. Cow characteristics such as parity, DIM and fertility sub-index were also assessed.

5.2.5 Capturing of Non-Return Rate Data

Insemination and NRR data were captured using the ICBF database by cross-referencing the technician name with the bull code and semen type used on each date within the trial period. Obvious errors were extracted from the dataset and data were then interrogated to remove animals based on the following criteria: cows which were not at first AI, cows which received two inseminations from two different bulls or treatments, or cows which were not of a dairy breed. However, if a cow received two inseminations from the same bull with the same treatment within five days of each other, the record was kept and the second date was assumed to be correct. Post editing, a total of 106,738 and 19,477 inseminations remained for Experiment 1b and 2b, respectively.

Cow characteristics such as parity, DIM and fertility sub-index were included in the model. Fertility sub index is a key component of the EBI comprising ~35% of the total EBI (ICBF 2017a). The EBI is an estimate of the economic value of an animal's genetic merit. It was established to combat a decline in reproductive performance by providing farmers with a profit index enabling the selection of elite sires to breed replacement heifers with increased milk yield, reproductive performance and improved health traits (Berry et al. 2005).

5.3 Statistical Analysis

Data from Experiment 1a and 2a were examined for homogeneity of variance and analysed using the GLM repeated-measures procedure with a compound symmetry covariance structure in SPSS. In Experiment 1b and 2b, the NRR data were compared using Pearson's chi-squared procedure in SPSS. The dependent variable in the analysis was NRR (1 = pregnant, 0 = not pregnant). In addition, a GLM for binomial data was used to assess a number of fixed effects on NRR including temperature, bull, parity, breed, fertility sub-index, DIM, herd and technician. Each fixed effect was assessed for an interaction with temperature treatment. All post-hoc tests were carried out using the Bonferroni test. Results are reported as the mean \pm the standard error of the mean (sem) in Experiment 1a and 2a and as the estimated marginal means in Experiment 1b and 2b, to adjust for imbalance between numbers of inseminations in each treatment. Values were considered to differ significantly at $P < 0.05$.

5.4 Results

5.4.1 Experiment 1a: Effect of Storing Liquid Semen Stored at a Constant or Fluctuating Storage Temperatures on Sperm Motility and Kinematic Parameters

There was an effect of storage temperature and day on both total and progressive motility of liquid semen (Figure 5.3; $P < 0.01$), however, there was no temperature by day interaction ($P > 0.05$). From Day 0 to Day 4 across all treatments the percentage of total and progressively motile sperm declined linearly. Semen held at a constant 15 °C had a higher total motility score throughout the duration of storage

compared to semen held at 5, 28 and semen fluctuated between 5 and 28 °C ($P < 0.05$); however, this did not differ from semen fluctuated between 5 and 15 °C ($P > 0.05$). Semen held at 5, 15 and fluctuated between 5 and 15 °C had a higher progressive motility score than semen held at 28 and fluctuated between 5 and 28 °C ($P < 0.01$) but did not differ from each other ($P > 0.05$). There was an effect of bull on total and progressive motility ($P < 0.01$) with bulls ranging from 48.5 to 79.7% and from 43.6 to 71.1% for total and progressive motility, respectively. There was no bull by day or bull by temperature interaction on total and progressive motility ($P > 0.05$). Semen held at a constant 28 °C resulted in the lowest total and progressive motility score for all days of storage ($P < 0.01$) and also resulted in a large proportion of agglutinated sperm, the percentage of which increased dramatically with increased duration of storage (data not recorded). Semen maintained at 5 °C and fluctuated between 5 and 15 °C, although not differing from each other in relation to total and progressive motility ($P > 0.05$), recorded greater total and progressive motility scores than semen fluctuated between 5 and 28 °C ($P < 0.01$). Overall, semen held at a constant 28 °C exhibited poor motility with a low VCL and VAP, however, surprisingly recorded high progressive motion values with the highest LIN, STR, wobble (WOB) and lowest ALH values compared to storage temperatures of 5, 15, fluctuated between 5 and 15 and fluctuated between 5 and 28 °C ($P < 0.01$; Table 5.1). Sperm stored in all other storage temperatures were exhibited slightly hyper motility indicated by the high VCL and ALH values and did not differ in motility kinematics between each other ($P > 0.05$). There was no effect of treatment on VSL or on proximal and distal droplets ($P > 0.05$).

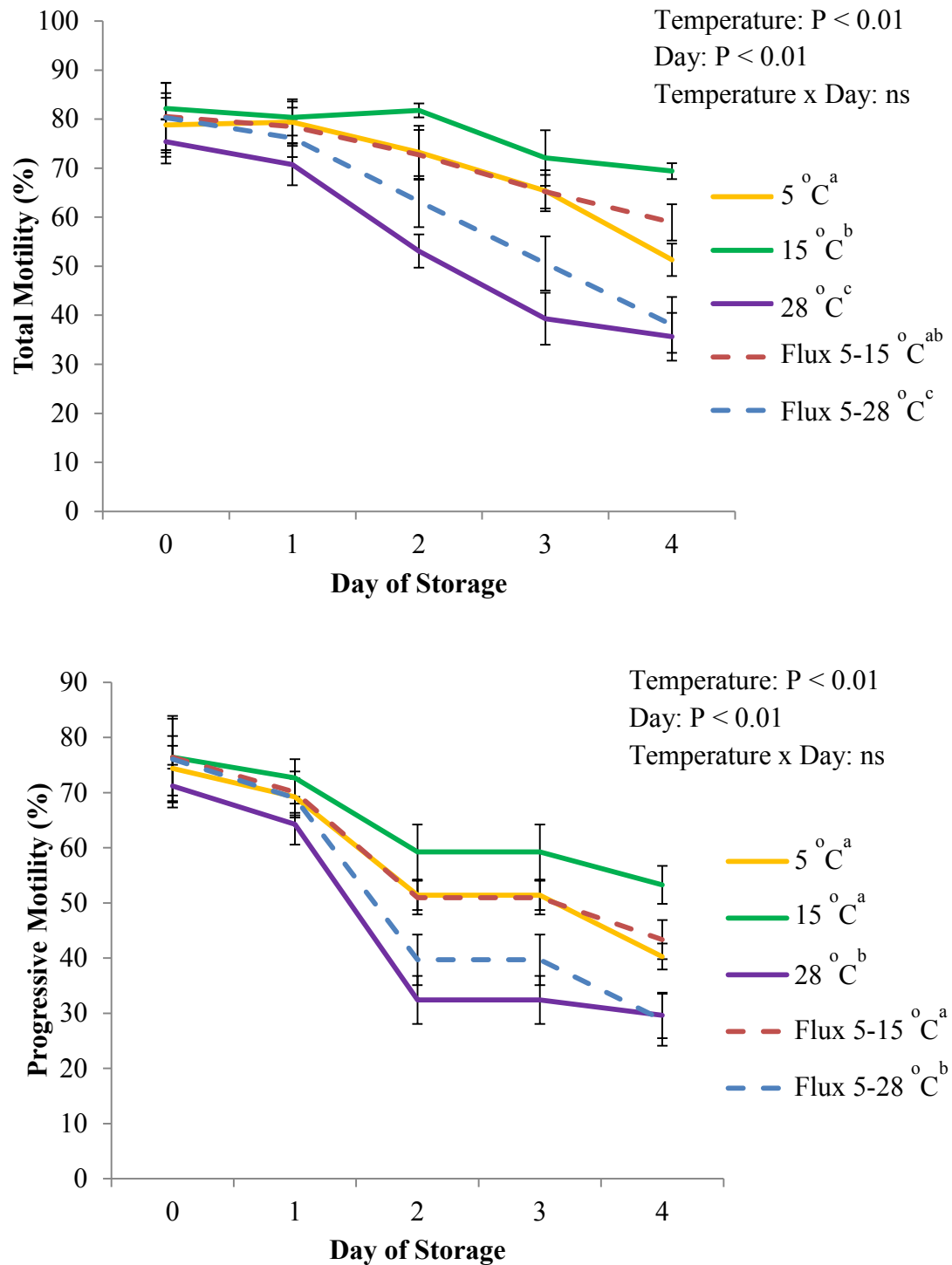


Figure 5.3: The effect of storage temperature on total motility (upper panel) and progressive motility (lower panel) of liquid bull semen on Days 0, 1, 2, 3 and 4 post collection (Experiment 1a) as assessed using computer assisted sperm analysis. Vertical bars represent sem. ^{abcd}Temperatures with different superscripts differ significantly (P < 0.01). ns = non-significant.

Table 5.1: The overall effect of storage temperature on total and progressive motility and kinematic parameters as assessed using computer assisted sperm analysis in bovine semen stored at 5, 15, 28, fluctuated between 5 and 15 or fluctuated between 5 and 28 °C (Experiment 1b). ^{abc}Values with different superscripts differ significantly within row (P < 0.01; values are mean ± sem).

Parameter	Storage Temperature (°C) mean ± sem					P Value Effect of treatment
	5	15	28	Fluctuating 5 and 15	Fluctuating 5 and 28	
Total Motility (%)	68.7 ± 2.8 ^a	79.2 ± 1.25 ^b	52.4 ± 2.98 ^c	69.9 ± 2.32 ^{ab}	59.2 ± 3.57 ^c	P < 0.01
Progressive Motility (%)	57.0 ± 2.59 ^a	66.9 ± 1.68 ^a	46.9 ± 2.57 ^b	58.2 ± 2.66 ^a	50.4 ± 3.59 ^b	P < 0.01
ALH (µm)	11.3 ± 0.22 ^a	10.7 ± 0.21 ^a	8.4 ± 0.21 ^b	11.3 ± 0.19 ^a	11.1 ± 0.21 ^a	P < 0.01
BCF (Hz)	23.5 ± 0.47 ^a	27.7 ± 0.62 ^b	29.3 ± 0.42 ^b	24.6 ± 0.45 ^a	25.4 ± 0.42 ^a	P < 0.01
LIN (%)	36.1 ± 1.24 ^a	40.1 ± 0.99 ^a	46.2 ± 0.76 ^b	36.6 ± 1.30 ^a	37.8 ± 1.27 ^a	P < 0.01
STR (%)	70.2 ± 1.28 ^a	74.5 ± 1.11 ^a	82.4 ± 0.61 ^b	71.0 ± 1.46 ^a	73.3 ± 1.34 ^a	P < 0.01
VAP (µm/s⁻¹)	116.7 ± 1.58 ^a	123.2 ± 1.15 ^a	105.0 ± 2.67 ^b	118.2 ± 1.60 ^a	117.6 ± 1.82 ^a	P < 0.01
VCL (µm/s⁻¹)	235.5 ± 4.04 ^a	240.6 ± 3.22 ^a	198.8 ± 5.69 ^b	239.2 ± 2.91 ^a	239.3 ± 3.96 ^a	P < 0.01
VSL (µm/s⁻¹)	82.5 ± 2.23	91.9 ± 1.85	87.1 ± 2.27	84.7 ± 2.75	86.9 ± 2.65	ns
WOB (%)	50.6 ± 0.77 ^a	52.7 ± 0.59 ^a	55.0 ± 0.56 ^b	50.5 ± 0.76 ^a	50.5 ± 0.76 ^a	P < 0.01
Proximal Droplets (%)	3.9 ± 2.31	3.2 ± 1.73	4.2 ± 2.45	3.8 ± 2.03	3.9 ± 2.19	ns
Distal Droplets (%)	4.4 ± 0.25	3.6 ± 0.18	3.7 ± 0.22	4.3 ± 0.23	4.2 ± 0.24	ns

ALH = amplitude of lateral head displacement, BCF = beat cross frequency, LIN = linearity, STR = straightness, VAP = average path velocity, VCL = curvilinear velocity, VSL = straight line velocity, WOB = wobble, ns = non-significant

5.4.2 Experiment 2a: Effect of Straw Source on Total and Progressive Motility of Liquid Semen Diluted in INRA96

Liquid semen stored in IMV straws had a higher progressive motility score than Minitube straws ($P > 0.05$; Figure 5.4); however there was no effect of straw type on total motility ($P > 0.05$). There was an effect of day on total and progressive motility ($P < 0.01$) as motility declined with increased duration of storage ($P < 0.01$). There was no effect of bull, bull by day and or bull by straw type interaction on total and progressive motility ($P > 0.05$).

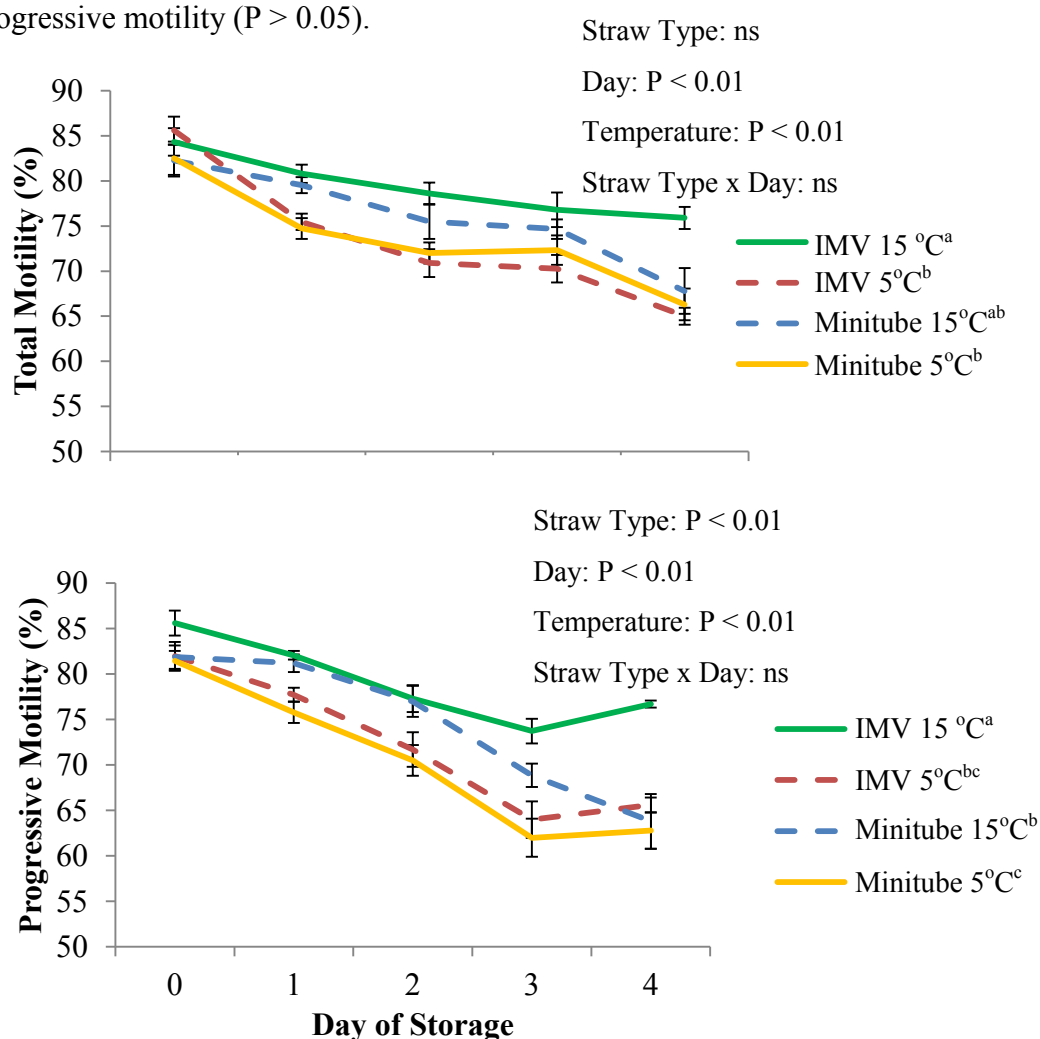


Figure 5.4: The effect of straw source on total motility (upper panel) and progressive motility (lower panel) of liquid bull semen on Days 0, 1, 2, 3 and 4 post collection (Experiment 2a). Vertical bars represent sem. ^{abc}Temperatures with different superscripts differ significantly ($P < 0.01$). ns = non-significant

5.4.3 Experiment 1b: Effect of Storage Temperature on Field Fertility of Liquid Semen Diluted in INRA96

There was a treatment by day interaction as semen stored at a constant 5 °C on Day 1 and 2 of storage had a reduced NRR compared to all other treatments ($P < 0.01$; Figure 5.5); however, there was no difference in NRR between frozen-thawed semen or any other temperature on Day 1 or Day 2 of storage ($P > 0.05$). Semen stored at a constant 5 °C had a reduced 60-day NRR compared to semen stored at a constant 15 °C, unregulated and frozen-thawed semen (74.4%). Overall, insemination with liquid semen on Day 1 post collection resulted in similar NRR (74.4%) to frozen-thawed semen (74.4%; $P > 0.05$); however, inseminations with liquid semen on Day 2 of storage resulted in a lower NRR (73.2%) compared to semen used on Day 1 ($P < 0.05$) as well as frozen-thawed semen ($P < 0.01$).

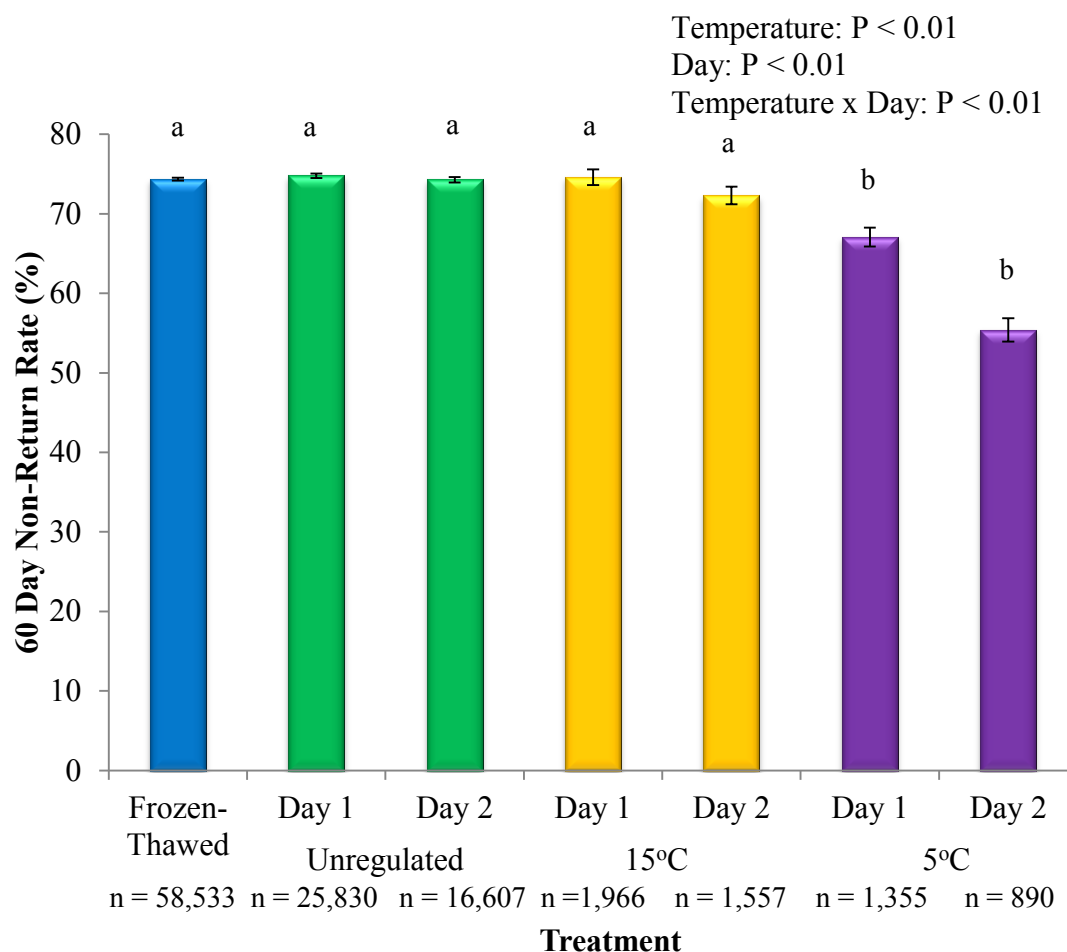


Figure 5.5: The effect of storage temperature and day of storage on 60 Day non-return rate in dairy cows and heifers (Experiment 2). Vertical bars represent 95% confidence intervals. ^{ab}Values with different superscripts differ significantly ($P < 0.01$). n = number of inseminations per treatment per day.

5.4.3.1 Effect of Bull on Non-Return Rate

There was an effect of bull on NRR ($P < 0.01$) with NRR for individual bulls varying from 69.9 to 78.7%. There was a bull by treatment interaction as all bulls had a lower NRR for semen stored in 5 °C compared to all other treatments ($P < 0.01$) with the exception of bulls K and L ($P > 0.05$). A bull by day interaction ($P < 0.01$) was observed, explained by bulls F and H having a higher NRR on Day 1 than liquid

semen inseminated on Day 2 ($P < 0.05$). Bulls C, F, H and K had a higher NRR when frozen-thawed semen was used in comparison to liquid semen on Day 2 ($P < 0.05$) but did not differ to liquid semen inseminated on Day 1 ($P > 0.05$). Bulls E and M had a reduced NRR when frozen-thawed semen was used in comparison to liquid semen inseminated on Day 2 ($P < 0.05$), while bull N had a reduced NRR in frozen-thawed semen compared to liquid semen on Day 1 and Day 2 ($P < 0.01$). There was no effect of semen type (fresh versus frozen-thawed) on NRR; however there was a bull by semen type interaction ($P < 0.01$; Figure 5.6). Bulls A, C, F, K and H had a higher NRR when used as frozen-thawed semen compared to liquid semen ($P < 0.05$), while, bulls E, G, M and N had a higher NRR when used as liquid semen compared to frozen-thawed semen ($P < 0.01$); however, there was no difference in NRR between the remaining bulls ($P > 0.05$).

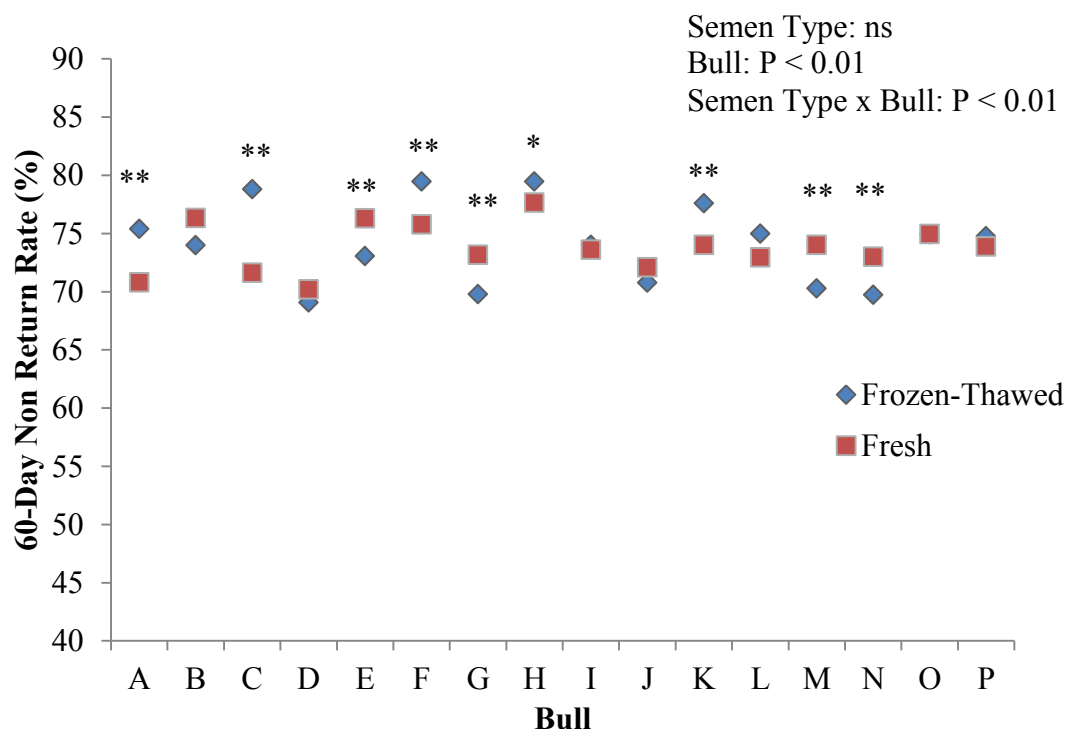


Figure 5.6: The interaction of bull and semen type on 60 Day non-return rate in dairy cows and heifers (Experiment 2). **Asterisk represents differences between semen type within bull ($P < 0.01$). ns = non-significant.

5.4.3.2 Effect of Cow Characteristics on Non-Return Rate

There was an effect of parity, cow fertility sub-index and DIM on NRR ($P < 0.01$). Maiden heifers had a higher NRR (87.2%) than primiparous and multiparous dairy cows (73.6 and 71.8%, respectively; $P < 0.01$). Cows with a fertility sub-index of greater than €70 recorded a higher NRR ($P < 0.01$) in comparison to cows with a fertility sub-index of less than €70 (77.9 vs 73.3%, respectively). There was a linear increase in NRR with increasing DIM ($P < 0.01$). As expected, NRR varied between individual herds and technicians ($P < 0.01$). There was no effect of cow breed, nor was there a breed, parity, cow fertility sub-index, DIM, herd or technician by storage temperature interaction ($P > 0.05$).

5.4.4 Experiment 2b: Effect of Straw Source on Field Fertility of Liquid Semen Diluted in INRA96

There was no effect of straw type on 60-day NRR as semen stored in IMV and Minitube straws had similar NRRs of 72.6 and 72.7%, respectively (Figure 5.7; $P > 0.05$). Overall, insemination with liquid semen on Day 1 post collection resulted in similar NRR (73.8%) in comparison to frozen-thawed semen (74.1%; $P > 0.05$); however, insemination with liquid semen on Day 2 of storage resulted in a lower NRR (70.1%) compared to Day 1 and frozen-thawed semen ($P < 0.01$). There was a treatment by day interaction as semen stored in IMV straws on Day 2 of storage had a reduced NRR compared to frozen-thawed semen ($P < 0.01$); however there was no difference compared to semen stored in Minitube straws on Day 2 ($P > 0.05$). There was an effect of bull on NRR ($P < 0.01$) with NRR for individual bulls varying from 69.4 to 76.8%. There was an effect of parity, cow fertility sub-index and DIM on NRR ($P < 0.01$). Maiden heifers had a higher NRR (86.1%) than primiparous and

multiparous dairy cows (72.3 and 70.3%, respectively; $P < 0.01$). Cows with a fertility sub-index of greater than €70 recorded a higher NRR (78.9%) in comparison to cows with a fertility sub-index of less than €70 ($P < 0.01$). There was a linear increase in NRR with increasing DIM ($P < 0.01$). As expected, NRR varied between individual herds and technicians ($P < 0.01$). There was no effect of breed, nor was there a bull, breed, cow fertility sub-index, DIM, herd or technician by straw source interaction ($P > 0.05$).

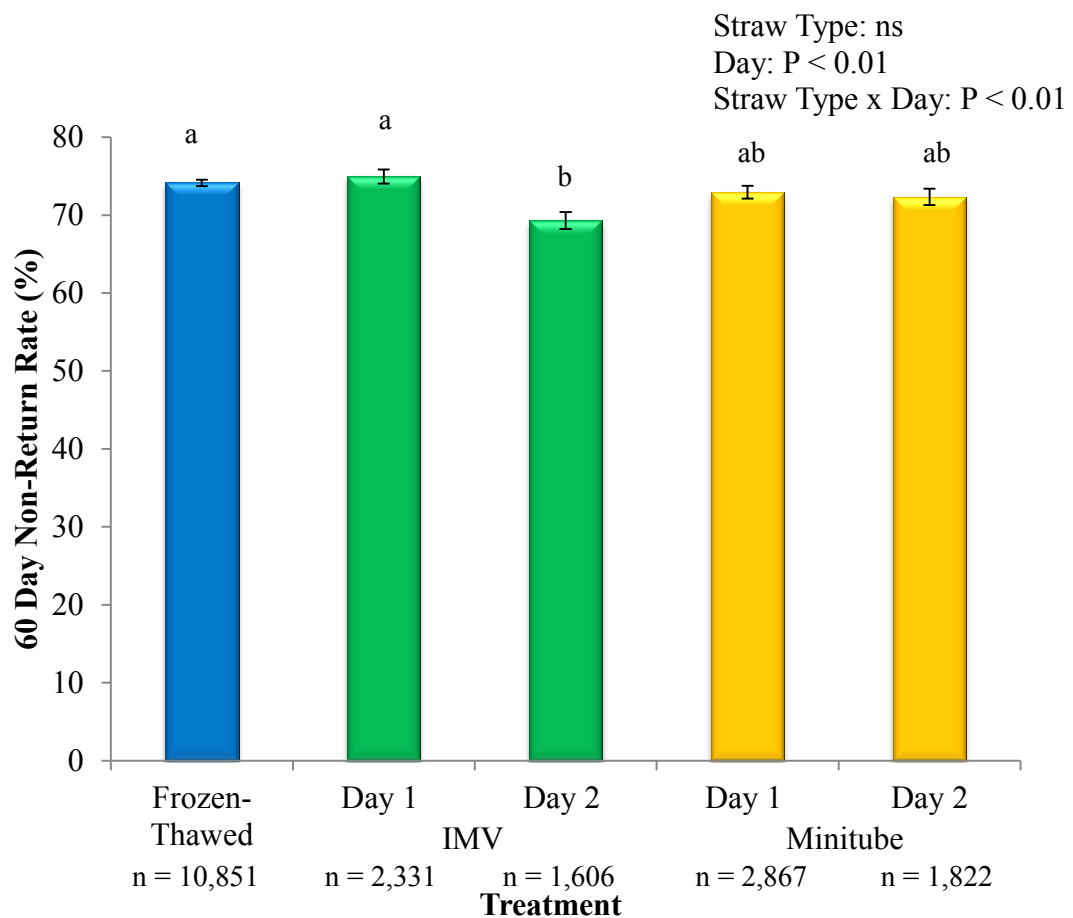


Figure 5.7: The effect of straw source and day of storage on non-return rate in dairy cows and heifers (Experiment 2b). ^{ab}Values with different superscripts differ significantly ($P < 0.01$). n = number of inseminations per treatment per day, ns = non-significant.

5.5 Discussion

This study illustrates the importance of matching the storage conditions to the diluent used. We recently reported that INRA96, a milk-based diluent, could be used as an alternative to the industry standard Caprogen for the storage of liquid bovine semen, with the advantage of being ready to use off-the-shelf. Here, we have taken the approach of using split ejaculates and a combination of *in vitro* and *in vivo* assessments in a comprehensive attempt to identify the optimal semen storage temperature for liquid bovine semen stored in INRA96. The main findings of the study were: (i) semen stored in INRA96 at a constant 15 °C resulted in greater sperm quality than semen stored at 5, 28 or fluctuating between 5 and 28 °C (ii) straw source affected progressive motility but had no effect on total motility or field fertility and (iii) semen stored at a constant 15 °C resulted in greater NRR on Days 1 and 2 of storage in comparison to semen stored at 5 °C but did not differ to liquid semen stored at unregulated temperature or frozen-thawed semen.

Motility assessment constitutes an integral part of semen quality control with the use of CASA systems allowing an objective assessment of sperm motility kinematics (Verstegen et al. 2002). It is widely accepted that regardless of storage temperature, sperm motility and fertility declines over an extended period of time with bull sperm reported to exhibit a gradual decline in motility for up to 4 weeks while there is a sharp decline in NRR after 5 days of semen storage (Vishwanath and Shannon 2000). In agreement, the results of the current study demonstrate that semen quality measured in terms of total and progressive motility declined with increased duration of storage, regardless of storage temperature or straw source. Although straw source had an effect on progressive motility with IMV straws recording a higher progressive

motility score compared to Minitube straws, the difference was not commercially significant and additionally there was no effect of straw source on total motility. A number of studies have reported a correlation between sperm motility kinematics and fertility (Oliveira et al. 2013, Nagy et al. 2015, Kathiravan et al. 2008), however, Amann and Waberski (2014) and Amann et al. (2017) suggest that sperm kinematic characteristics are not an accurate predictor of fertilising potential but instead could be used to provide important information relating to the quality assurance of semen. Surprisingly, in the current study, semen held at 28 °C recorded higher progressive motion values than any other storage temperature, however, storing semen at extreme high temperatures of 28 °C was detrimental to sperm as they exhibited reduced overall motility and velocity values. All other storage temperatures recorded similar kinematic parameters. The results of this study highlight that sperm are quite tolerant to a variation in temperature in terms of sperm quality, retaining acceptable *in vitro* standards between storage temperatures of 5 and 15 °C, while storing semen at a constant 15 °C resulted in the best semen quality throughout the duration of storage. Therefore, it could be postulated that the components of INRA96 interact similarly with semen at different storage temperature conditions. The results of this study support the findings of Murphy et al. (2016) who previously reported semen stored in Caprogen at 15 °C had greater motility compared to semen stored at 5, 22, 32 °C or fluctuating temperatures between 5 and 15, 5 and 22 and 5 and 32 °C.

Although milk-based extenders are more widely used at storage conditions of between 4-8 °C, INRA96 has also been shown to be beneficial in the preservation of equine sperm stored at 15 °C. The results of the current study demonstrate that bovine semen diluted in INRA96 resulted in greater NRR on Day 1 and Day 2 of

storage when semen was stored at a constant 15 °C or unregulated temperature compared to storage at 5 °C. Furthermore, INRA96 was effective in protecting sperm from temperature fluctuations under unregulated field conditions and supports the *in vitro* findings of Murphy et al. (2017). The current results are similar to the *in vitro* findings of Batellier et al. (1997) and the fertility findings of Cuervo-Arango et al. (2014) and Batellier et al. (2001) who reported better *in vitro* survival and fertility of equine sperm stored in INRA96 at 15 compared to 4 °C, respectively. Surprisingly, in the current study, semen stored at a constant 5 °C also had a reduced NRR on both Day 1 and Day 2 of storage compared to semen stored at unregulated temperature and frozen-thawed semen. All bulls, with the exception of two (Bulls K and L), performed relatively poorly when semen was stored at a constant 5 °C compared to any other storage conditions. A possible explanation for the poor fertility performance of liquid semen stored at 5 °C may be due to the inability of sperm from these bulls to adapt to the lower storage temperature, increasing the incidence of cold shock injuries, which could result in a decline in sperm membrane integrity due to a loss of phospholipids, thus, causing membrane impairment and a reduction in fertility (Batellier et al. 2001). However, no evidence of cold shock injuries were observed when assessing samples *in vitro*. In addition, the *in vitro* results of the current study highlight that fluctuating storage conditions between 5 and 28 °C resulted in a significant loss of sperm motility. Therefore, it could be postulated that exposure to such daytime/night-time temperature fluctuations typically observed in the field could result in a decline in membrane integrity as a consequence of membrane changes, consistent with the lipid phase transition (Drobnis et al. 1993). However, the fertility results of the current study do not support this notion as

storage at a constant 5 °C was more detrimental to NRR than unregulated temperature storage conditions.

In the current study, semen type (liquid versus frozen-thawed) was found not to affect NRR or to negate the effects observed of cow characteristics. The use of liquid semen has many advantages in that it promotes and maximises the utilisation of genetically superior sires, due to the reduced sperm concentration per straw and therefore generates a greater number of straws per ejaculate compared with frozen-thawed semen. This facilitates the acceleration of genetic gain through more intensive sire utilisation and provides a distinct advantage to AI centres, particularly in relation to young genomically-selected superior sires, as the advent of genomics has placed additional pressure on AI centres to better utilise this valuable semen. While young sires are now in high demand they produce lower semen volumes compared to their mature counterparts (Brito et al. 2002), thus, the use of liquid semen provides a significant advantage to AI centres as semen production can be maximised. However, it is widely acknowledged that fertility from individual bulls varies by ~20-28%, despite semen meeting minimum routine quality control standards (Kastelic and Thundathil 2008, Holden et al. 2017). The results of the current study also highlights that bull variation exists between semen type with some bulls having a higher NRR when used for liquid versus frozen-thawed semen or vice versa. Anzar et al. (2002) reported that the number of apoptotic cells in liquid semen differs between bulls, while Murphy et al. (2016) and Murphy et al. (2017) previously reported that sperm of some bulls are more susceptible to sperm aging effects when stored in liquid semen. The production of ROS, which ultimately leads to an apoptotic cascade in which sperm lose their motility, DNA integrity and

vitality (Aitken et al. 2012), may be linked to the aging effect leading to a reduction in fertility. The results of the current study would agree with this sperm aging affect as although only 3 bulls had a significant decline in NRR on Day 2 of storage of liquid semen, 62.5% of bulls had a numerical decline in NRR on Day 2 of storage compared to Day 1 of storage. Thus, indicating that some bulls are better able to maintain semen longevity in terms of prolonged storage days without a drop in fertility. Furthermore, while it is widely accepted that cow characteristics such as parity, fertility sub-index and DIM play a role in fertility (Murphy et al. 2016, Murphy et al. 2017), storage temperature does not nullify the effects of these as no interaction between storage temperature and cow characteristics were observed. Consistent with previous reports (Gabriel et al. 2011, Pursley et al. 1997), maiden heifers had a significantly higher NRR compared to primiparous and multiparous dairy cows with an increase in NRR of ~14% and 15%, respectively.

In conclusion, bovine semen held at a constant 15 °C had the highest total and progressive motility score over the duration of storage; however, the results also highlight that sperm are quite tolerant to variation in storage temperature and can retain acceptable motility between temperatures of 5 and 15 °C. Semen held at a constant 15 °C resulted in similar NRR to semen stored in unregulated storage conditions but NRR was significantly reduced at storage of 5 °C. In climatic conditions where there is large day to night time temperature fluctuations, a stricter temperature regulation regimen should be put in place for liquid semen with a storage temperature of 15 °C being most desirable. Nevertheless, in circumstances or field conditions where maintaining a constant temperature is not possible, unregulated storage conditions attain acceptable fertility however; provisions should

be put in place to avoid exposure of liquid semen to extreme temperatures. Furthermore, coloured Minitube liquid semen straws are a suitable alternative to the standard clear IMV liquid semen straws.

Chapter Six

Effect of Increasing Equilibration Time of Diluted Bull Semen up to 72 Hours Prior to Freezing on Sperm Quality Parameters and Calving Rates Following Artificial Insemination

Abstract

An equilibration period of approximately 3-4 h prior to semen cryopreservation is standard practice for maintaining membrane integrity and motility of bull sperm. However, a number of studies indicate that an overnight equilibration period prior to freezing results in improved post-thaw semen quality thus optimising pregnancy rates. The aim of this study was to assess the effect of increasing the equilibration time of bull semen up to 72 h before freezing on sperm quality parameters and CR following AI with frozen-thawed semen. The effect of holding semen at 4 °C for 6, 24, 48 or 72 h post dilution before freezing on subsequent post-thaw total and progressive motility (Experiment 1) and field fertility (n = 1,640 inseminations, Experiment 2) of frozen-thawed semen was assessed. Equilibration time did not affect post-thaw total and progressive motility ($P > 0.05$). In addition, there was no effect ($P > 0.05$) of equilibration time on field fertility with a CR of 53.3, 50.5, 51.3 and 47.3 for the 6, 24, 48 and 72 h treatments, respectively. In conclusion, increasing the equilibration time of diluted bull semen from 6 - 72 h had no significant effect on CR, within the expected range of fertility outcomes, thus providing semen processing centres with flexibility in the time which semen can be held prior to freezing.

6.1 Introduction

The use of AI facilitates the rapid dissemination of genetic material from a relatively small number of superior sires to a large number of females (Vishwanath and Shannon 1996). Thus, AI is considered to be the single most important technique devised to facilitate the genetic improvement of animals (Howley et al. 2012). The widespread use of frozen-thawed semen in cattle is partly attributed to the

development of suitable cryopreservation protocols and a number of investigative avenues have been undertaken in order to improve the freeze-thaw process (Holt 2000, Vishwanath and Shannon 2000). Bull semen used for cryopreservation is typically diluted in an egg yolk-based extender as egg yolk is known to be one of the best cryoprotectant components for the preservation of post-thaw sperm function and subsequent fertility (Vishwanath and Shannon 2000). The components within egg-yolk, in particular LDLs, bind to the sperm membrane during the freeze-thaw process, increasing chilling tolerance and preventing loss of membrane phospholipids (Muiño et al. 2007). Typically, extenders used for the cryopreservation of bull semen differ in composition from fresh semen extenders as greater emphasis is placed on stabilising the cell membrane. The addition of cryoprotectants, which are classified as either penetrating (glycerol; 3-6%) or non-penetrating (egg yolk; containing LDLs and cholesterol; (Purdy 2006), minimises the physical and chemical stresses associated with cryopreservation, thus reducing membrane damage during freezing (Bathgate et al. 2006). As the name suggests, a non-penetrating cryoprotectant, such as egg-yolk, cannot cross the sperm membrane and thus only acts extracellularly to modify the sperm membrane (Amann 1999). On the other hand, penetrating cryoprotectants, the most common of which is glycerol, are membrane permeable and so act both intra- and extracellularly causing dehydration of the sperm and membrane lipid and protein rearrangement resulting in increased membrane fluidity and a decrease in the freezing point of the cell (Holt 2000).

Semen cryopreservation involves several steps including cooling, equilibration, freezing and subsequent thawing (Ahmad et al. 2015). Typically, freezing protocols for bull semen include cooling to 4 - 5 °C followed by a variable duration of equilibration (0 - 24 h) at this temperature prior to freezing (Leite et al. 2010). Equilibration allows sperm to adapt to cooler temperatures (Muiño et al. 2007), facilitates the movement of the cryoprotectant across the cell membrane (in the case of penetrating cryoprotectants) and enables the movement of water out of the cell, thus minimizing damage sustained by ice crystal formation during the freeze-thaw process (Muiño et al. 2007). Equilibration time was first believed to be important in allowing glycerol sufficient time to penetrate the sperm membrane (Leite et al. 2010). However, Berndtson and Foote (1972) reported that glycerol penetration in bull sperm is rapid, taking no more than 5 min; therefore, it is now suggested that a period of equilibration is necessary to allow sperm membranes sufficient time to adapt to cooler temperatures (Muiño et al. 2007). Studies aimed at identifying the optimum equilibration time have been conducted on semen from a number of species including sheep (Câmara et al. 2011), goats (Deka and Rao 1986) and cattle (Fleisch et al. 2017). Although the majority of cryopreservation protocols for bovine semen involve an equilibration period of 4 h, a wide range of equilibration times have been reported: 0 h (Leite et al. 2010), 1- 4 h (Arifiantini and Yusuf 2010), 18 - 24 h (Foote and Kaproth 2002) and 24 - 72 h (Fleisch et al. 2017, Crespilho et al. 2014). Increasing equilibration time from 8 to 18 h (Michel et al. 2016) or from 4 to 72 h (Fleisch et al. 2017) increased the quality of bull sperm motility and viability, and did not affect field fertility.

Implementing a shorter equilibration time such as 4 h can result in processing difficulties within a commercial AI centre, as semen has to be frozen on the same day of collection. A number of studies have found that using an equilibration time of 24 h resulted in an increase in semen quality (Fleisch et al. 2017, Foote and Kaproth 2002) and therefore may be more convenient for the working schedule in AI centres which involves the collection of semen from a large number of bulls on a daily basis or where semen has to be transported over long distances to a central processing laboratory. In particular, to provide flexibility over weekends, it would be useful if semen collected on a Friday or Saturday could be processed the following Monday. Therefore, the aim of this study was to assess the effects of increasing the equilibration time up to 72 h post dilution on sperm motility *in vitro* and on *in vivo* fertility following artificial insemination.

6.2 Materials and Methods

6.2.1 Experiment 1: The Effect of Equilibration Time on the *In Vitro* Quality of Frozen-Thawed Semen

The aim of this experiment was to assess the effects of holding time (6, 24, 48 and 72 h post dilution) on total and progressive motility of bull sperm pre-freeze and post-thawing. Semen was collected from Holstein Friesian bulls (n = 8) via artificial vagina at a commercial AI centre (National Cattle Breeding Centre, Enfield, Co Meath, Ireland). The raw ejaculate was placed into a 15 mL tube and was partially diluted immediately after collection in 10 mL pre-warmed BullXcell (37 °C; IMV Technologies, Normandy, France) by slowly pouring the extender into the 15 mL tube containing the raw ejaculate. Typically this dilution rate was 2:1 diluent to raw

ejaculate ratio. The samples were transported in a temperature-regulated cooler box at 18 °C to the laboratory (within 3 h). Upon arrival, the ejaculate was assessed for sperm concentration using a coulter counter (Z Series, Beckman Coulter, Clare, Ireland), as well as an initial score of total motility (%) and gross motility on a 5-point subjective scale (1 = twitching/no forward progressive motility; 5 = excellent forward progressive motile sperm) to ensure all semen samples were of a commercial standard (results not shown). Initial quality control cut-off values were a total and gross motility of <70% and a score of <3, respectively, and any ejaculates failing to meet these criteria were rejected. Three ejaculates (replicates) were assessed per bull and semen from each ejaculate was kept separate throughout processing.

Following initial *in vitro* assessment, the ejaculate was fully extended in BullXcell, based on initial sperm concentration, to achieve a concentration of 15×10^6 sperm per 0.25 mL insemination dose. Semen straws (IMV Technologies) were filled as per routine procedures using the IS4 machine (IMV Technologies), placed in an insulated box (to slow the temperature drop) and stored in a fridge at 4 °C. Straws from each ejaculate (n = 20 straws per equilibration time point) were frozen at 6, 24, 48 or 72 h post dilution as follows: -5 °C per min from +4 °C to -10 °C, -40 °C per min from -10 °C to -100 °C and thereafter -20 °C per min from -100 °C to -140 °C (Murphy et al. 2017) in a programmable freezer (Digitcool, IMV Technologies), followed by submersion and storage in liquid nitrogen at -196 °C until use. Each ejaculate was split so that each bull was represented in each treatment in each replicate. Samples from the different treatments were assessed in a randomised sequence to remove bias as a result of sampling order. The evaluator was blinded to

the treatment. Total and progressive motility was assessed *in vitro* just prior to freezing at 6, 24, 48 and 72 h post dilution as well as immediately post-thawing via standard phase-contrast microscopy (described below) and CASA (Hamilton Thorne IVOSII, IMV Technologies). In addition, sperm viability and acrosomal integrity were assessed using flow cytometry. Within each replicate, at each assessment time (both pre- and post-freezing), five straws from each bull for each of the equilibration times were assessed.

6.2.1.1 Standard Microscopic Analysis of Frozen-Thawed Semen Frozen at 6, 24, 48 and 72 h post Dilution

Sperm motility (total and progressive) was assessed subjectively pre-freeze and post-thaw using a phase contrast microscope (CX31; Olympus, Centre Valley, PA, USA) at a magnification of 400 X. Frozen straws were thawed at 35 °C for 30 sec. Each straw was dried fully, to remove any excess water, cut at the sealed end and separately placed into a pre-warmed eppendorf. The plug end of each straw was then cut to expel the contents of the straw into the eppendorf and the semen sample was mixed thoroughly to ensure homogeneity. A droplet of diluted semen (5 µL) was placed on a pre-warmed glass slide, covered with a pre-warmed coverslip (37 °C) and assessed by counting a minimum of 100 sperm, over at least five different fields of view, for each treatment. Total motility was expressed as a percentage of the total sperm population (motile and non-motile). A sperm was deemed to display progressive motility if it moved in a linear fashion (ie; movement of sperm in a straight or curvilinear motion); progressive motility was expressed as the percentage of live and progressively motile sperm in the total sperm population.

6.2.1.2 Computer Assisted Sperm Analysis of Frozen-Thawed Semen Frozen at 6, 24, 48 and 72 h post Dilution

Motility of frozen-thawed sperm samples was assessed using the IVOS-II CASA system driven by software version 14 (Hamilton Thorne Inc, Beverly, USA). Straws (n = 5 per ejaculate) were thawed at 37 °C for 30 sec and a drop (3 µL) of diluted semen was placed in a pre-warmed chamber (37 °C; Leja counting chambers, depth 20 µm; Microptics, Barcelona, Spain) and analysed for sperm motion and kinematic characteristics immediately post-thaw. A minimum of 1000 sperm were analysed in at least eight microscopic fields with 30 frames acquired per field at a frame rate of 60 Hz. Objects incorrectly identified as sperm were edited out using the playback function. The CASA-derived motility and kinematic characteristics assessed were total motility (%), progressive motility (%), proximal and distal droplets (%), as well as VAP above 10 µm/s, VSL, VCL, LIN, STR, ALH and BCF (Mortimer 2000). Regarding analysis settings, the CASA was set to standard factory settings for bull semen; sperm with straightness of >80% and VAP >50 µm/s were considered progressively motile.

6.2.1.3 Flow Cytometric Analysis of Frozen-Thawed Semen Frozen at 6, 24, 48 and 72 h post Dilution

Before flow cytometric analysis, semen samples were diluted to a final working concentration of 300×10^5 sperm/mL in Beltsville Thawing Solution (BTS; 37 °C). Samples were analysed on a flow cytometer (Guava easyCyte 6HT-2L, Merck Millipore, Billerica, MA, USA) equipped with both a Krypton (642 nm) and an Argon laser (488 nm). Appropriate single colour controls were prepared to establish the respective fluorescent peaks of the individual stains. These were used in

conjunction with the forward scatter and side scatter signals to discriminate sperm from debris (P0.1 Population). Fluorescent events were recorded using GuavaSoft (Version 2.7; Merck Millipore) and all variables were assessed using logarithmic amplification. In each sample, a minimum of 10,000 gated events were captured.

5.2.1.3.1 Assessment of Viability

Viability was assessed using fluorescent probes. SYTO 16 (Ex/Em: 488/518; Life Technologies, Carlsbad, CA USA) is a cell-permeant probe which fluoresces upon binding to nucleic acids and propidium iodide (PI; Ex/Em: 535/617; Life Technologies) is selectively taken up by membrane-compromised cells, thus indicating a loss of viability (Murphy et al. 2016). SYTO 16 (5 μ L) was added to diluted sperm (300×10^5 sperm/mL) at a final concentration of 100 nM and incubated at 37 °C in the dark for 10 min. Subsequently, PI was added at a final concentration of 15 μ M and incubated for a further 5 min. SYTO 16 emission was detected via the Green photomultiplier (PMT; 525/30 nm BP filter) and PI was read with the Red1 PMT (690/50 nm BP filter); no compensation was required. Viability was defined as the percentage of sperm positive for SYTO 16 but negative for PI and all percentages were calculated as part of the total gated sample, P0.1 Population.

5.2.1.3.2 Assessment of Acrosomal Integrity

Acrosomal integrity was assessed by incubating sperm with SYTO 16 and PI, as described above, followed by incubation with Alexa Fluor 647 (AF647; Ex/Em: 650/668; Life Technologies) to identify live sperm which had undergone the acrosome reaction (Murphy et al. 2016). Acrosomal integrity can be verified cytometrically using glycoprotein markers such as lectin (Graham et al. 1990).

Pisum sativum agglutinin (PSA) is an agglutinin which can bind to glycol-conjugates from the acrosome and has an affinity for terminal residues of glycoproteins, binding specifically to α -mannosidase (Cross et al. 1986). PSA is routinely tagged to either FITC or Alexa Fluor probes and fluoresces upon contact with acrosome reacted sperm (Casey et al. 1993). Alexa Fluor 647 (3 μ L) was added to diluted sperm (300 x 10⁵ sperm/mL) to a final concentration of 4.6 μ M and incubated in the dark for 15 min at 37 °C. AF647 positive events were read on the Red2 PMT (661/19 nm BP filter) and no compensation was required. The percentage of acrosome-intact sperm in the live population was expressed as a percentage of the sperm negative for Alexa Fluor 647 and positive for SYTO 16 as part of the total gated sample, P0.1 Population.

6.2.2 Experiment 2: The Effect of Equilibration Time on Field Fertility of Frozen-Thawed Semen

The aim of this experiment was to assess the effect of equilibration time of frozen-thawed semen (15 x 10⁶ sperm per 0.25 mL insemination dose) on CR following AI. Semen was collected from Holstein Friesian bulls (n = 5) at a commercial AI centre from early February to the end of March 2015. There were 7 collection days in total, with all five bulls collected on each collection day (total of 35 ejaculates). Following initial assessment for volume, concentration and motility (as described in Experiment 1), each acceptable ejaculate was diluted and filled in straws which were then printed and sealed as per Experiment 1. Straws were stored for 6, 24, 48 or 72 h prior to freezing. Each batch of semen was clearly labelled, assessed post-thaw to ensure samples were of a commercial standard, and distributed for insemination after 30 days of quarantine as per European regulations (Irish Statute Book, 2004).

Inseminations were carried out in May 2016 (coinciding with the peak dairy breeding season) in Irish dairy herds ($n = 284$). The majority of inseminations were in Holstein Friesian animals ($n = 1,582$) but a small number of other breeds were represented including Jersey ($n = 20$), Montbeliarde ($n = 12$), Norwegian Red ($n = 9$) and Others ($n = 17$; includes Ayrshire and Shorthorn). Technicians ($n = 24$) were blind to treatments and received equal numbers of straws from each of the four equilibration times from each bull. For each insemination, the technician recorded the bull code, cow tag number and the straw code on an electronic handheld device. Inseminations and CR data were captured using the Irish Cattle Breeding Federation (ICBF; Bandon, Co. Cork, Ireland) database by cross-referencing the technician name with the bull code and semen type used on each date within the trial period. Obvious errors were extracted from the dataset and data were then interrogated to remove animals ($n = 382$) based on the following criteria: cows which were not at first AI, cows which received two inseminations from two different bulls or equilibration time treatments, or cows which were not of a dairy breed. However, if a dairy cow received two inseminations from the same bull with the same time treatment within five days of each other, the record was kept and the second date was assumed to be correct. Post editing, a total of 1,640 inseminations (1,537 cows and 103 heifers) remained. Calving rate was measured using a cut-off value of 275 and 290 days from date of insemination to calving date (Murphy et al. 2016)

Cow characteristics such as parity, DIM and fertility sub-index were also assessed. Fertility sub index is a key component of the EBI comprising ~35% of the total EBI (ICBF 2017a). The EBI is an estimate of the economic value of an animal's genetic merit. It was established to combat a decline in reproductive performance by

providing farmers with a profit index enabling the selection of elite sires to breed replacement heifers with increased milk yield, reproductive performance and improved health traits (Berry et al. 2005).

6.3 Statistical Analysis

Data from Experiment 1 were examined for normality of distribution, homogeneity of variance and analysed using the GLM repeated-measures procedure with a compound symmetry covariance structure in Statistical Package for the Social Sciences (SPSS, Version 22.0; IBM, Chicago, USA). In Experiment 2, CR data were compared using Pearson's chi-squared procedures in SPSS. The dependent variable in the analysis was CR (1 = calved, 0 = not calved). In addition, a GLM for binomial data was used to assess a number of fixed effects on CR, including equilibration time, bull, parity, breed, fertility sub-index, DIM, herd and technician. Each fixed effect was assessed for an interaction with equilibration time treatment. All post-hoc tests were carried out using the Bonferroni test. Results are reported as the mean \pm the sem in Experiment 1 and as the estimated marginal means in Experiment 2, to adjust for imbalance between numbers of inseminations in each treatment. Values were considered to differ significantly at $P < 0.05$.

6.4 Results

6.4.1 Experiment 1: The Effect of Equilibration Time on the *In Vitro* Quality of Frozen-Thawed Semen

Equilibration time had no effect on pre-freeze total and progressive motility ($P > 0.05$) as subjectively assessed via standard microscopic techniques or objectively using CASA (Table 6.1). There was an effect of equilibration time on viability ($P < 0.01$) and acrosomal integrity ($P < 0.01$; Table 6.1). The percentage of live sperm post-thaw increased from 6 to 72 h ($P < 0.01$) while the percentage of live sperm with intact acrosomes post-thawing declined in the 72 h equilibration treatment ($P < 0.01$; Table 6.1). Semen equilibrated for 72 h exhibited inferior kinematic motility parameters compared to an equilibration time of 6 and 24 h ($P < 0.01$) with the exception of ALH and VCL ($P > 0.05$; Table 6.1). All CASA motility characteristics, with the exception of ALH and VCL deteriorated when equilibration time was increased from 6 to 72 h (106.3 ± 3.56 versus $97.3 \pm 1.16 \mu\text{m/s}^{-1}$, 31.1 ± 0.77 versus 24.9 ± 0.26 Hz, 41.6 ± 0.45 versus $35.4 \pm 0.83\%$, 76.6 ± 0.98 versus $70.2 \pm 1.14\%$, 81.7 ± 3.28 versus $67.9 \pm 0.72 \mu\text{m/s}^{-1}$ and 53.3 ± 0.48 versus $49.6 \pm 0.39\%$ for VAP ($P < 0.05$), BCF, LIN, STR, VSL and WOB, respectively; $P < 0.01$).

Table 6.1: The effect of equilibration time on viability and acrosomal integrity as assessed via flow cytometry as well as computer assisted sperm analyser post-thaw total and progressive motility and kinematic parameters in bull semen extended for 6, 24, 48 and 72 h post dilution prior to freezing (Experiment 1). ^{abc}Values with different superscripts differ significantly within row ($P < 0.01$; values are mean \pm sem).

Parameters	Equilibration Time (h)				P value
	mean \pm sem				
	6	24	48	72	Effect of Treatment
Viability (%)	53.4 \pm 2.76 ^a	60.5 \pm 2.16 ^{ab}	61.3 \pm 3.72 ^b	61.3 \pm 2.06 ^b	P < 0.01
Acrosomal Integrity (%)	92.3 \pm 0.57 ^a	93.1 \pm 0.54 ^a	90.6 \pm 0.63 ^{ab}	89.7 \pm 0.89 ^b	P < 0.01
Total Motility (%)	44.4 \pm 2.64	53.7 \pm 2.30	50.1 \pm 3.16	48.9 \pm 2.80	ns
Progressive Motility (%)	34.7 \pm 1.80	42.8 \pm 1.90	38.0 \pm 2.47	36.4 \pm 2.12	ns
ALH (μm)	8.7 \pm 0.19 ^a	9.3 \pm 0.18 ^{ab}	9.7 \pm 0.21 ^b	9.8 \pm 0.16 ^b	P < 0.01
BCF (Hz)	31.1 \pm 0.77 ^a	28.9 \pm 0.73 ^a	26.0 \pm 0.46 ^b	24.9 \pm 0.26 ^b	P < 0.01
LIN (%)	41.6 \pm 0.45 ^a	39.4 \pm 0.79 ^{ab}	36.5 \pm 0.95 ^{bc}	35.4 \pm 0.83 ^c	P < 0.01
STR (%)	76.6 \pm 0.98 ^a	75.6 \pm 1.03 ^{ab}	71.5 \pm 1.18 ^{bc}	70.2 \pm 1.14 ^c	P < 0.01
VAP ($\mu\text{m}/\text{s}^{-1}$)	106.3 \pm 3.56 ^a	105.9 \pm 0.94 ^a	101.7 \pm 0.91 ^{ab}	97.3 \pm 1.16 ^b	P < 0.05
VCL ($\mu\text{m}/\text{s}^{-1}$)	202.3 \pm 7.04	209.4 \pm 1.17	207.0 \pm 3.13	201.9 \pm 3.17	ns
VSL ($\mu\text{m}/\text{s}^{-1}$)	81.7 \pm 3.28 ^a	80.5 \pm 1.57 ^{ab}	72.5 \pm 1.11 ^{bc}	67.9 \pm 0.72 ^c	P < 0.01
WOB (%)	53.3 \pm 0.48 ^a	51.3 \pm 0.46 ^{ab}	50.2 \pm 0.62 ^b	49.6 \pm 0.39 ^b	P < 0.01
Proximal Droplets (%)	3.8 \pm 0.97	3.8 \pm 1.04	4.1 \pm 1.22	3.9 \pm 1.35	ns
Distal Droplets (%)	4.6 \pm 0.32	3.9 \pm 0.36	4.4 \pm 0.38	4.4 \pm 0.33	ns

ALH = amplitude of lateral head displacement, BCF = beat cross frequency, LIN = linearity, STR = straightness, VAP = average path velocity, VCL = curvilinear velocity, VSL = straight line velocity, WOB = wobble, ns = non-significant.

6.4.2 Experiment 2: The Effect of Equilibration Time on Field Fertility of Frozen-Thawed Semen

While CR declined numerically with increased equilibration time (53.3, 50.5, 51.3 and 48.3% for 6, 24, 48 and 72 h, respectively), the difference between treatments was not significantly different ($P > 0.05$; Figure 6.1). There was no bull, parity, cow fertility sub-index, DIM, herd or technician by treatment interaction on CR ($P > 0.05$). CR varied from 42.7 to 56.8% for individual bulls ($P < 0.01$). There was a positive linear increase in CR with increasing cow fertility sub-index and DIM ($P < 0.01$). Cows with a fertility sub-index greater than €90 had a higher CR in comparison with cows with a sub-index of less than €90 ($P < 0.05$). Cows which were less than 40 DIM had a reduced CR (27.8%) in comparison to cows which were greater than 40 days in milk prior to insemination. Maiden heifers had a numerically higher CR (59.2%) than primiparous (52.5%) and multiparous cows (49.4%) but this was not significant ($P > 0.05$). For herds and technicians with greater than 15 and 20 recorded inseminations, CR varied between individual herds and technicians ($P < 0.01$).

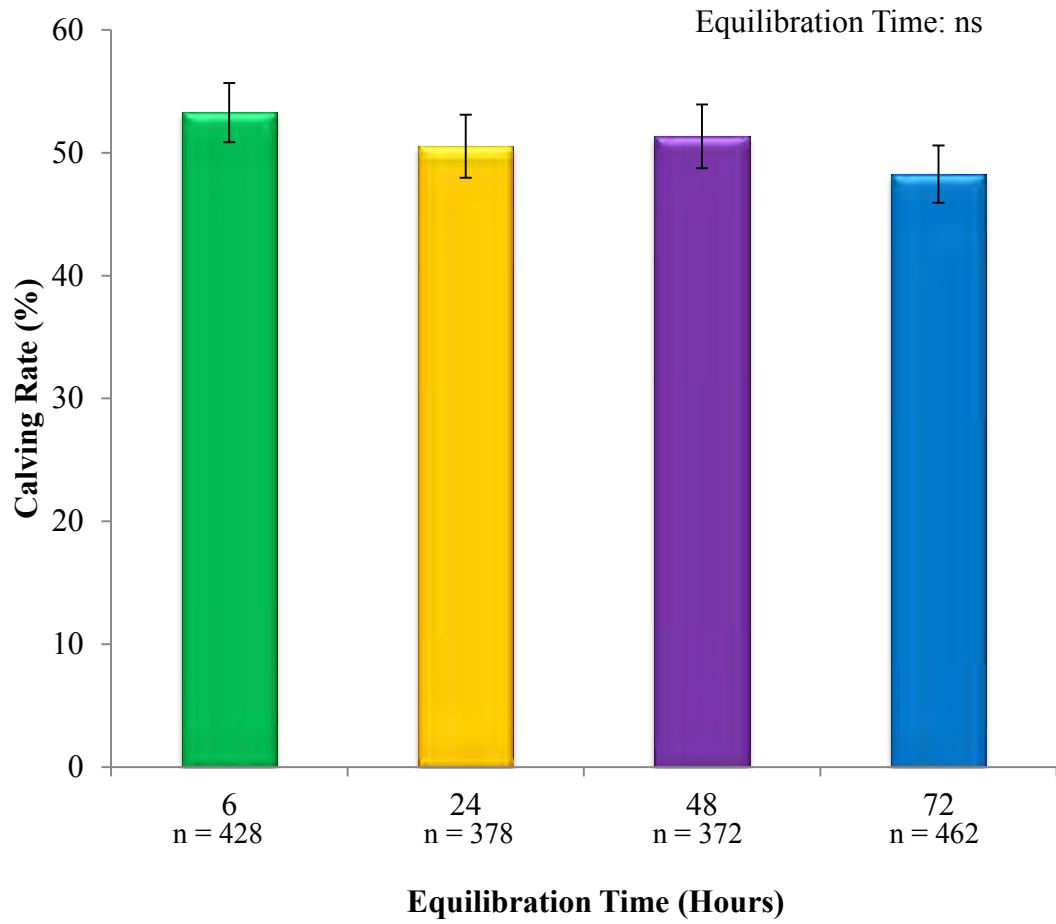


Figure 6.1: The effect of equilibration time on calving rate following AI of bull semen extended for 6, 24, 48 and 72 h post dilution prior to freezing (Experiment 2) including expected fertility outcomes for each treatment group.

6.5 Discussion

The main findings of this study were that: (i) increasing equilibration time from 6 to 72 h does not detrimentally affect sperm quality *in vitro* with 24 h equilibration resulting in the highest numerical post-thaw total and progressive motility scores, (ii) although there was a numerical decline in CR with increased equilibration time, equilibration up to 72 h did not significantly affect CR, (iii) cows with a higher fertility sub-index had a higher CR than those with a lower sub-index, and (iv) cows

with a greater number of DIM (>40 days) at AI, had a higher CR than those which were inseminated closer to their calving date (<40 days).

Motility is one of the most important characteristics associated with fertilising potential of a sperm as it is indicative of sperm viability and structural integrity (Nagy et al. 2015). Therefore, motility assessment constitutes an integral part of semen quality analysis with the use of CASA systems allowing an objective accurate assessment of sperm motility kinematics (Verstegen et al. 2002). A number of studies have correlated motility kinematics of bull sperm with field fertility (Nagy et al. 2015, Kathiravan et al. 2008, Amann 1989, Budworth et al. 1988); however, many of these studies are conflicting in terms of the relative importance of different motility characteristics. Oliveira et al. (2013) reported a correlation between a combination of CASA parameters and bull fertility such as total and progressive motility, ALH and BCF, based on 191 inseminations. In contrast, Amann and Waberski (2014) suggested that sperm kinematic characteristics are not an accurate predictor of fertilising potential but instead could be used to provide important information relating to the quality assurance of semen. This is supported by Oliveira et al. (2012) who concluded that while there was no effect of sperm characteristics on field fertility, sires producing lower semen quality had lower pregnancy rates. In the current study, semen quality initially increased with increasing equilibration time from 6 to 24 h but declined gradually thereafter *in vitro* up to 72 h. Although not significant, post-thaw total and progressive motility scores were higher for 24 h compared with 6, 48 and 72 h. All CASA motility parameters, with the exception of ALH and VCL, deteriorated significantly with increasing equilibration time from 6 to 72 h. Furthermore, the percentage of viable sperm increased when equilibration

time was increased from 6 to 24 h. Thus, this study highlights the importance and beneficial effect of prolonging equilibration of semen at 4 °C before freezing up to 24 h in order to attain optimal post-thaw semen quality. This is in agreement with Fleisch et al. (2017) and Rickenbacher (2009) who reported higher semen quality values after 24 h equilibration of bovine semen compared to their retrospective shorter equilibration time of 4 and 1.5 h, respectively. Similarly, Foote and Kaproth (2002) and Anzar et al. (2011) achieved greater motility of bovine semen after 18 and 24 h in comparison to equilibration time of 2 and 4 h, respectively.

Generally, most bull semen freezing protocols involve an equilibration period of 3 - 4 h, resulting in semen being frozen on the day of collection. However, an extensive review of bovine fertility trials by Pickett and Berndtson (1978) established that a prolonged period of equilibration of 18 h was required in order to obtain maximum fertility. In AI centres, where daily collection schedules involve the collection of a large number of bulls and/or where semen is being transported over long distances for processing, a prolonged period of equilibration (up to 24 h) would be more convenient for the working operations of the centre as semen could be frozen the subsequent day or even after a weekend. The results of the current study indicate that increasing equilibration time from 6 to 72 h has no effect on CR. This finding is supported by Fleisch et al. (2017) who reported that increasing equilibration time from 4 to 72 h had no effect on 90-day NRR, while no difference in 56-day NRR was reported when bull semen was equilibrated between 4 and 28 h (Foote and Kaproth 2002). This indicates that semen frozen on the day following collection should be of better quality and yield comparable fertility compared to semen frozen

on the day of collection, thus, creating greater flexibility within the working environment of a commercial AI centre.

It is widely acknowledged that cow characteristics such as fertility sub-index and DIM play a role in fertility (Amann 1989, Murphy et al. 2017). The current study, followed the same trend, whereby, cows with a greater fertility sub-index (≥ 110) and DIM (> 80 days) had a higher CR than cows with lower fertility sub-indices and less DIM. Animals in the lowest categories for both characteristics (< 50 and < 40 DIM, respectively) had lower CR than animals of greater than ≥ 90 and ≥ 40 DIM, respectively. While this study demonstrates that a higher CR can be achieved through increasing the number of DIM before insemination, it also illustrates that late calving cows can be inseminated with reasonable success.

In conclusion, increasing equilibration time up to 72 h post collection, whilst resulting in a numerical decline, did not significantly affect field fertility within the expected range of fertility outcomes. Furthermore, an equilibration period of 24 h resulted in numerically greater post-thaw total and progressive motility scores. Therefore, implementing an equilibration period of 24 h may be more suitable to the working environment and collection schedules of AI centres, while confidence can also be gained knowing that in circumstances where it is required (e.g., mechanical failure, avoidance of weekends), allowing an equilibration time of 3 days will not negatively impact on field fertility.

Chapter Seven

Comparison of Plant- and Egg Yolk-
Based Semen Diluents on the *In Vitro*
Sperm Kinematics and *In Vivo* Fertility of
Frozen-Thawed Bovine Semen

Abstract

The use of extenders with components of animal origin for the dilution of bovine semen poses a potential risk of microbial contamination, leading to increased health concerns over their use in semen diluents. Alternative diluents using components of plant origin have been developed but it is unclear if they have the same level of *in vivo* fertility as traditional egg yolk-based diluents. The aim of this study was to assess the effect of semen diluent on 60-day NRR following AI with frozen-thawed bull semen. The effect of semen dilution in 1 of 3 different commercial diluents (BullXcell – egg yolk-based, OptiXcell – plant-based or AndroMed – plant-based) on post-thaw total and progressive motility as well as kinematic parameters (Experiment 1) and field fertility (Experiment 2, n = 1,480 inseminations) was assessed. Semen stored in OptiXcell had higher post-thaw total and progressive motility than AndroMed ($P < 0.05$) but did not differ from semen diluted in BullXcell. Semen stored in BullXcell had a higher beat cross frequency and straight line velocity compared to semen stored in AndroMed ($P < 0.05$) but did not differ to OptiXcell; while semen stored in OptiXcell and AndroMed did not differ from each other ($P > 0.05$). There was no difference in any other sperm kinematic parameters ($P > 0.05$). There was no effect of diluent on 60-day NRR (71.5, 67.8 and 70.6% for BullXcell, OptiXcell and AndroMed, respectively). In conclusion, while diluent significantly affected post-thaw sperm motility and kinematics, no effect on 60-day NRR was observed. Given that OptiXcell and AndroMed are animal protein-free media they may be a suitable alternative to BullXcell for the storage of frozen-thawed bull semen as their use eliminates the risk associated with extenders which contain components of animal origin.

7.1 Introduction

The extensive use of AI within the dairy industry can be attributed in part to the development of suitable diluents for both fresh and frozen-thawed semen (Foote 2002). Cryoprotectants, predominately glycerol and egg-yolk, are added to extenders in order to protect sperm from damage during the cryopreservation process. Since the discovery of the protective properties of egg yolk in relation to the preservation of bull semen (Phillips and Lardy 1940), the addition of egg yolk (a non-permeable cryoprotectant) is regarded as one of the most essential components of diluents (Vishwanath and Shannon 2000, Crespilho et al. 2012). It is widely acknowledged that LDLs are the main component in egg yolk extenders offering protection, primarily acting by increasing the cholesterol/phospholipid ratio, thus preventing a loss of membrane phospholipids, increasing chilling tolerance and reducing cold shock injuries (Medeiros et al. 2002, Muiño et al. 2007).

The use of egg yolk, however, is not without its disadvantages as it renders microscopic semen assessment more difficult, particularly when using computer-assisted sperm analysis techniques (CASA; Singh et al. 2012). Furthermore, being a protein of animal origin, egg yolk may introduce the risk of exotic disease transmission, such as avian influenza (Yildiz et al. 2013), or microbial contamination leading to increased widespread health concerns over its use in semen diluents (Aires et al. 2003). Moreover, there is growing demand for full product tractability and increasing emphasis on biosecurity issues in government legislation regarding animal based products (Layek et al. 2016). In addition, egg yolk is difficult to standardise, with significant potential for variation from batch to batch (Bousseau et al. 1998), posing problems for quality assurance in the laboratory. Therefore,

alternatives to components of animal origin in semen extenders such as soya-lecithin in plant-based diluents are now of interest (Akhter et al. 2012, Ansari et al. 2016, Gil et al. 2003), primarily due to their traceability and the reduced health risk associated with animal protein-free media and would represent a valuable contribution to the AI industry, however they are still not universally accepted due to concerns over reduced fertility (Layek et al. 2016, Leite et al. 2010).

Plant-based extenders contain a natural mixture of phosphatidylcholine and a number of fatty acids such as stearic, oleic and palmitic acid which are known to confer structural stability to cells (Oke et al. 2010, Chaudhari et al. 2015). Due to this composition, plant-based extenders have been used to substitute for egg yolk extenders as alternative diluents for commercial semen with studies reporting comparable *in vitro* assessment results in a number of species including bovine (Miguel et al. 2008, Stradaioli et al. 2007, Aires et al. 2003), ovine (Forouzanfar et al. 2010, Gil et al. 2003) and equine (Papa et al. 2010), In addition, comparable fertility rates have been reported in buffalo (Akhter et al. 2012). However, a number of other studies have reported a reduction in semen quality when comparing plant-based and egg yolk-based extenders (Muiño et al. 2007) with some studies also reporting a reduction in fertility (Crespilho et al. 2012, Van Wagtendonk-de Leeuw et al. 2000). The exact mechanism through which plant-based extenders protect sperm from cryo-injury is not yet well understood. It is believed that exogenous phospholipids and liposomes composed of different lipids protect sperm by reversibly binding lipids and phospholipids as well as fusing liposomes with the sperm plasma membrane, thus stabilising the membrane during the freezing and subsequent thawing process (Ansari et al. 2016). Furthermore, Zeron et al. (2002)

reported that the fusion of liposomes with sperm membranes decrease the lipid phase transition of bovine sperm resulting in decreased sensitivity of sperm to cryopreservation. Therefore, the aim of this study was to compare three commercially available diluents for frozen-thawed bovine semen, one egg yolk-based and two plant-based, in terms of sperm functional parameters *in vitro* and *in vivo* fertility following AI. Importantly, ejaculates were split such that each treatment was represented in each ejaculate, eliminating any potential confounding effects.

7.2 Materials and Methods

7.2.1 Semen Collection and Processing

Semen was collected from Holstein-Friesian bulls (n = 5) at a commercial AI centre on four different occasions (occasion = replicate; total of 20 ejaculates) from early April to the end of April 2017. Upon collection, the raw ejaculate was split into three equal parts and partially diluted in 2 mL (approximately 1:1) of each of the pre-warmed (37 °C) diluents, namely, BullXcell (egg yolk-based; IMV Technologies, L'Aigle, France), OptiXcell (plant-based; IMV Technologies) and AndroMed (plant-based; Minitube, Tiefenbach, Germany) for transport. All diluents were prepared as per the manufacturer's instructions. Semen from each bull was kept separate throughout processing and ejaculates were split such that each bull was represented in each treatment. The ejaculate was then placed into a temperature-regulated cooler box at 18 °C and transported to the laboratory (approximately 3 h transport). On arrival, the ejaculate was assessed for weight, sperm concentration using a coulter counter (Z Series, Beckman Coulter, Clare, Ireland), total motility (%) and gross motility on a 5-point scale (1 = twitching/no forward progressive motility; 5 =

excellent forward progressive motile sperm) to ensure all semen samples were of a commercial standard (results not shown). Microscopic assessments were conducted by the same experienced technician and initial quality control cut-off values were a total and gross motility of $\geq 70\%$ and a score of ≥ 3 , respectively; any ejaculates failing to meet these criteria were rejected and thus not used in the study.

Following *in vitro* assessment, each acceptable ejaculate was fully extended in the respective diluents to achieve a concentration of 15×10^6 sperm per 0.25 mL insemination dose. The final dilution ratio was dependent upon the ejaculate volume and sperm concentration per mL within each ejaculate. Semen straws were filled, printed and sealed as per routine procedures, gradually cooled to 4 °C and frozen using the following protocol: -5 °C per min from +4 °C to -10 °C, -40 °C per min from -10 °C to -100 °C and thereafter -20 °C per min from -100 °C to -140 °C (Murphy et al. 2017) in a programmable freezer (IMV Technologies), followed by submersion and storage in liquid nitrogen at -196 °C until use.

7.2.2 Experiment 1: *In Vitro* Analysis of the Effects of Semen Diluent on Frozen-Thawed Semen

The aim of this experiment was to assess the effects of three commercially available diluents on the motility and kinematics parameters of frozen-thawed bull sperm using the IVOS-II CASA system driven by software version 14 (Hamilton Thorne Inc, Beverly, USA). Samples from the three treatments were assessed in a randomised sequence to remove bias as a result of sampling order. Straws (n = 4 per ejaculate) were thawed at 37 °C for 30 sec and each sample was diluted at a 1:3 ratio in EasyBufferB (IMV Technologies). A drop (3 μ L) of diluted semen was placed in

a pre-warmed chamber (37 °C; Leja counting chambers, depth 20 µm; Microptics, Barcelona, Spain) and analysed for sperm motion and kinematic characteristics immediately post-thaw. A minimum of 1000 sperm were analysed in at least eight microscopic fields with 30 frames acquired per field at a frame rate of 60 Hz. Objects incorrectly identified as sperm were edited out using the playback function. The CASA-derived motility and kinematic characteristics assessed were total motility (%), progressive motility (%), proximal and distal droplets (%), as well as VAP above 10 µm/s, VSL, VCL, LIN, STR, ALH and BCF; (Mortimer 2000). Regarding analysis settings, the CASA was set to standard factory settings for bull semen and sperm with straightness of >80% and VAP >50 µm/s were considered progressively motile.

7.2.3 Experiment 2: Field Fertility of Frozen-Thawed Semen Diluted in BullXcell, OptiXcell and AndroMed

The aim of this experiment was to assess the effect of frozen-thawed bovine semen diluent on 60-day NRR following AI. Semen from the same Holstein-Friesian bulls (n = 5) and same batches of semen as in Experiment 1 (15 x 10⁶ sperm per 0.25 mL dose) were used in the field trial. Each batch of semen was clearly labelled and distributed for insemination after 30 days of quarantine as per European regulations (Irish Statue Book, 2014). Inseminations were carried out in May 2017 (coinciding with the peak dairy breeding season) in Irish dairy herds (n = 255). The majority (97%) of inseminations were in Holstein-Friesians (n = 1,433) but a small number of other breeds were represented including Jersey, MRY, Ayrshire, Montbeliarde, Norwegian Red and Shorthorn. Technicians (n = 22) were blind to treatments and received equal number of straws from each of the three treatments from each bull.

For each insemination, the AI technician recorded the bull code, cow tag number and the straw code on an electronic handheld device. Inseminations and NRR data were captured using the ICBF database by cross-referencing the technician name with the bull code and semen type used on each date within the trial period. Obvious errors ($n = 115$) were extracted from the dataset and data were then interrogated to remove animals based on the following criteria: cows which were not at first AI, cows which received two inseminations from two different bulls or treatments, or cows which were not of a dairy breed. However, if a dairy cow or heifer received two inseminations from the same bull with the same diluent treatment within five days of each other, the record was kept and the second date was assumed to be correct. Post editing, a total of 1,480 inseminations ($n = 576, 547$ and 357 for BullXcell, OptiXcell and AndroMed, respectively) consisting of 280 heifers and 1,200 multiparous dairy cows remained.

Cow characteristics such as parity, DIM and fertility sub-index were also included in the model. Fertility sub index is a key component of the EBI comprising ~35% of the total EBI (ICBF 2017a). The EBI is an estimate of the economic value of an animal's genetic merit. It was established to combat a decline in reproductive performance by providing farmers with a profit index enabling the selection of elite sires to breed replacement heifers with increased milk yield, reproductive performance and improved health traits (Berry et al. 2005).

7.3 Statistical Analysis

Data from Experiment 1 were examined for homogeneity of variance and analysed using the GLM repeated-measures procedure with a compound symmetry covariance

structure in SPSS (Version 22.0; IBM, Chicago, USA). In Experiment 2, NRR data were compared using Pearson's chi-squared procedures in SPSS. The dependent variable in the analysis was NRR (1 = pregnant, 0 = not pregnant). In addition, a GLM for binomial data was used to assess the influence of a number of fixed effects on NRR including diluent treatment, bull, parity, cow breed, cow fertility sub-index, DIM, herd and technician. Each fixed effect was assessed for an interaction with treatment. All post-hoc tests were carried out using the Bonferroni test and results are reported as the mean \pm the standard error of the mean (sem) in Experiment 1 and as the estimated marginal means in Experiment 2, to adjust for imbalance between the number of inseminations in each treatment. Data were considered to differ significantly at $P < 0.05$.

7.4 Results

7.4.1 Experiment 1: *In Vitro* Effects of Semen Diluent on Frozen-Thawed Semen

There was an effect of diluent on post-thaw total and progressive motility as assessed by CASA ($P < 0.01$; Table 7.1). Semen diluted in OptiXcell had greater post-thaw total and progressive motility (59.0 ± 4.52 and $45.7 \pm 4.09\%$, respectively) scores than that diluted in AndroMed ($P < 0.05$) but did not differ from BullXcell which were intermediate ($P > 0.05$; Table 7.1). There was an effect of treatment on BCF and VSL ($P < 0.05$) as semen diluted in BullXcell exhibited superior BCF and VSL compared to semen diluted in AndroMed ($P < 0.01$) but did not differ from OptiXcell ($P > 0.05$). Semen diluent did not affect any other kinematic motility parameter (ALH, LIN, STR, VAP, VCL and WOB) or the percentage of sperm with proximal and distal droplets ($P > 0.05$; Table 7.1).

Table 7.1: The effect of semen diluent on post-thaw motility and kinematic parameters in bovine semen as assessed by computer assisted sperm analysis (Experiment 1). ^{abc}Values with different superscripts differ significantly within row ($P < 0.01$; values are mean \pm sem).

Parameters	Treatment			P value
	mean \pm sem			
	BullXcell	OptiXcell	AndroMed	Effect of Treatment
Total Motility (%)	51.9 \pm 1.76 ^{ab}	59.0 \pm 4.52 ^b	41.93 \pm 3.61 ^a	P < 0.05
Progressive Motility (%)	41.8 \pm 1.82 ^{ab}	45.7 \pm 4.09 ^b	31.7 \pm 2.97 ^a	P < 0.05
ALH (μm)	7.5 \pm 0.85	6.6 \pm 0.58	7.2 \pm 0.67	ns
BCF (Hz)	29.2 \pm 0.02 ^a	25.3 \pm 1.22 ^{ab}	24.5 \pm 1.24 ^b	P < 0.05
LIN (%)	43.1 \pm 1.51	44.0 \pm 1.48	39.2 \pm 1.70	ns
STR (%)	78.7 \pm 0.99	79.8 \pm 1.52	75.1 \pm 2.21	ns
VAP ($\mu\text{m}/\text{s}^{-1}$)	90.5 \pm 5.68	75.5 \pm 4.86	72.6 \pm 4.19	ns
VCL ($\mu\text{m}/\text{s}^{-1}$)	174.3 \pm 13.76	141.8 \pm 10.41	143.7 \pm 8.32	ns
VSL ($\mu\text{m}/\text{s}^{-1}$)	71.0 \pm 4.26 ^a	61.1 \pm 4.29 ^{ab}	55.4 \pm 4.11 ^b	P < 0.05
WOB (%)	53.7 \pm 1.24	54.3 \pm 0.97	51.4 \pm 0.75	ns
Proximal Droplets (%)	2.2 \pm 0.33	1.6 \pm 0.36	2.5 \pm 0.33	ns
Distal Droplets (%)	0.7 \pm 0.14	0.5 \pm 0.10	0.8 \pm 0.08	ns

ALH = amplitude of lateral head displacement, BCF = beat cross frequency, LIN = linearity, STR = straightness, VAP = average path velocity, VCL = curvilinear velocity, VSL = straight line velocity, WOB = wobble, ns = non-significant.

7.4.2 Experiment 2: Field Fertility of Frozen-Thawed Semen Diluted in BullXcell, OptiXcell and AndroMed

Diluent did not affect field fertility of frozen-thawed semen, with a 60-day NRR of 71.5, 67.8 and 70.6% for BullXcell, OptiXcell and AndroMed, respectively; Figure 7.1). There was no bull, breed, parity, cow fertility sub-index, DIM, herd or technician by treatment interaction with NRR ($P > 0.05$). Cows which were less than 60 DIM had a reduced NRR (65.4%) in comparison with those greater than 60 DIM prior to insemination (76.3%). There was no difference in NRR between maiden heifers (71.4%), primiparous (72.5%) and multiparous dairy cows (68.5%; $P > 0.05$). As expected, NRR varied between individual herds and technicians ($P < 0.01$). There was no effect of bull, breed, cow fertility sub-index or parity on NRR ($P > 0.05$).

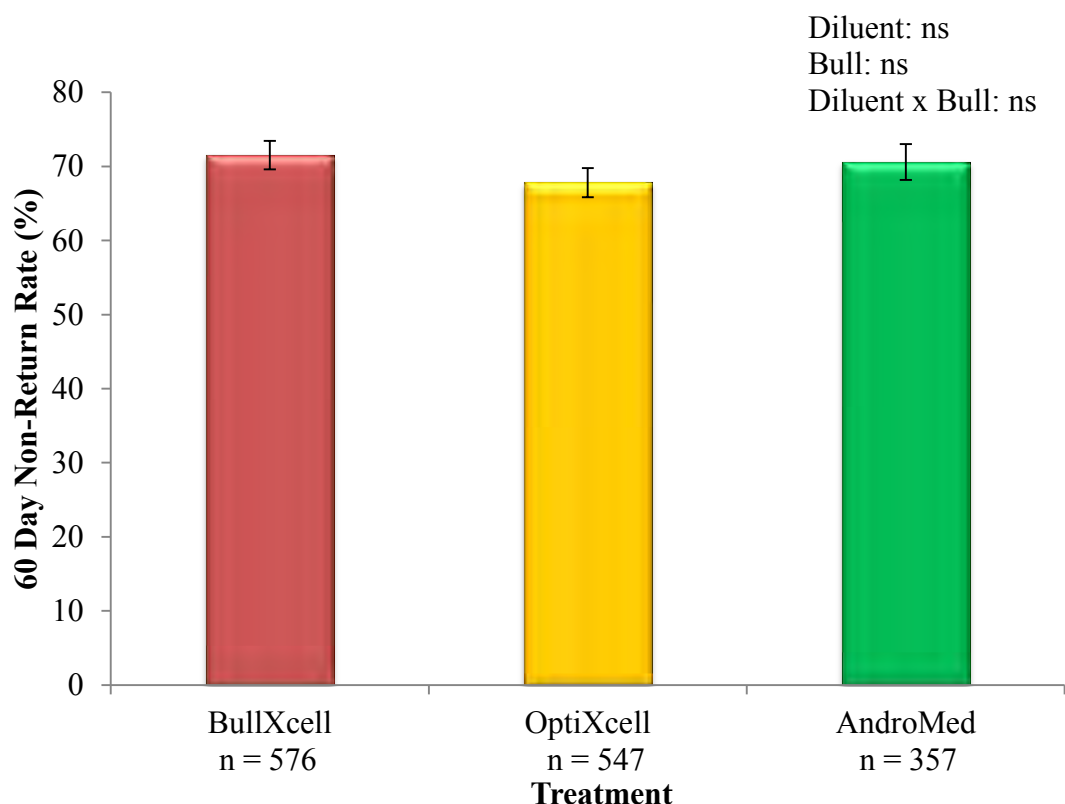


Figure 7.1: The effect of semen diluent on 60-day non-return rate of frozen-thawed semen in dairy cows and heifers (Experiment 2). Vertical bars represent 95% confidence intervals. n = number of inseminations, ns = non-significant.

7.5 Discussion

The continuous health concerns within the AI industry relating to the use of animal proteins in semen extenders have led to the development of alternative diluents free of animal-derived products. Here, we used split ejaculates and a combination of *in vitro* and *in vivo* assessments in a comprehensive attempt to identify the optimal semen diluent for frozen-thawed bovine semen. The main findings of this study were that semen diluted in plant-based extenders OptiXcell and AndroMed resulted in similar total and progressive motility *in vitro* and NRR following AI compared to egg yolk-based extender BullXcell.

Motility is one of the most important parameters associated with semen fertilising capacity and has therefore been recognised as essential for sperm transport and fertilisation in the female reproductive tract (Verstegen et al. 2002). However, the presence of egg yolk globules have been shown to interfere with microscopic evaluation (Vishwanath and Shannon 2000) with the use of plant-based extenders resulting in better sample visualisation and lower bacterial load (Meena et al. 2010). Therefore, there are clear advantages in using plant-based over animal-based extenders. However, the efficacy of the use of plant-based extenders is still a matter of debate with studies reporting contradictory *in vitro* and *in vivo* results. Some studies report no difference when comparing plant-based versus egg-yolk extenders (Gil et al. 2003, Bousseau et al. 1998) whereas others found beneficial (Chaudhari et al. 2015, Aires et al. 2003) or damaging effects (Veerabramhaiah et al. 2015, Crespilho et al. 2012). In the current study, diluent did not affect the majority of sperm kinematic parameters (ALH, LIN, STR, VAP, VCL and WOB), nevertheless, it is noteworthy that extender had a significant effect on VSL and BCF with

BullXcell having a higher value than semen diluted in AndroMed. A higher BCF indicates that BullXcell may be more effective at preserving flagellar structures or stimulating ATP production and consequently beat frequency (Celeghini et al. 2008) compared to AndroMed; but there was no difference between BullXcell and OptiXcell. Ansari et al. (2016) and Aires et al. (2003) reported that sperm motility increased when semen was diluted in OptiXcell and AndroMed, respectively, compared to an egg yolk-based diluent. Furthermore, Kumar et al. (2015) reported that semen diluted in plant-based liposome extenders had improved kinematic parameters than semen diluted in egg yolk extenders while Crespilho et al. (2012) reported that egg yolk-based extenders recorded higher total and progressive motility scores compared to lecithin-based extender. The results of the current study would contradict these findings as there was no overall significant difference observed between plant-based and egg yolk-based extenders in sperm motility.

Continuous debate exists surrounding the cryo-protective capabilities of plant-based extenders compared to egg yolk-based extenders (Layek et al. 2016, Leite et al. 2010). Previous studies have reported a reduction in motility, viability and membrane integrity when semen was diluted in soy-lecithin-based extenders compared to egg yolk-based extenders (Beran et al. 2012, Crespilho et al. 2012, Papa et al. 2010, Celeghini et al. 2008) as well as better protective capacity of egg yolk extenders resulting in higher *in vivo* fertility (Veerabramhaiah et al. 2015, Thun et al. 2002). In contrast other studies have reported higher sperm total motility, acrosomal integrity (Chaudhari et al. 2015, Amirat et al. 2005, Aires et al. 2003) and higher (Akhter et al. 2012) or similar (Gill et al. 2003, Bousseau et al. 1998) fertility rates when cryopreserved semen was diluted in lecithin-based extenders as opposed to egg

yolk extenders. It has also previously been reported that higher viscosity and the presence of particulate debris in egg yolk-based extenders may cause reduced fertility (Van Wagendonk-de Leeuw et al. 2000); however, in the current study there was no difference in 60-day NRR of semen diluted in either egg yolk- or lecithin-based diluents. The results also contradict Ansari et al. (2016) and Aires et al. (2003), based on approximately 100 and 9,000 inseminations per treatment, respectively, who reported higher fertility rates when semen was diluted in OptiXcell and AndroMed compared to egg yolk extenders. The absence of a diluent effect on NRR observed in this study suggests that plant-based diluents are just as effective at protecting sperm cells during cryopreservation process as to egg-yolk extenders. While it is widely accepted that cow characteristics such as parity, fertility sub-index and DIM play a role in fertility (Murphy et al. 2017, Murphy et al. 2016), semen diluent did not negate the effects of these on NRR.

In conclusion, the chemically defined plant-based commercial extender, OptiXcell, was more efficient than the egg yolk-based extender BullXcell in conserving post-thaw total and progressive motility; however, there was no effect of diluent on the 60-day NRR. Given that the use of plant-based extenders such as OptiXcell or AndroMed provides substantial advantages compared to egg-yolk extenders by reducing health risks, increasing standardisation and ease of preparation and assessment, the use of plant-based extenders should be considered as a viable alternative to egg yolk based diluents.

Chapter Eight

Discussion

8.1 Final Discussion

Excellent reproductive performance in both males and females is fundamental to profitable dairy and beef production systems (Berry et al. 2014). While NRR is the predominant phenotype used for assessing male fertility in the field (Clay and McDaniel 2001, Kuhn and Hutchison 2008), reporting pregnancy rate, which is strongly correlated with NRR (Berry et al. 2011), or CR gives a more precise and accurate assessment of fertility. The advent of genomics in the dairy industry has resulted in the intensive use of young genomically selected sires and so has placed additional pressure on AI centres to rapidly identify, purchase and collect semen from these peri-pubertal bulls. Although selecting bulls at a younger age has enabled the industry to shorten the generation interval (Bouquet and Juga 2013), these bulls present a number of challenges to AI centres as semen yield from these young bulls is reduced compared to their mature counterparts (Brito et al. 2002a). Thus, there is ever growing pressure on AI centres to maximise semen production and quality from these bulls so as to meet consumer demands. However, as demands greatly out way semen production, an evaluation of the semen production processes within AI centres with regards both liquid and frozen-thawed semen is paramount so as to optimise production processing procedures with the aim to facilitate greater utilisation of this valuable semen. Thus, this thesis sought to optimise semen processing procedures of both liquid and frozen-thawed semen within a commercial AI centre through the implementation and assessment of a variety of concepts that could potentially be adopted by the AI centre for the prospective use with young genomically selected sires. Namely, it sought to (i) identify factors influencing semen production and quality in Holstein Frisian bulls (ii) investigate semen diluents in both liquid and frozen-thawed semen so as to prolong the fertile lifespan and

increase cyro-survival, respectively, (iii) define the optimal sperm concentration in fresh semen so as to maximise the use of young genetically superior sires, (iv) determine the optimum storage temperature of liquid semen, and (v) ascertain the optimal equilibration time for frozen-thawed semen in order to achieve optimum semen quality.

As the economic return of an AI centre largely depends on the production ability of a small number of elite bulls to produce fertile sperm, understanding the relationship of key factors affecting semen production and quality from young and mature bulls is paramount so that management practises can be adapted to optimise production. Semen production in terms of ejaculate volume and TSN was found to increase with increasing bull age (Brito et al. 2002a, Mathevon et al. 1998, Everett and Bean 1982); however sperm concentration remained constant after one year of age. Not surprisingly, bulls aged less than one year had the lowest production values (Al-Kanaan et al. 2015) as it is widely acknowledged that peri-pubertal bulls have lower ejaculate volumes than mature bulls (Perumal 2014, Karabinus et al. 1990, Mathevon et al. 1998). The collection of subsequent ejaculates resulted in lower semen production values for bulls older than one year; however these semen production values did not differ for bulls less than one year of age, which might be due to the significantly lower semen production values associated with young bulls and the large variation within their analysis compared to more mature bulls. Critically, the collection of subsequent ejaculates maintained acceptable post-thaw *in vitro* semen quality, similar to the findings of (Boujenane and Boussaq 2013), although higher values were observed for first ejaculates (Fuerst-Waltl et al. 2006); therefore, highlighting that overall production of semen can increase with the collection of

multiple ejaculates and can be justified for bulls in high demand. While bulls are not typically considered seasonal breeders, there was a season by bull age interaction on semen production with bulls aged less than one recording poorest semen production values than any other season; however there was no clear biological pattern for any age category. Furthermore, while there was a significant seasonal difference in semen quality, with Winter recording the highest values, the difference between seasons was believed to be of no biological importance or have any impact on quality control analysis.

The use of liquid semen provides AI centres with a more practical and economical advantage compared to frozen-thawed semen due to the considerably reduced sperm numbers required to achieve comparable fertility. Thus, the use of liquid semen maximise semen production as approximately three times more insemination doses can be produced. Regardless of semen diluent, sperm concentration, N₂ gassing or storage temperature motility of liquid semen declined with increased duration of storage. Caprogen diluent has long been regarded as the industry gold standard for the dilution and storage of liquid semen (Vishwanath and Shannon 2000), however, the continual advancement of semen dilution technology and increased health concerns of the use of egg-yolk in semen extenders has led to the development of alternative liquid and frozen-thawed semen diluents which are of milk or plant-based origin (Stradaioli et al. 2007, Bergeron et al. 2007, Akhter et al. 2012). Caprogen and INRA96 maintained sufficient sperm quality *in vitro* when semen was stored at either a constant or fluctuating temperature, and resulted in similar CRs, suggesting that both diluents are efficient at protecting sperm from damage sustained by during

storage. Thus, the protective properties of milk casein micelles are analogues to the protective action of LDLs in egg-yolk (Manjunath 2012, Bergeron et al. 2007).

BioXcell displayed an ability to protect sperm when semen was stored at a constant temperature. However, during temperature fluctuations (such as those exhibited in field conditions) its protection was inferior as evidenced by a reduction of approximately 30 and 20% in total and progressive motility on Day 3 of storage, respectively, and by the reduction in CR in comparison to Caprogen and INRA96. Thus, BioXcell appears less tolerant of temperature fluctuations. These results contradict Akhter et al. (2011) who reported that BioXcell had similar protective capabilities in terms of maintaining semen quality comparable to those exhibited by both egg-yolk and milk-based extenders. Given the complex nature relating to the preparation of Caprogen and that INRA96 involves no additional components; the use of INRA96 is more convenient to the working schedule of an AI centre.

In order to increase the rate of genetic gain and to optimise the use of young genomically selected bulls, it is imperative to maximising the number of inseminations doses produced per ejaculate. Although Ireland and New Zealand have comparable NRRs of between 65-74% using liquid semen, the later benefits by employing a maximum sperm concentration of 2×10^6 sperm per dose, used on Day 3 of storage (Xu 2014). Therefore, highlighting the potential of AI centres in Ireland to reduce sperm numbers in a liquid semen dose by over 50%. However, the effect of such a reduction in sperm concentration on semen quality and fertility is unknown with previous studies reporting beneficial (Murphy et al. 2013, Prathalingam et al. 2006) and detrimental *in vitro* effects of excessive dilution (Garner et al. 1997). Additionally, fertility studies have reported similar contradictory results with reports

of a reduction (Murphy et al. 2016, Shannon and Vishwanath 1995) or no significant reduction (Shannon and Curson 1984) of fertility with reduced sperm numbers. Semen diluted to a concentration of 3, 4 or 5 x 10⁶ sperm per dose maintained acceptable total and progressive motility throughout the duration of storage. However, reducing sperm concentration from the standard 5 x 10⁶ sperm per dose to 3 and 4 x 10⁶ resulted in a reduction in CR on Day 2 of storage compared to frozen-thawed semen when stored under unregulated field conditions; but this did not differ to semen diluted to 5 x 10⁶ sperm per dose. Additionally, CR of liquid semen treatments did not differ from each other on Day 1 of storage. This suggests that sperm numbers could be increased for targeted use on subsequent days post collection; namely 4 and 5 x 10⁶ sperm per dose for insemination on Day 1 and 2 post collection, respectively, leading to an increase in the use of individual sires.

It is widely acknowledged that liquid semen has a finite fertile lifespan of only 2.5 to 3 days post collection with a reduction in fertility reported thereafter due to the detrimental effects of metabolism on sperm survival (Vishwanath and Shannon 2000). Lower storage temperatures and or N₂ gassing of media at ambient temperatures have been shown to reduce the metabolic activity of sperm (Shannon et al. 1984, Shannon 1965). Bubbling N₂ gas into the media did not significantly affect sperm motility up to Day 3 of storage, suggesting that diluents may provide sufficient support for sperm motility over prolonged periods (up to 3 days of storage) regardless of the oxygenated state of the media and that the benefits of N₂ gassing are not observed by assessing motility alone. Although sperm metabolic activity has previously been shown to increase with increased storage temperature (Setchell 1998), thus, increasing the generation of ROS, sperm were found to be quite tolerant

to temperature variation in relation to sperm quality retaining acceptable *in vitro* motility standards between storage temperatures of 5 and 15 °C. However, exposing semen to extreme high temperatures of 28 °C was detrimental to sperm as they exhibited reduced overall motility and velocity parameters, suggesting that the unregulated liquid semen storage conditions applied in the field may negatively impact fertility. These results supported the findings of Murphy et al. (2016) and Krzyzosiak et al. (2001) who reported that sperm quality was retained between temperatures of 5 – 22 °C and N₂ gassing did not affect *in vitro* fertility. Conversely, while semen held at a constant 15 °C resulted in superior semen quality throughout the duration of storage and resulted in greater NRR on Day 1 and Day 2 of storage compared to semen held at a constant 5 °C; NRRs of semen held at 15 °C did not differ to unregulated storage conditions. Nonetheless, in climatic conditions where a large day to night time temperature fluctuation is likely, provisions should be put in place for the storage of liquid semen in order to avoid exposure to extreme temperatures.

The use of frozen-thawed semen in AI is one of the most common management tools applied in the cattle breeding industry with 95% of inseminations worldwide conducted with the use of frozen-thawed semen (Murphy et al. 2016). This can be attributed to the in-depth investigation of semen cryopreservation processes (Shah et al. 2016, Liu et al. 1998, Muiño et al. 2008) which has resulted in the development of species specific cryopreservation protocols. Although bovine semen freezing protocols typically included a short equilibration of 4 – 6 h, a prolonged equilibration period of greater than 24 h has been shown to result in better semen quality (Fleisch et al. 2017, Anzar et al. 2011). The beneficial effect of implementing

a prolonged equilibration period up to 24 h was evident in this study as increasing the equilibration period from 6 to 24 h resulted in optimising *in vitro* post-thaw semen quality; however, this was not translated in field conditions as increasing equilibration time from 6 – 72 h yielded comparable fertility. While Pickett and Berndtson (1978) established that an equilibration period of 18 h was optimum to achieve maximum fertility, the results of this thesis supported the findings of Fleisch et al. (2017) and Foote and Kaproth (2002) as increasing equilibration time from 4 – 72 h or 4 – 28 h had no effect on 90-day or 56-day NRR, respectively. However, while there was no effect on fertility, a prolonged equilibration resulted in superior semen quality and allows for greater flexibility within the working environment of a commercial AI centre particularly with regards the collection of a large number of bulls, semen transportation over long distances, mechanical failure and/or staff shortages.

The use of plant-based extenders for the preservation of frozen-thawed semen is becoming increasingly popular (Akhter et al. 2012, Ansari et al. 2016, Gil et al. 2003) primarily due to the health risks associated with animal-derived extenders (Aires et al. 2003). However, the debate surrounding the cryo-protective capabilities of plant-based compared to egg yolk-based extenders still exists (Layek et al. 2016, Leite et al. 2010) with contradictory *in vitro* and *in vivo* results reported. Previous studies report no difference between extenders (Bousseau et al. 1998, Gil et al. 2003) while others reported beneficial (Aires et al. 2003, Chaudhari et al. 2015, Ansari et al. 2016) or damaging effects (Crespilho et al. 2012, Veerabramhaiah et al. 2015). The results of this thesis showed that semen diluted in either BullXcell, OptiXcell or AndroMed resulted in similar *in vitro* semen quality in terms of total and progressive

motility and kinematic parameters. Furthermore, there was no difference in NRR between the egg-yolk or plant-based extenders; suggesting that plant-based diluents are just as effective at protecting sperm cells during cryopreservation process as to egg-yolk extenders.

Throughout this thesis bull variation in fertility was reported in all studies, despite semen meeting routine minimum quality control standards. Additionally, cow characteristics were also reported to play a role in fertility which is in agreement with a number of studies (Forde et al. 2015, Fouz et al. 2011, Hillers et al. 1984, Murphy et al. 2017). Semen type cannot negate the effects of bull or cow characteristics on fertility and results of this thesis reported the existence of a bull by semen type interaction. This is evident by some bulls having higher fertility rates when used for liquid versus frozen-thawed semen or vice versa. It has previously been reported (Murphy et al. 2016) and further highlighted in this thesis that some bulls are more susceptible to sperm aging effects when stored in liquid semen, possibly due to a lack of a repair mechanism leading to a reduction in fertility. This further suggests that some bulls may be more suitable for use in frozen-thawed semen rather than liquid semen programs. With the abolition of milk quotas and the predicted 50% increase in milk production by the year 2020, the need for accelerated genetic gain and expansion within the Irish dairy herd has placed huge pressure on AI centres to maximise reproductive technologies so as to produce the best quality semen. Therefore, devising strategies to identify bulls with the potential to perform better in liquid semen compared to frozen-thawed semen must be undertaken in order to maximise fertility from individual bulls.

8.2 Conclusion

In conclusion, this work offers AI centres a number of novel insights into optimising liquid and frozen-thawed semen production and processing procedures that could potentially enhance the utilisation of mature and young genomically-selected bulls. In seasonal grass based production systems whereby profitability is driven by fertility, the use of liquid semen has the potential to maximise the utilisation of young genomically selected sires and thus maximise the rate of genetic gain. However, extending the finite fertile lifespan of liquid semen beyond Day 3 of storage is still elusive. Research identifying bulls which have inferior fertility when used in liquid semen compared to frozen-thawed semen programs is essential and methods of combating this reduction in fertility would be hugely beneficial to AI centres.

8.3 Key Findings

The main findings of this thesis are:

1. Factors such as bull age, collection frequency and season affect semen production and quality in Holstein Friesian bulls. Semen production and quality increased with increasing bull age but there was no clear biological pattern of season.
2. The collection of subsequent ejaculates resulted in a reduction in semen production from mature bulls but did not negatively affect semen production and quality in bulls of less than one year. Critically, subsequent collections had no effect on post-thaw semen quality.
3. INRA96 is efficient at maintaining liquid semen quality and resulted in similar CRs to Caprogen and frozen-thawed semen.
4. N₂ bubbling of the media or reducing sperm concentrations had no effect on semen quality *in vitro*.
5. Reducing sperm numbers to 3 and 4 million sperm per dose in liquid semen had a reduced CR on Day 2 of storage compared to frozen-thawed semen but did not differ to the standard sperm concentration of 5 million sperm per dose.

6. Bull sperm are versatile with respect to storage temperature during liquid semen storage between 5 and 15 °C but were detrimentally affected by extreme temperatures of 28 °C.
7. Liquid semen stored at a constant 5 °C resulted in a reduced NRR compared to semen stored at a constant 15 °C or unregulated field storage conditions; however, the fertility of semen stored at a constant 15 °C or unregulated storage conditions did not differ from each other.
8. Minitube coloured liquid semen straws are a suitable alternative to the clear IMV liquid semen straws as there was no commercially significant effect of straw source on sperm motility or on NRR.
9. Prolonging the equilibration period of diluted semen up to 72 h did not detrimentally affect semen quality or CR, with an equilibration period of 24 h resulting in superior semen quality.
10. OptiXcell and/or AndroMed are suitable alternatives to the egg-yolk extender BullXcell for the preservation of frozen-thawed semen as there was no effect of diluent on NRR.
11. A semen type by bull interaction is evident with bulls performing better when used as liquid or frozen-thawed semen; however, semen type does not negate the effects of bull or cow characteristics on fertility.

8.4 Future Work

Future work should focus on:

1. Implement managerial practises to hasten the onset of puberty allowing for the routine collection of semen from young genomically selected sires at an even early age.
2. Devising strategies to identify bulls with the potentially to perform better in liquid semen compared to frozen-thawed semen in order to maximise fertility from individual sires.
3. Reduce sperm concentrations of frozen-thawed semen so as to optimise production.

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Appendices

Preparation of Caprogen

Caprogen diluent was composed of four main constituents; 1) glycerol-antibiotic solution, 2) buffer solution, 3) catalase, caproic acid and citric acid and 4) egg yolk.

Glycerol – antibiotic solution

Penicillin G potassium (0.2943 g) was dissolved in distilled water (dH₂O; 1 mL), while streptomycin sulphate (0.4348 g) was diluted separately in H₂O (1 mL). Following this, linco-spectin (2.17 mL) was added, and the total volume was brought to 5.5 mL using dH₂O. Pure glycerol (4.5 mL) was added to the solution which was then mixed using a vortex. The glycerol – antibiotic solution can be stored at 4 °C for four weeks.

Buffer solution

Sodium citrate (8 g), glycine (4 g) and D-glucose (1.2 g) were added to dH₂O (150 mL). Refrigerated dH₂O was added to bring the total volume to 380 mL. Glycerol – antibiotic solution (10 mL) was added to the buffer, which was brought to 400 mL using dH₂O. The pH of the buffer was then brought to 7.4. Buffer solution can be stored at 4 °C for up to one week.

Catalase, Caproic Acid and Citric Acid

Liquid bovine liver catalase (100 µL) was added in 5 mL of buffer solution. Caproic acid (125 µL) was added to buffer solution (4.88 mL). Citric acid (1.4 g) was dissolved in buffer solution (100 mL) and aliquoted into 1.5 mL eppendorfs. Diluted catalase and caproic acid can be stored at 4 °C for one week, while citric acid can be stored at -20 °C for one year.

Egg yolk

Egg yolk (1 litre) was provided twice weekly (O'Brien Country Fresh Foods, Dublin, Ireland). A 5% egg yolk Caprogen diluent was prepared (400 mL) by pouring buffer solution (368 mL) into a graduated cylinder and adding catalase (4 mL), caproic acid (4 mL) and citric acid (4 mL). Egg yolk (20 mL) was added last to the solution. The diluent was then purged in fresh food grade N₂ gas for 1 h, to dispel any oxygen in the media and used on the day of preparation.

Preparation of BullXcell

The content of 1 bottle of BullXcell (250 mL) was added to 750 mL dH₂O. Egg yolk (250 g) was then added to the solution to create a final solution of 1250 mL.

Preparation of BioXcell

The content of 1 bottle of BioXcell (100 mL) was added to 400 mL dH₂O to create a final solution of 500 mL.

Preparation of OptiXcell

The content of 1 bottle of OptiXcell (250 mL) was added to 500 mL dH₂O to create a final solution of 750 mL.

Preparation of AndroMed

The content of 1 bottle of AndroMed (200 mL) was added to 800 mL dH₂O to create a final solution of 1000 mL.

Preparation of NutriXcell

The content of 1 packet of NutriXcell (1 litre pack) was added to 1000 mL dH₂O to create a final solution of 1000 mL.

Preparation of SYTO 16

The contents of one vial of SYTO 16 (250 µL) were diluted in dimethylsulfoxide (DMSO; 2.25 mL) to give a 100 µM SYTO 16 reagent stock solution. A sample of this stock solution was diluted in a 1:10 ratio with BWW (10 µM) and then added to sperm in a 1:100 ratio, to give a working concentration of 100 nM. SYTO 16 is excited at 488 nm and emits at 518 nm. Aliquots of SYTO 16 were stored at – 20 °C and protected from light.

Preparation of PI

PI was supplied as a 1.5 mM solution. A sample of this stock solution was added to sperm in a 1:100 ratio, to give a working concentration of 15 µM. PI is excited at 535 nm and emits at 617 nm. Aliquots of PI were stored at 4 °C and protected from light.

Preparation of AF647

The contents of one vial of AF647 (100 mg) were dissolved in dH₂O (1 mL) to give a 0.77 mM AF647 reagent stock solution. A sample of this stock solution was added to sperm in a 1:167 ratio to sperm, to give a working concentration of 4.5 µM. AF647 is excited at 650 nm and emits at 668 nm. Aliquots of AF647 were stored at – 20 °C and protected from light.

Publications

First Authored International Journal Papers

Murphy, E. M., Murphy, C., O'Meara, C., Dunne, G., Eivers, B., Lonergan, P. and Fair, S. (2017) 'A comparison of semen diluents on the *in vitro* and *in vivo* fertility of liquid bull semen', Journal of Dairy Science, 100, 1541-1554.

Murphy, E. M., Eivers, B., O'Meara, C., Lonergan, P. and Fair, S. (2018) 'Effect of increasing equilibration time of diluted bull semen up to 72 hours prior to freezing on sperm quality parameters and calving rate following artificial insemination', Theriogenology, 108, 217-222.

Murphy, E. M., Eivers, B., O'Meara, C., Lonergan, P. and Fair, S. (2018) 'Effect of storage temperature, nitrogen gassing and sperm concentration on the *in vitro* semen quality and *in vivo* fertility of liquid bull semen stored in INRA96', Theriogenology, 108, 223-228.

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Co-authored International Journal Papers

Holden, S. A., Fernandez-Fuertes, B., Murphy, E. M., Lonergan, P. and Fair, S. (2017) 'Effects of seminal plasma from high- and low-fertility bulls on cauda epididymal sperm function', In Press, *Reproduction, Fertility and Development*, DOI: <http://dx.doi.org/10.1071/RD17136>

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Submitted Papers

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Conference Publications

Murphy, E. M., Eivers, B., O'Meara, C., Lonergan, P. and Fair, S. (2017) 'Effect of increasing equilibration time of diluted bull semen up to 72 hours prior to freezing on sperm quality parameters and calving rate following artificial insemination'. 21st Annual Conference of the European Society for Domestic Animal Reproduction (ESDAR), Aug 24 – 26, Bern, Switzerland (Oral Presentation)

Murphy, E. M., Murphy, C., O'Meara, C., Dunne, G., Eivers, B., Lonergan, P. and Fair, S. (2017) 'A comparison of semen diluents on the *in vitro* and *in vivo* fertility of liquid bull semen'. 18th International Congress on Animal Reproduction (ICAR), Jun 26 – 30, Tours, France (Poster Presentation)



A comparison of semen diluents on the *in vitro* and *in vivo* fertility of liquid bull semen

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ABSTRACT

The aim of this study was to assess the effect of semen diluent on calving rate (CR) following artificial insemination with liquid bull semen stored for up to 3 d postcollection. In experiment 1, the effect of storing liquid semen maintained at a constant ambient temperature in 1 of 7 different diluents [Caprogen (homemade), OptiXcell, BioXcell, BullXcell, INRA96, NutriXcell, or AndroMed (all commercially available)] on total and progressive motility was assessed on d 0, 1, 2, and 3 postcollection. In experiment 2, the field fertility of liquid semen diluted in Caprogen, BioXcell, or INRA96 and inseminated on d 1, 2, or 3 postcollection was assessed in comparison to frozen-thawed semen (total of $n = 19,126$ inseminations). In experiment 3, the effect of storage temperature fluctuations (4 and 18°C) on total and progressive motility following dilution in Caprogen, BioXcell, and INRA96 was assessed on d 0, 1, 2, and 3 postcollection. In experiment 1, semen stored in Caprogen, BioXcell, and INRA96 resulted in the highest total and progressive motility on d 1, 2, and 3 of storage compared with OptiXcell, BullXcell, NutriXcell, and AndroMed. In experiment 2, an effect of diluent on CR was found as semen diluted in BioXcell had a lower CR on d 1, 2, and 3 of storage (46.3, 35.4, and 34.0%, respectively) in comparison with Caprogen (55.8, 52.0, and 51.9%, respectively), INRA96 (55.0, 55.1, and 52.2%, respectively), and frozen-thawed semen (59.7%). Effects were found of parity, cow fertility sub-index, as well as the number of days in milk on CR. In experiment 3, when the storage temperature of diluted semen fluctuated between 4 and 18°C, to mimic what occurs in the field (nighttime vs.

daytime), BioXcell had the lowest total and progressive motility in comparison to Caprogen and INRA96. In conclusion, diluent significantly affected sperm motility when stored for up to 3 d. Semen diluted in INRA96 resulted in a similar CR to semen diluted in Caprogen and to frozen-thawed semen, whereas that diluted in BioXcell resulted in a decreased CR. Consistent with this finding, semen diluted in BioXcell was less tolerant of temperature fluctuations than that stored in Caprogen or INRA96. Given that it can be used directly off the shelf, INRA96 may be a suitable alternative to Caprogen for the storage of liquid bull semen.

Key words: sperm, liquid semen, bovine, artificial insemination, calving rate

INTRODUCTION

Artificial insemination is the single most important technique devised to facilitate the genetic improvement of animals as it facilitates the widespread use of elite males (Black, 2006; Oliveira et al., 2013). Currently, within the Irish dairy industry, 95% of AI is conducted using frozen-thawed semen, with liquid (i.e., fresh, non-cryopreserved) semen accounting for only 5% of inseminations (Murphy et al., 2015). However, Ireland has a seasonal grass-based production system, and during the peak breeding season, from mid-April to early June, the use of liquid semen can increase to approximately 25% of inseminations to accommodate the large demand (Al Naib et al., 2011). In the Irish AI industry, a typical dose of liquid semen contains 5 million sperm (Murphy et al., 2013) in comparison to 15 to 20 million sperm for a typical frozen-thawed semen dose (Vishwanath et al., 1996). Liquid semen processing yields more doses per ejaculate, thereby, facilitating the greater utilization of genetically superior sires. This is particularly beneficial for young genomically selected sires as these sires are in high demand but produce lower semen volumes in comparison to more mature bulls (Brito et al., 2002).

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The extensive use of AI in the dairy industry can be partly attributed to the development of suitable diluents for both liquid and frozen-thawed semen (Foote, 2002). In Ireland, liquid semen is typically stored at ambient temperature in thermo-insulated containers to reduce the effects of the natural day to nighttime temperature fluctuations. Murphy et al. (2015) reported that although fluctuating storage temperature between 5 and 32°C had no effect on viability, total progressive motility was greatest for liquid semen stored at 15°C compared with other temperatures assessed. In addition to dilution of the semen, diluents provide protective compounds such as BSA, antioxidants, and antibiotics to maintain sperm function (Rehmana et al., 2013; van den Berg et al., 2014). Despite this, liquid semen has a limited shelf life and semen stored in Caprogen (the gold standard for liquid semen dilution) is principally used for only 2.5 to 3 d postcollection as a reduction in pregnancy rates has been reported thereafter (Vishwanath and Shannon, 2000). Several studies have been conducted on liquid bull semen diluents with the aim of combating the reduction in fertility associated with increased duration of storage (Vishwanath and Shannon, 2000; Verberckmoes et al., 2004; Murphy et al., 2015). Many of these studies have focused on reducing the metabolic activity of sperm cells as the survival of sperm for extended periods of time has been shown to be inversely related to their metabolic activity (Vishwanath and Shannon, 2000). Approaches taken have included storing semen at 5°C (Saha et al., 2014), reducing sperm concentration (Murphy et al., 2013), altering the pH (Ferdinand et al., 2014), as well as N₂ gassing and modifying the diluent composition (Shannon, 1968). The ability to extend the shelf life of liquid semen is important as (1) the distribution of liquid semen would be simplified if it could be used for more days, and (2) the work load involved in collecting and processing semen would be greatly reduced as bulls used for liquid semen would have a reduced collection schedule.

Apart from Caprogen, several other diluents have been developed for the storage of semen for a variety of domestic species. BioXcell is an animal protein-free medium (soy lecithin-based extender), which has been routinely used for the cryopreservation and preservation at chilled temperatures of buffalo (Akhter et al., 2010, 2011), ram (Kulaksiz et al., 2012), and bull semen (Stradaoli et al., 2007). Although the benefits of using lecithin-based extenders on the cryopreservation of bull semen have been reported by several authors (van Wagendonk-de Leeuw et al., 2000; Aires et al., 2003; Crespilho et al., 2014), many of these studies have focused on in vitro analysis only. INRA96 is a

milk-based diluent that was primarily developed to maintain the fertility of stallion semen during chilled storage at 4 or at 15°C for up to 72 h (Batellier et al., 1997) but has also been used for the cryopreservation of equine semen (Fayrer-Hosken et al., 2008; Pillet et al., 2008). In recent years, INRA96 has also been used as a storage medium for the sperm of other species including rabbit (De Amicis et al., 2004), dog (Sahashi et al., 2011), goat (López-Fernández et al., 2011), and sheep (Olivera-Muzante et al., 2011) and has yielded acceptable fertility in vivo in sheep following cervical AI after 24 h of storage (O'Hara et al., 2010). Other commercially available extenders include OptiXcell, a protein-free egg yolk-like medium for frozen and liquid bull semen; NutriXcell, a long-term extender primarily used for the preservation of boar semen up to 6 d (Kaeoket et al., 2010); AndroMed, an egg-yolk-free, soy lecithin-based medium for freezing of bull (Maxwell et al., 2007), ram (Fukui et al., 2008), buffalo (Herold et al., 2004), and goat semen (Gacitua and Arav, 2005); and BullXcell, an egg-yolk Tris extender used for bull semen cryopreservation. Although all of the aforementioned diluents have been used for the preservation of semen from domestic species, many of these studies have solely reported in vitro data and there is a dearth of published studies on the in vivo fertility of bull semen stored in the majority of these diluents.

Using a combination of in vitro assessments and a large-scale commercial field trial, the objectives of this study were to assess the effect of (1) liquid semen diluent on total and progressive sperm motility, (2) liquid semen diluent on CR, and (3) temperature fluctuation of liquid semen on total and progressive motility of liquid bull semen. To our knowledge, this is the first report to examine the effect of a large number of diluents on liquid bull semen during in vitro storage, where each treatment was prepared from the same ejaculate. This eliminates any potential confounding effects arising from the collection of diluent treatments from different bulls or ejaculates and thus provides clear and reliable data.

MATERIALS AND METHODS

Experiment 1: Storing Liquid Semen Maintained at a Constant Ambient Temperature

Experimental Design. The aim of this experiment was to assess the ability of 7 liquid semen diluents (1 homemade and 6 commercially available) to sustain total and progressive motility of bull sperm for up to 3 d postcollection. Semen was collected from Holstein Friesian bulls (n = 6) at a commercial AI center on

3 different occasions (occasion = replicate). The raw ejaculate was placed into a 50-mL tube and transported in a temperature-regulated cooler box at 18°C to the laboratory (up to 3 h transport). On arrival, the ejaculate was assessed for weight, sperm concentration using a Coulter counter (Z Series, Beckman Coulter, Clare, Ireland), total motility (%), and progressive motility on a 5-point scale (1 = twitching/no forward progressive motility; 5 = excellent forward progressive motile sperm) to ensure all semen samples were of a commercial standard. Microscopic assessments were conducted by the same technician and initial quality control cut-off values were a total and progressive motility of $\geq 70\%$ and a score of ≥ 3 , respectively, and any ejaculates failing to meet these criteria were rejected.

The raw ejaculate was split and diluted to achieve a concentration of 5×10^6 sperm per 0.25-mL insemination dose in 1 of 7 different diluents, namely Caprogen (homemade as per Vishwanath and Shannon 2000), OptiXcell (IMV Technologies, Normandy, France), BioXcell (IMV Technologies), BullXcell (IMV Technologies), INRA96 (IMV Technologies), NutriXcell (IMV Technologies), and AndroMed (Minitube, Tiefenbach, Germany). Prior to dilution, the Caprogen diluent was purged in food fresh nitrogen gas (BOC, Dublin, Ireland) as per standard preparation procedures to dispel oxygen from the media and create an anaerobic environment, limiting the metabolic activity of sperm during liquid storage (Vishwanath and Shannon, 2000). All other diluents were prepared as per the manufacturers' instructions. Semen from each bull was kept separate and ejaculates were split such that each bull was represented in each treatment. Semen straws were filled as per routine procedures and placed in a temperature-regulated cooler box at 18°C. Samples from the different treatments were assessed in a randomized sequence to remove bias as a result of sampling order. Total and progressive motility; $n = 3$ replicates) were assessed in vitro on d 0, 1, 2, and 3 postcollection (d 0 = 4 h after collection). Within each replicate, on each assessment day, 2 straws from each bull ($n = 6$) for each diluent ($n = 7$) were assessed.

Assessment of Sperm Motility. Sperm motility (total and progressive) in liquid semen was assessed on d 0, 1, 2, and 3 postsemen collection using a phase contrast microscope (CX31, Olympus, Centre Valley, PA) at a magnification of 400 \times . A droplet of diluted semen (5 μ L) was placed on a pre-warmed glass slide, covered with a pre-warmed coverslip (37°C), and assessed by counting a minimum of 100 sperm, over at least 5 different fields of view, for each treatment on each assessment day. Total motility was expressed as a percentage of the total sperm population (motile and

nonmotile). A sperm was deemed to display progressive motility if it moved in a linear fashion; progressive motility was expressed as the percentage of live and motile sperm that displayed forward progressive motion.

Experiment 2: Field Fertility of Liquid Semen Diluted in Caprogen, BioXcell, and INRA96

Experimental Design. The aim of this experiment was to assess the effect of 3 liquid semen diluents, selected based on the outcome of experiment 1, on CR following AI. Semen was collected from Holstein Friesian bulls ($n = 8$; denoted A–H) at a commercial AI center from early May to the end of May 2015. There were 11 collection days in total, with 2 bulls used per collection day (total of 22 ejaculates). Following assessment for volume, concentration, and motility (as described in experiment 1), each acceptable ejaculate was split into 3 equal volumes and diluted directly to a final concentration of 5×10^6 sperm per 0.25-mL insemination dose in 1 of 3 diluents; namely, Caprogen, BioXcell, or INRA96 (Figure 1). All 3 diluents were prepared as per experiment 1. Each batch of liquid semen was clearly labeled and distributed for insemination on the day of collection. Liquid semen was used for up to 3 d post-collection on both heifers ($n = 192$) and multiparous ($n = 9,611$) dairy cows. Due to logistical constraints, frozen-thawed semen doses were derived from previously collected ejaculates from the same 8 bulls, which were processed and frozen using routine procedures ($n = 9,323$ inseminations consisting of 526 heifers and 8,797 multiparous dairy cows). Upon collection of semen samples for cryopreservation, the raw ejaculate was partially diluted in 10 mL of pre-warmed BullXcell (37°C). Semen samples were assessed for volume, sperm concentration, and total and progressive motility as described in experiment 1. Only ejaculates achieving a total motility score of $\geq 70\%$ and a progressive motility score of ≥ 3 were used for cryopreservation. Following in vitro assessments, the semen was fully extended with pre-warmed BullXcell to achieve a concentration of 15×10^6 sperm per 0.25-mL insemination dose. Straws were frozen to -140°C as follows: -5°C per min from $+4$ to -10°C , -40°C per min from -10 to -100°C and thereafter -20°C per min from -100 to -140°C in a programmable freezer (IMV Technologies), followed by submersion and storage in liquid nitrogen at -196°C until use.

Field Inseminations. Inseminations were carried out in May 2015 (coinciding with the peak dairy breeding season) in Irish dairy herds ($n = 2,490$). The majority of inseminations were in Holstein Friesian cows ($n = 18,304$), but small numbers of cows of other breeds were

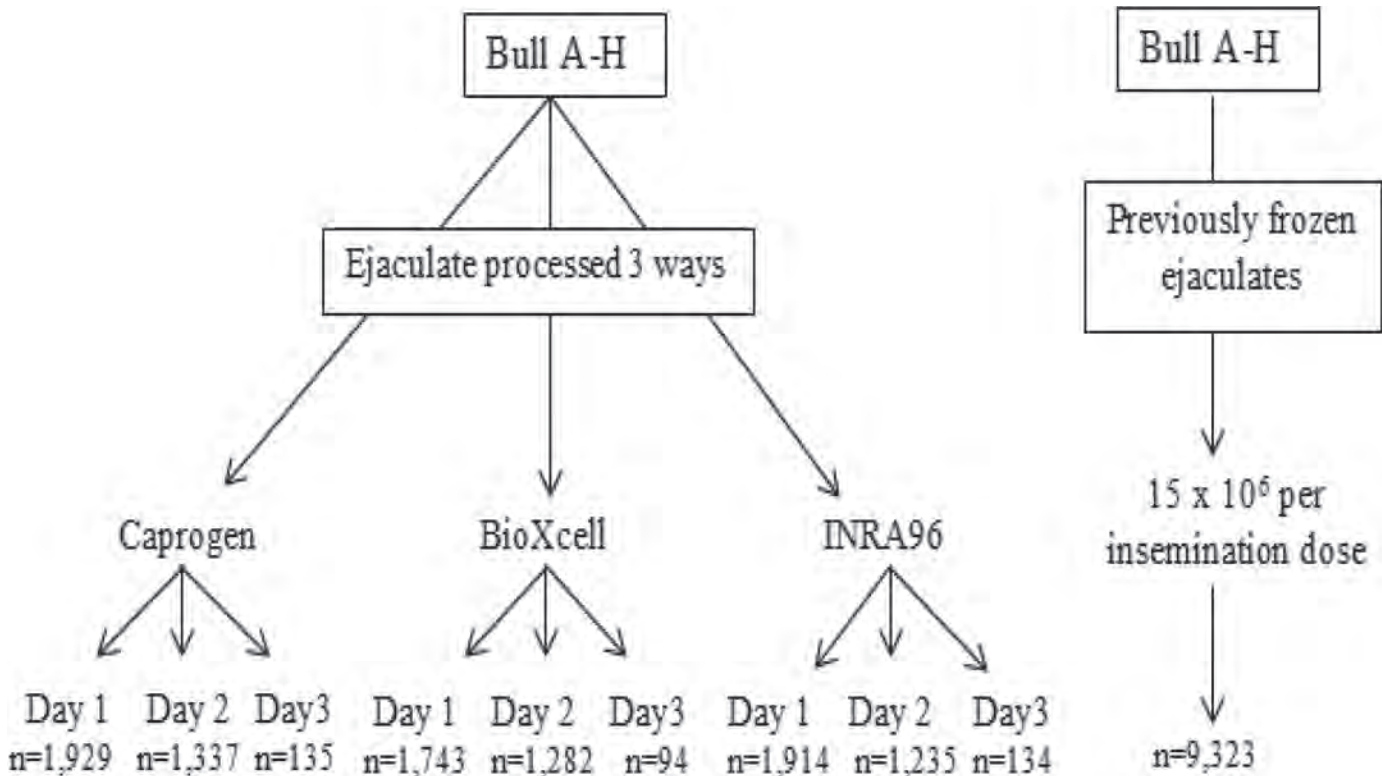


Figure 1. Experimental design for experiment 2. Ejaculates were split for the liquid semen treatments, and previously frozen ejaculates from the same bull were sourced for the frozen-thawed treatment (n = the total number of inseminations per diluent per day). Caprogen (homemade as per Vishwanath and Shannon, 2000), BioXcell (IMV Technologies, Normandy, France), and INRA96 (IMV Technologies).

represented: Jersey (n = 375), Montbeliarde (n = 113), Norwegian Red (n = 268), Swedish Red cows (n = 10), and other (n = 56; includes Normande, Rotbunte, Danish Red, and Red Poll). Technicians (n = 108) were grouped into geographical areas and treatments were rotated on each collection day to ensure that technicians received different diluent treatments from each of 2 bulls on each day (Figure 2). Technicians were blind to treatments. For each insemination, the AI technician recorded the bull code, cow tag number, and straw code on a handheld electronic device. Insemination and CR data were captured using the Irish Cattle Breeding Federation (Bandon, Co. Cork, Ireland) database by cross-referencing the technician name with the bull code and semen type used on each date within the trial period. Obvious errors were extracted from the data set and data were then interrogated to remove animals based on the following criteria: cows which were not at first AI, cows which received 2 inseminations from 2 different bulls or diluent treatments, or cows that were not of a dairy breed. However, if a cow received 2 inseminations from the same bull with the same diluent treatment within 5 d of each other, the record was

kept and the second date was assumed to be correct. Postediting, a total of 19,126 inseminations remained. The CR was measured using a cut-off value of 275 and 290 d from the date of insemination to calving date.

Experiment 3: Effect of Temperature Fluctuations on Semen

Based on the outcome of experiment 2, we hypothesized that the effect of fluctuating temperatures experienced in practice during storage of liquid semen would be different for semen diluted in Caprogen, BioXcell, and INRA96. Semen was collected from Holstein Friesian bulls (n = 6) at a commercial AI center on 3 occasions (occasion = replicate). Semen from each bull was kept separate and ejaculates were assessed and diluted in Caprogen, BioXcell, and INRA96 (as per experiment 2). After packaging, straws were placed in a polystyrene box and stored at 18°C during the day and gradually brought to 4°C during the night to mimic the unregulated temperature fluctuations to which liquid semen is typically subjected when stored in thermo-insulated containers in practice (Murphy et al., 2015). Samples

from the different treatments were assessed on d 0, 1, 2, and 3 postcollection in a randomized sequence to remove bias as a result of sampling order. Total and progressive motility was assessed in vitro as described in experiment 1 using a computer-assisted sperm analyzer (IVOS II, IMV Technologies).

Statistical Analysis

Data from experiments 1 and 3 were examined for normality of distribution and homogeneity of variance and analyzed using the general linear model (GLM) repeated-measures procedure with a compound sym-

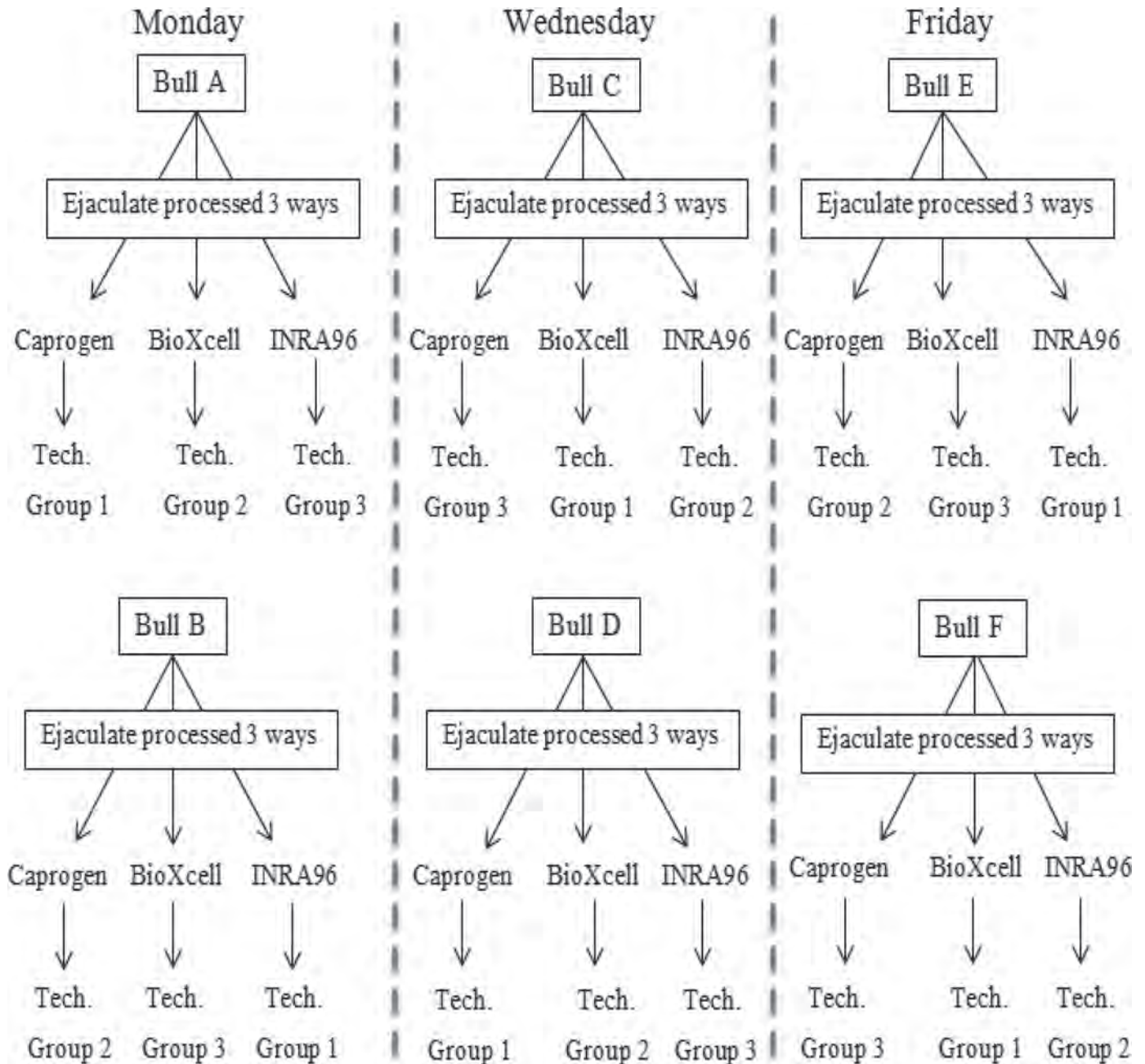


Figure 2. Experimental design for experiment 2, highlighting the distribution of different liquid semen treatments to technicians within a week (displaying bulls A-F only). Each technician received 2 different treatments each day. Frozen-thawed semen was also inseminated throughout the period. Caprogen (homemade as per Vishwanath and Shannon, 2000), BioXcell (IMV Technologies, Normandy, France), INRA96 (IMV Technologies).

metry covariance structure in Statistical Package for Social Science (SPSS, version 22.0, IBM, Chicago, IL). The final model included the main effects of diluent treatment, day, and their interaction. In experiment 2, the CR data from the field trial were assessed using Pearson's chi-squared procedure in SPSS to compare CR between diluent treatments. Data were cross checked using an ANOVA model. The dependent variable in the analysis was CR (1 = calved, 0 = not calved). In addition, using a general linear model for binomial data CR was evaluated and correlations were investigated with several fixed effects, namely, diluent treatment, bull, parity number, cow breed, cow fertility sub-index, DIM, herd, and technician. Each fixed effect was assessed for an interaction with diluent treatment. All post-hoc tests were carried out using the Bonferroni test and results are reported as the mean \pm the standard error of the mean in experiments 1 and 3 and as the estimated marginal mean in experiment 2, to adjust for imbalance between the number of inseminations in each treatment. Data were considered to differ significantly at $P < 0.05$.

RESULTS

Experiment 1: Storing Liquid Semen Maintained at a Constant Ambient Temperature

An effect was observed of diluent ($P < 0.01$) and day ($P < 0.05$) on both total and progressive motility; from d 0 to 3 across all treatments the percentage of motile sperm declined linearly. No diluent by day interaction ($P > 0.05$) was found. Caprogen, BioXcell, and INRA96 maintained d 3 sperm total motility (64.0 ± 2.66 , 58.5 ± 2.83 , and $58.0 \pm 2.35\%$, respectively) and progressive motility (59.2 ± 3.18 , 47.5 ± 2.36 , and $56.8 \pm 1.59\%$, respectively) at the highest levels. Sperm stored in BullXcell and AndroMed had intermediate total and progressive motility scores, whereas NutriXcell and OptiXcell had the lowest total and progressive motility on d 3, respectively (Figure 3).

Experiment 2: Field Fertility of Liquid Semen Diluted in Caprogen, BioXcell, and INRA96

Effect of Diluent on Calving Rate. Overall, insemination with liquid semen on d 1, 2, and 3 postcollection resulted in a lower CR (52.7, 47.3, and 47.5%, respectively) in comparison to frozen-thawed semen (59.7%, $P < 0.01$; Figure 4). However, this was attributed to the poor CR recorded for BioXcell on d 1, 2, and 3 of storage, as semen diluted in Caprogen and INRA96 and stored for up to 3 d had a similar CR compared with frozen-thawed semen. Semen diluted in BioXcell

had a lower CR following storage for 1, 2, or 3 d after collection in comparison with Caprogen, INRA96, and frozen-thawed semen ($P < 0.01$; Figure 5).

Effect of Bull on Calving Rate. An effect was observed of bull on CR ($P < 0.01$) with the CR for individual bulls varying from 54.7 to 67.3%. A bull by day interaction ($P < 0.01$) was observed, represented by bulls B and D having a higher CR on d 1 than liquid semen on d 2 ($P < 0.05$) but did not differ from d 3 ($P > 0.05$). Bull F had a higher CR for liquid semen on d 1 than liquid semen on d 3 ($P < 0.05$) but did not differ from liquid semen inseminated on d 2 ($P > 0.05$). Although mean CR following AI with liquid semen on d 2 was reduced in comparison to d 1 in all bulls with the exception of bull E, this reduction was only statistically significant in 2 bulls (B and D; Table 1). However, bulls B and G had a higher CR when frozen-thawed semen was used in comparison to liquid semen on d 1 and 2, respectively ($P < 0.01$; Table 1), but this was primarily a result of the poor CR recorded for BioXcell.

Effect of Cow Characteristics, Herd, and Technician on Calving Rate. An effect was found of parity, cow fertility sub-index, and DIM on CR ($P < 0.01$). The CR varied between individual herds and technicians, for herds and technicians with greater than 20 and 100 recorded inseminations, respectively ($P < 0.01$). A parity by diluent treatment interaction was found but with no clear biological pattern ($P < 0.01$). A cow fertility sub-index by treatment interaction ($P < 0.01$) was observed as the CR of cows and heifers inseminated with BioXcell, INRA96, and frozen-thawed semen increased with increasing cow fertility sub-indexes, whereas this trend was present but not statistically significant for semen diluted in Caprogen (Figure 6). No effect was found of breed or a breed, herd, or technician by diluent interaction ($P > 0.05$).

Experiment 3: Effect of Temperature Fluctuations on Semen

When temperature was fluctuated, diluent ($P < 0.01$) and day ($P < 0.01$) had an effect on both total and progressive motility. Semen stored in Caprogen and INRA96 recorded a greater total and progressive motility score than BioXcell ($P < 0.01$) but did not differ from each other ($P > 0.05$; Figure 7). From d 0 to 3 across all treatments, the percentage of sperm displaying total and progressive motility declined linearly, but no diluent by day interaction was found ($P > 0.05$). Fluctuation of temperature between 4 and 18°C was detrimental to motility of sperm stored in BioXcell as both total and progressive motility declined with increased duration of storage from 62.3 ± 4.61 to $37.4 \pm 10.0\%$, and 54.4 ± 18.7 to $35.4 \pm 10.2\%$, respectively,

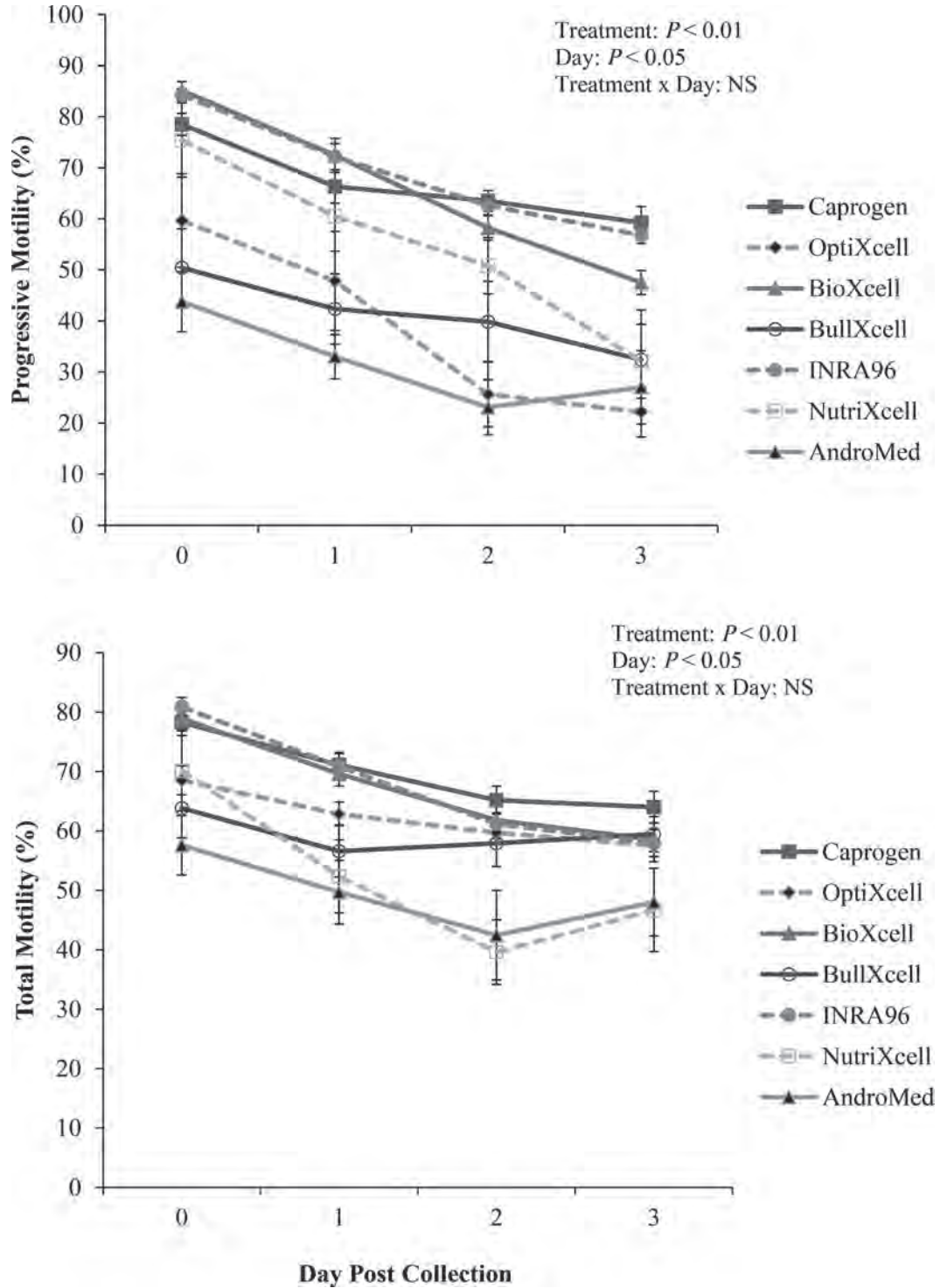


Figure 3. The effect of diluent on progressive motility (upper panel) and total motility (lower panel) of liquid bull semen on d 0, 1, 2, and 3 postcollection (experiment 1). Vertical bars represent SEM. Caprogen (homemade as per Vishwanath and Shannon, 2000), OptiXcell (IMV Technologies, Normandy, France), BioXcell (IMV Technologies), BullXcell (IMV Technologies), INRA96 (IMV Technologies), NutriXcell (IMV Technologies), and AndroMed (Minitube, Tiefenbach, Germany).

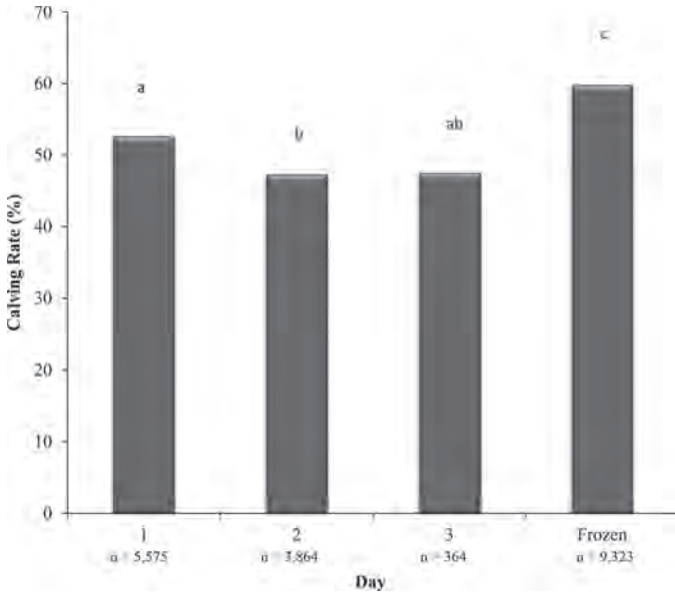


Figure 4. The effect of day of storage of liquid bull semen on calving rate in dairy cows and heifers (experiment 2). Values with different superscripts differ significantly ($P < 0.01$).

from d 0 to 3 of storage. Sperm stored in Caprogen and INRA96 maintained total (65.7 ± 1.98 and $65.7 \pm 5.02\%$, respectively) and progressive motility ($52.5 \pm 3.58\%$ and $59.6 \pm 5.04\%$, respectively) up to 3 d of storage (Figure 7).

DISCUSSION

Despite the importance of AI to the dairy industry, there is a dearth of published studies comparing the field fertility of semen stored in different diluents. We have taken the approach of using split ejaculates and a combination of in vitro and in vivo assessments in a comprehensive attempt to identify the optimal semen diluent for liquid bull semen. The main findings of this study were that (1) liquid semen diluted in Caprogen or INRA96 resulted in a similar CR to frozen-thawed semen; (2) liquid semen stored in BioXcell resulted in a reduced CR in comparison to semen diluted in Caprogen or INRA96 and frozen-thawed semen; and (3) temperature fluctuation between 4 and 18°C was detrimental to sperm motility when diluted in BioXcell but not in Caprogen or INRA96.

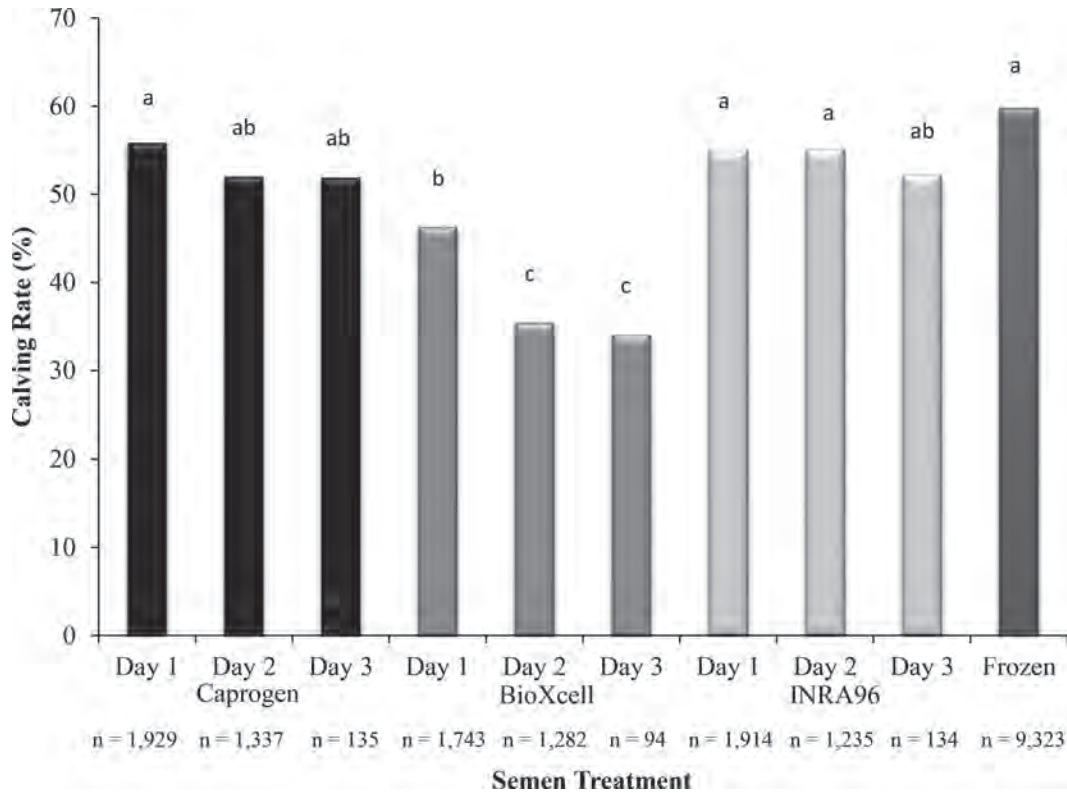


Figure 5. The effect of liquid semen diluent and day of storage on calving rate in dairy cows and heifers (experiment 2). Values with different superscripts differ significantly ($P < 0.01$). Caprogen (homemade as per Vishwanath and Shannon, 2000), BioXcell (IMV Technologies, Normandy, France), and INRA96 (IMV Technologies).

Table 1. The effect of liquid bull semen inseminated on d 1, 2, or 3 postcollection and frozen-thawed semen on calving rate in dairy cows and heifers (experiment 2)

Bull	Liquid d 1, % calving rate (n)	Liquid d 2, % calving rate (n)	Liquid d 3, % calving rate (n)	Frozen-thawed, % calving rate (n)
A	53.3 (880)	47.2 (697)	46.2 (13)	51.9 (376)
B	53.4 ^a (1,836)	47.4 ^b (1,453)	73.1 ^{abc} (26)	60.0 ^c (1,106)
C	55.1 (234)	52.2 (113)	68.4 (19)	64.4 (877)
D	52.3 ^a (457)	41.5 ^b (415)	54.6 ^{ab} (11)	57.7 ^a (2,593)
E	44.4 ^a (531)	51.1 ^a (276)	43.9 ^a (139)	61.3 ^b (741)
F	54.8 ^a (765)	47.9 ^{ab} (386)	42 ^b (150)	59.8 ^a (801)
G	34.2 ^a (79)	36.8 ^a (57)	0 (0)	61.1 ^b (2,272)
H	54.9 ^{ab} (793)	50.1 ^a (467)	83.3 ^{ab} (6)	59.4 ^b (557)
Overall	52.6 ^a (5,575)	47.3 ^b (3,864)	47.5 ^{ab} (364)	59.7 ^c (9,323)

^{a-c}Values in the same row with different superscripts differ significantly ($P < 0.01$).

Regardless of storage temperature, sperm motility, and thus fertility, declines over an extended period of time (Vishwanath and Shannon, 2000; Akhter et al., 2011). In agreement, the results of this study demonstrated that irrespective of semen extender, semen

quality, measured in terms of total and progressive motility, declined with increased duration of storage. When liquid semen was stored at a constant temperature, Caprogen, BioXcell, and INRA96 maintained better total and progressive motility than AndroMed,

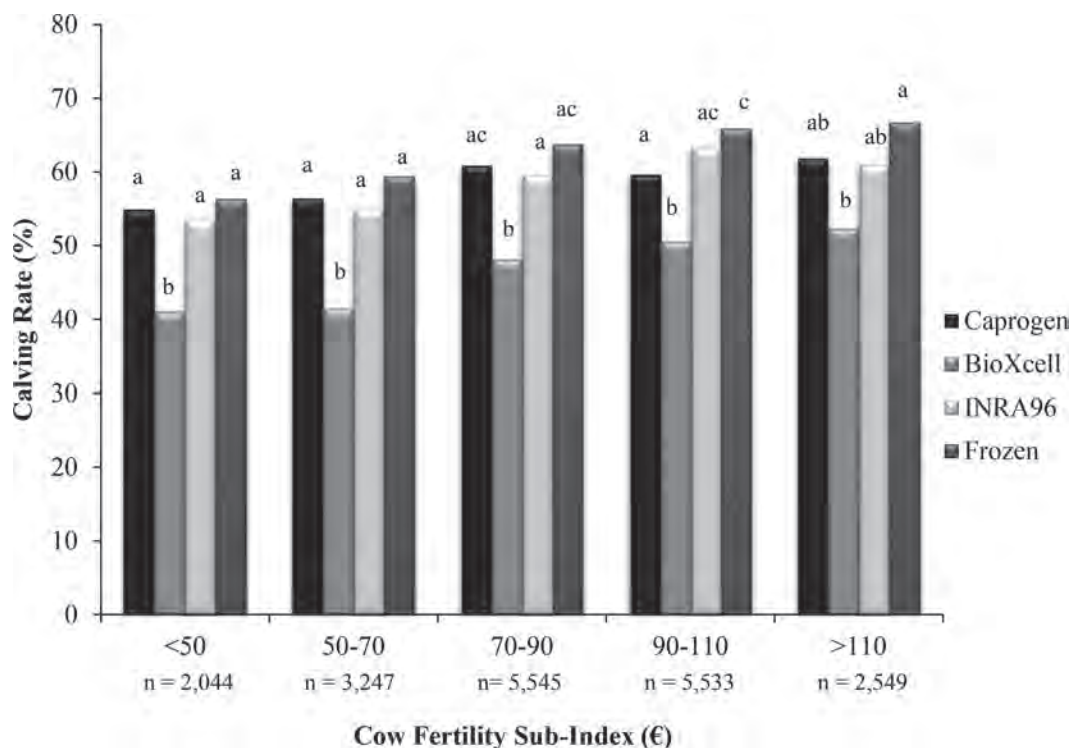


Figure 6. The effect of cow fertility sub-index by treatment interaction on calving rate in dairy cows and heifers (experiment 2). Values with different superscripts differ significantly within fertility sub-indices ($P < 0.01$). Caprogen (homemade as per Vishwanath and Shannon, 2000), BioXcell (IMV Technologies, Normandy, France), and INRA96 (IMV Technologies).

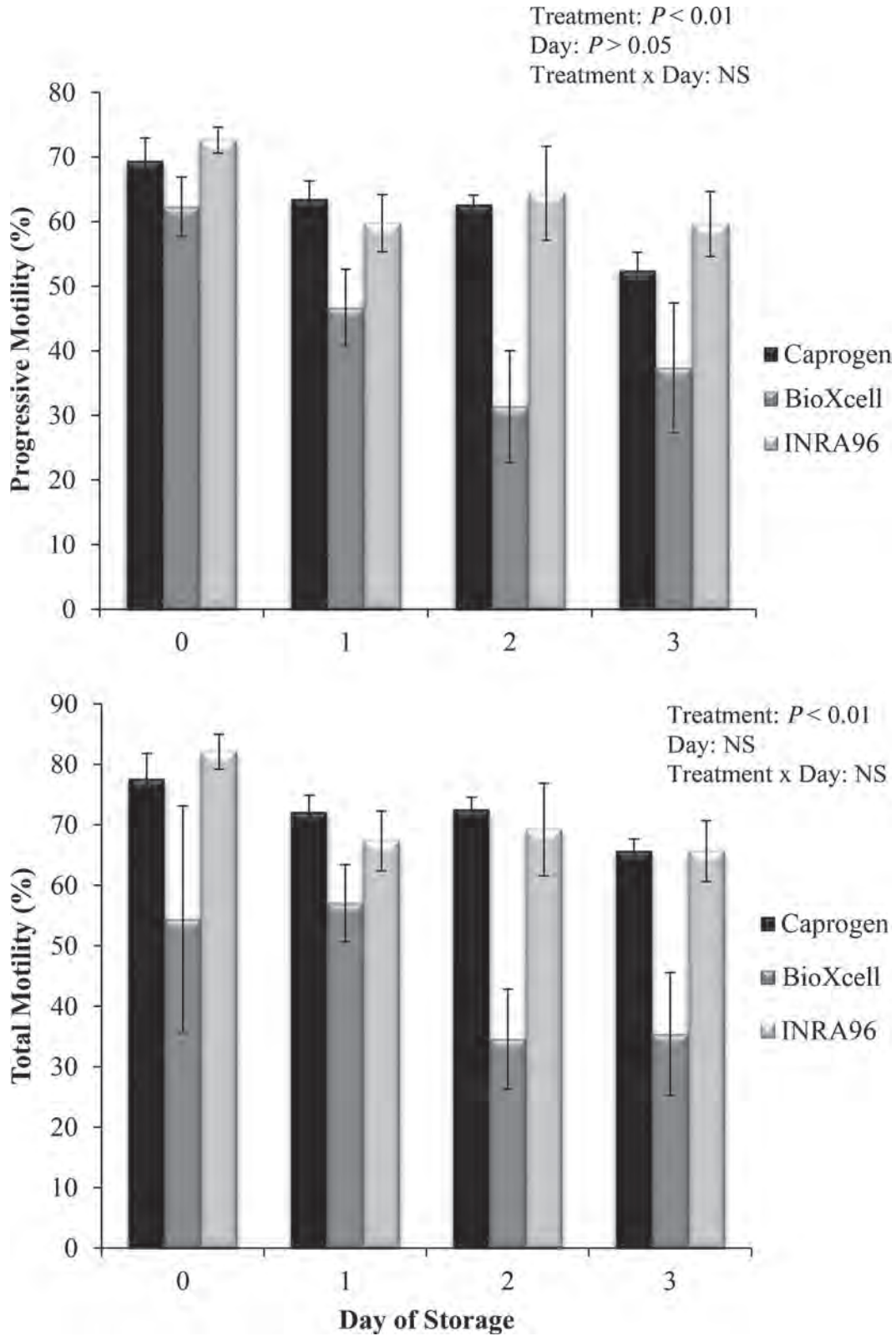


Figure 7. The effect of temperature fluctuation on progressive motility (upper panel) and total motility (lower panel) of liquid bull semen stored in 3 diluents and assessed on d 0, 1, 2, and 3 postcollection (experiment 3). Vertical bars represent SEM. Caprogen (homemade as per Vishwanath and Shannon, 2000), BioXcell (IMV Technologies, Normandy, France), and INRA96 (IMV Technologies).

BullXcell, NutriXcell, and OptiXcell. Caprogen, the industry standard liquid bull semen diluent, although initially developed for the dilution of semen at 5°C (Shannon 1965), has been shown to maintain sperm motility for extended periods and result in higher fertility rates when stored at an ambient temperature of 18°C (Shannon et al., 1984). The results of this study support this finding as Caprogen resulted in the highest total motility score on d 3 of storage when stored at ambient temperature. INRA96, although not primarily used for the preservation of bull semen, performed well in comparison to Caprogen and had the highest total and progressive motility score when temperatures were fluctuated between 4 and 18°C. O'Hara et al. (2010) reported that storing ram semen in INRA96 at a constant 15°C resulted in significantly reduced sperm motility but when stored at a constant 5°C INRA96 performed well both in terms of *in vitro* and *in vivo* fertility. In the current study, INRA96 recorded the second highest and highest total and progressive motility score, respectively, on d 3 compared with the other diluents when semen was stored at a constant temperature of 18°C.

BioXcell (soy lecithin-based extender) has been used routinely as a cryopreservation medium for buffalo, ram, and bull semen, and several studies have compared its use on frozen-thawed semen against other commercially available diluents in these species (Gil et al., 2003; Stradaoli et al., 2007; Sharafi et al., 2009; Chaudhari et al., 2015). Both Gil et al. (2003) and Sharafi et al. (2009) reported a lack of difference in motility and capacitation status between ram semen diluted in BioXcell and the control extender (L1G7 and milk-egg yolk based extender, respectively). Similarly, Gil et al. (2003) reported no difference in fertility, suggesting that although BioXcell did not improve sperm quality *in vitro* or *in vivo* it could offer a safer alternative to preserving ram semen due to the reduced health risk associated with animal protein-free media. Stradaoli et al. (2007) reported BioXcell to be superior in preserving post-thaw bull sperm motility as well as lowering the proportion of acrosome-reacted and capacitated sperm in comparison to a traditional egg-yolk Tris glycerol extender. An *in vitro* study on liquid stored buffalo semen by Akhter et al. (2011) demonstrated that semen quality parameters for BioXcell were comparable to both milk and egg-yolk-based extenders up to d 3 of storage but were higher for BioXcell on d 5 when semen was stored at a constant temperature of 5°C (the manufacturer's recommended storage temperature of fresh semen diluted in BioXcell). Kasimanickam et al. (2011) stated that soy-based extenders had similar protection capabilities in liquid ram semen as an egg-yolk extender and were superior to liquid semen diluted in a milk-based diluent. In contrast to these studies, in the

current study, although BioXcell performed well up to d 3 of storage when maintained at a constant ambient temperature, the *in vivo* fertility results were inferior to those recorded by both Caprogen and INRA96.

The reduction in fertility associated with BioXcell observed in this study would indicate that BioXcell is less tolerant of temperature fluctuations and thus would not be a suitable substitute for an egg-yolk or milk-based extender if liquid semen was subjected to temperature variations. One possible explanation for this reduction is that as semen temperature fluctuates, morphological membrane changes consistent with a lipid phase transition occurring (Drobnis et al., 1993). This results in compacting or relaxing the packing of the phospholipid bilayer, causing membrane destabilization and ultimately cell death (Crowe et al., 1990). Thus, maintaining semen at a constant ambient temperature results in regulating this transition phase and reduces the damage sustained to sperm cells (Crowe et al., 1998). As well as this, a family of lipid-binding proteins (bovine seminal plasma proteins; **BSP**) found in seminal plasma are among the principal causes of damage to sperm cells during storage (Bergeron and Manjunath, 2006). It is believed that low density lipoproteins found in egg-yolk and casein micelles found in milk interact with BSP proteins to reduce the lipid loss from the sperm membrane, thus stabilizing the membrane and maintaining sperm function during storage (Bergeron et al., 2007; Lusignan et al., 2011; Manjunath, 2012). These low-density lipoproteins are also believed to be important in protecting sperm cells during the lipid phase transition (Holt, 2000) and as the protective action of casein micelles on sperm is thought to be analogous to the protective action of lipoproteins in egg yolk (Bergeron et al., 2007), it could be postulated that casein micelles are the effective component in milk-based diluents also protecting cells from the damage arising from the lipid phase transition.

Caprogen and INRA96 resulted in similar CR, suggesting that both diluents are capable of efficiently protecting the sperm membrane from excessive BSP protein binding, which results in the loss of cholesterol and phospholipids, resulting in a deleterious effect on sperm function (Bergeron and Manjunath, 2006). The results also highlight that both Caprogen and INRA96 are efficient at maintaining sperm quality when semen is stored at either a constant or fluctuating temperature, suggesting that they are both capable of stabilizing the sperm membrane during the lipid phase transition. BioXcell displayed an ability to protect sperm cells when semen was stored at a constant temperature; however, during temperature fluctuations its protection was inferior to both Caprogen and INRA96, as evidenced by a reduction of approximately 30 and

20% in total and progressive motility on d 3 of storage, respectively, in comparison to Caprogen and INRA96. The reason for this is unclear, but as soy lecithin (a plant source) is used in animal-protein free medium to substitute for the phospholipids in egg yolk and casein micelles in milk-based diluents, plant-based diluents may be less efficient in protecting sperm cells from temperature variations. Further research is required to understand the exact mechanisms of protection offered to sperm cells by BioXcell.

AndroMed, also a soy-lecithin-based extender, has been reported to result in better postthaw sperm quality than milk-based extenders in the goat (Jiménez-Rabadán et al., 2012), yielded similar pregnancy rates in frozen-thawed semen compared with an egg-yolk extender in the ram (Fukui et al., 2008) and resulted in higher nonreturn rates in frozen-thawed semen when compared with a Tris-egg-yolk-based extender in the bull (Aires et al., 2003). de Paz et al. (2010) reported that liquid ram semen diluted in a soybean lecithin extender maintained higher sperm motility and viability at 5 and 15°C of storage in comparison to a control egg-yolk extender. These studies suggest that a soy lecithin-based extender can be just as effective in preserving semen, if not better, than egg-yolk and milk-based diluents. In the current study, however, semen diluted in AndroMed performed poorly in comparison to the egg yolk (Caprogen) and milk-based (INRA96) diluent used for liquid semen in this study. BullXcell is used routinely in the cryopreservation of bull semen and performed adequately when storing liquid semen at a constant temperature. However, Caprogen resulted in superior semen quality in terms of total and progressive motility in comparison to BullXcell. NutriXcell, which is primarily used for the preservation of boar semen, and OptiXcell, a protein-free egg-yolk-like diluent, were the least effective extenders in maintaining total and progressive motility of bull semen, respectively, when stored at a constant ambient temperature. Although these extenders have been used for the preservation of sperm from several species, limited data have been published investigating their use against other diluents using bull semen.

In the current study, sperm were stored at 5 million per dose in all treatments compared with 1 to 2 million sperm, which are reported to be the norm in New Zealand (Vishwanath and Shannon, 2000). It is acknowledged that sperm metabolic activity is inversely related to extended sperm survival (Vishwanath and Shannon, 2000) and that higher sperm numbers during storage of liquid semen results in increased oxidative stress due to the production of reactive oxygen species (**ROS**; Murphy et al., 2013). The addition of components such

as citric acid, catalase, and glycerol in a wide range of diluents play a role in reducing the levels of peroxide generated in the storage medium. Other unique factors such as the use of nitrogen gassing and modifying the percentage of egg yolk, milk, or lecithin are also effective in reducing sperm metabolic activity and the effects of ROS production. Previous studies have reported that the production of ROS may be linked to an aging effect on sperm as ROS production ultimately leads to an apoptotic cascade in which sperm lose their motility, DNA integrity, and vitality (Aitken et al., 2012). Murphy et al. (2015) reported a lower nonreturn rate on d 2 of storage; however, this was only evident in 50% of the bulls tested, suggesting that individual bulls may be more susceptible to the aging process. The current study supports the notion of a sperm aging effect as a similar reduction in CR was recorded on d 2 of storage in 2 bulls used in this study. However, no significant decline occurred in CR in the other 6 bulls from d 1 to 2 of storage.

It is well documented that the physiological status of nulliparous heifers differs from that of lactating cows (Murphy et al., 2015) and that cows with a greater number of DIM more likely to conceive (Hillers et al., 1984), which are both consistent with the findings of this study. As the Irish dairy industry is a seasonal grass-based production system, a high level of fertility is critical to maximize reproductive efficiency within dairy herds, contributing to reducing costs associated with reproductive inefficiencies such as increased calving intervals, involuntary culling (Esslemont et al., 2001), labor costs, as well as increased costs associated with repeated AI (Shalloo et al., 2014).

CONCLUSIONS

In conclusion, Caprogen, BioXcell, and INRA96 maintained total and progressive sperm motility for a longer period of storage in comparison to OptiXcell, BullXcell, NutriXcell, and AndroMed when diluted semen was stored at a constant temperature. Storing semen at fluctuating temperatures between 4 and 18°C had no effect on motility when semen was stored in Caprogen and INRA96, but compromised the motility of liquid bull semen stored in BioXcell, indicating that BioXcell has a reduced ability to protect sperm during temperature fluctuations. The dilution and storage of liquid bull semen in INRA96 resulted in a similar CR to semen diluted in the industry standard, Caprogen. Given that INRA96 can be used directly off the shelf, it may be a suitable alternative to Caprogen for the storage of liquid bull semen.

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Effect of storage temperature, nitrogen gassing and sperm concentration on the *in vitro* semen quality and *in vivo* fertility of liquid bull semen stored in INRA96

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ABSTRACT

The aim of this study was to assess the effect of storage temperature, nitrogen (N₂) gassing and sperm concentration on *in vitro* characteristics and calving rate (CR) following artificial insemination (AI) of liquid bull semen stored in INRA96. In Experiment 1 the effect of liquid bull semen diluted in either N₂ bubbled or non-bubbled INRA96 at a concentration of 5×10^6 sperm per 0.25 mL insemination dose and stored at 5 or 15 °C was assessed subjectively for total and progressive motility on Days 0, 1, 2, 3 and 4 post collection. In Experiment 2a, the effect of stored liquid semen at three sperm concentrations (3, 4 or 5×10^6 sperm per 0.25 mL insemination dose) on total and progressive motility was assessed subjectively on Days 0, 1 and 2 post collection. In Experiment 2b, the field fertility of liquid semen stored at ambient temperature at a concentration of 3, 4 or 5×10^6 sperm per 0.25 mL dose and inseminated on Days 1 or 2 post collection was assessed in comparison to frozen-thawed semen (total of n = 5742). In Experiment 1, total and progressive motility decreased with increased duration of storage ($P < 0.01$); however, there was no effect of N₂ bubbling on motility on Days 0, 1, 2, 3 and 4 of storage. There was an effect of temperature on total and progressive motility, regardless of treatment, as semen stored at 15 °C recorded higher motility values than semen stored at 5 °C ($P < 0.01$). In Experiment 2a, there was no effect of sperm concentration on total or progressive motility on Days 0, 1 or 2 of storage. There was a linear decrease in motility with increased duration of storage ($P < 0.01$); however, there was no sperm concentration by day interaction. In Experiment 2b, there was an effect of sperm concentration on CR ($P < 0.01$); semen diluted to 3 and 4×10^6 sperm per dose resulted in a lower CR after 2 days of storage (41.1 and 44.7%, respectively) in comparison to frozen-thawed semen (55.2%) but did not differ to CR of semen diluted to 5×10^6 sperm per dose on Day 2 of storage. There was an effect of parity, fertility sub-index and days in milk (DIM) at AI on CR ($P < 0.01$). In conclusion, N₂ bubbling and sperm concentration had no effect on *in vitro* sperm motility of liquid semen, but this study demonstrated a reduction in CR on Day 2 of storage at lower sperm concentrations in comparison to frozen-thawed semen.

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1. Introduction

Seasonal grass-based dairy production systems depend on compact breeding during mid-April to early June in the Northern Hemisphere, in order to coincide milk production with grass growth [1]. Artificial insemination (AI) is the single most important

technique devised to facilitate the genetic improvement of animals [2,3] and currently, within the Irish dairy industry, 95% of AI is conducted using frozen-thawed semen, with liquid (i.e., fresh, non-cryopreserved) semen accounting for only 5% of annual inseminations [4]. However, the use of liquid semen increases to approximately 25% during the peak breeding season in order to accommodate the large demand [5]. Although liquid bull semen has traditionally been stored at ambient temperature in the egg-yolk based diluent, Caprogen, Murphy et al. [6] indicated that bull semen stored in INRA96 (a commercially available milk-based

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diluent) had a comparable calving rate (CR) to Caprogen but was more convenient for the busy working schedule of an AI centre as it could be used directly off the shelf.

In Ireland a typical liquid semen dose contains 5×10^6 sperm, irrespective of its usage day [7], in comparison to 15×10^6 sperm for a typical frozen-thawed semen dose [8]. Thus, liquid semen processing yields more doses per ejaculate, thereby facilitating the greater utilisation of genetically superior sires. This is particularly beneficial for young genomically-selected sires as these sires are in high demand but produce lower semen volumes in comparison to more mature bulls [9]. An added advantage is that unlike frozen-thawed semen, which must undergo a 30 day quarantine period in the European Union, liquid semen can be released for insemination on the day of collection. Despite its advantages, liquid semen has a limited shelf life and is normally used for only 2.5–3 days post collection as a reduction in pregnancy rates has been reported thereafter [10]. In order to combat this reduction in fertility a number of investigative avenues have been exploited with many studies focusing on reducing the metabolic activity of sperm, as sperm survival for extended periods of time has been shown to be inversely related to their metabolic activity [10]. Approaches taken include; altering storage temperatures [11], reducing sperm number in semen diluted in Caprogen [4] as well as N_2 gassing and modifying diluent composition [12], to name but a few.

It is widely acknowledged that reduced storage temperatures and N_2 gassing of media are two primary methods of reducing metabolic activity of sperm. However, storing semen at lower temperatures (ie: 5°C) can cause membrane damage and has been reported to cause a rise in intracellular levels of sodium to cytotoxic levels as the activity of the sodium-potassium pump declines [13]. Storage at ambient temperatures (ie: $15\text{--}20^\circ\text{C}$) avoids the damage sustained by reduced temperatures but it has been postulated that the production of reactive oxygen species is accelerated at higher compared to lower storage temperatures [15,16]. Protocols to reduce sperm metabolic activity at ambient temperature were devised such as N_2 gassing [14] to disperse oxygen in the media creating an anaerobic environment. INRA96, while marketed for use at 4°C in an aerobic environment, has demonstrated sufficient protection capabilities to bull sperm when stored at ambient temperature [6], however, whether the use of N_2 gassing of INRA96 is beneficial remains to be elucidated.

It has previously been reported that increasing the dilution rate, specifically when using an egg-yolk based diluent, was the primary reason for a decline in fertility of liquid semen at lower sperm numbers rather than a direct effect of a lower sperm concentration, as higher dilution rates adversely affect viability [17,18]. Excessive dilution can lead to permanent loss of motility, metabolic activity and fertilising capacity [19]. While a larger quantity of egg yolk provides more protection to sperm from the harmful effects of seminal plasma [20] it also provides a substrate for hydrogen peroxide (H_2O_2) production from dead sperm [21], thus contributing to increasing the generation of reactive oxygen species (ROS) which is detrimental to sperm [22]. Previous studies of liquid semen have reported beneficial *in vitro* effects of reducing sperm concentration such as increased sperm viability, reduced oxidative stress [7] as well as a decreased susceptibility to osmotic shock [23]. Shannon, Curson and Rhodes [24] reported no significant reduction in fertility of liquid semen when sperm concentration was reduced from 10 to 1.5×10^6 sperm per insemination dose. In contrast, Murphy et al. [4] demonstrated that excessive dilution of sperm had a negative effect on NRR of semen used on Day 2 of storage compared to frozen-thawed semen; however, this did not differ from fertility achieved with liquid semen used on Day 1.

Therefore, using a combination of *in vitro* assessments and a large-scale commercial field trial, the objectives of this study were

to assess the effect of N_2 gassing, storage temperature and reducing the sperm concentration in liquid bull semen diluted in INRA96.

2. Materials and methods

2.1. Experiment 1: Effect of diluting liquid semen in INRA96 with/without nitrogen at 5 or 15°C on total and progressive sperm motility

The aim of this experiment was to assess the effect of N_2 bubbling on total and progressive motility of liquid semen stored for up to 4 days post collection. Semen was collected from Holstein Friesian bulls ($n = 5$) on three occasions (each occasion/collection was one replicate) at a commercial AI centre in Ireland. The raw ejaculate was partially diluted in 10 mL pre-warmed INRA96 (IMV Technologies, L'Aigle, France) at 37°C and transported in a temperature-regulated cooler box at 18°C to the laboratory (1 h transportation). On arrival, the ejaculate was assessed for sperm concentration using a coulter counter (Z Series, Beckman Coulter, Co Clare, Ireland) as well as an initial score of total motility (%) and gross motility on a subjective 5-point scale (1 = twitching/no forward progressive motility; 5 = excellent forward progressive motile sperm) to ensure all semen samples were of a commercial standard (results not shown). Microscopic assessments were conducted by the same technician and initial quality control cut-off values were a total and gross motility of $\geq 70\%$ and a score of ≥ 3 , respectively, and any ejaculates failing to meet these criteria were rejected.

The ejaculate was then split into two equal parts and diluted using either N_2 bubbled or non-bubbled INRA96 to achieve a concentration of 5×10^6 sperm per 0.25 mL insemination dose. Prior to dilution, N_2 bubbled INRA96 (200 mL) was purged in food fresh Nitrogen gas (BOC, Dublin, Ireland) for 30 min [27] to disperse oxygen from the media and create an anaerobic environment, limiting the metabolic activity of sperm during liquid storage. Semen from each bull was kept separate and ejaculates were split such that each bull was represented equally in each treatment. Semen straws were filled as per routine procedures, placed in an insulated plastic container (to slow temperature shifts) and stored at one of two temperatures, 5°C (placed in fridge) and 15°C (placed in a temperature regulated box) [4,10,14]. Samples from the different treatments were assessed in a randomised sequence to remove bias as a result of sampling order. Total and progressive motility (%); ($n = 3$ replicates) were subjectively assessed *in vitro* on Days 0, 1, 2, 3 and 4 post-collection (Day 0 = 4 h after collection).

2.1.1. Assessment of sperm motility

Sperm motility (total and progressive) in liquid semen was assessed subjectively on Days 0, 1, 2, 3 and 4 post semen collection using a phase contrast microscope (CX31; Olympus, Centre Valley, PA, USA) at a magnification of $400\times$. A droplet of diluted semen ($5 \mu\text{L}$) was placed on a pre-warmed glass slide, covered with a pre-warmed coverslip ($18 \times 18 \text{ mm}$; 37°C) and assessed by counting a minimum of 100 sperm, over at least five different fields of view, for each treatment on each assessment day. Total motility was expressed as a percentage of the total sperm population (motile and non-motile). A sperm was deemed to display progressive motility if it moved in a linear fashion; progressive motility was expressed as the percentage of motile sperm.

2.2. Experiment 2a: Effect of storing liquid semen in INRA96 at varying sperm concentrations on total and progressive sperm motility

The aim of this experiment was to assess the effect of bull sperm concentration on total and progressive motility on Day 0, 1 and 2 of

storage. Semen was collected from Holstein Friesian bulls ($n = 6$; 7 collection days; 3 bulls used per collection day resulting in 3–4 ejaculates/replicates per bull) at a commercial AI centre. The raw ejaculate was collected, partially diluted (with 10 mL of INRA96), transported, assessed and processed as described above. The ejaculate was diluted via a two-step dilution, firstly to achieve a concentration of 60×10^6 sperm per mL and then to achieve the final concentrations of 3, 4 and 5×10^6 sperm per 0.25 mL insemination dose. Semen from each bull was kept separate and ejaculates were split such that each bull was represented equally in each treatment. Semen straws were filled as per routine procedures and stored in a temperature regulated cooler box at 15°C. Samples from the different treatments were assessed in a randomised sequence to remove bias as a result of sampling order. Total and progressive motility; ($n = 3$ replicates) were assessed *in vitro* as per Experiment 1 on Days 0, 1, 2 and 3 post-collection (Day 0 = 4 h after collection).

2.3. Experiment 2b: The field fertility of liquid semen diluted in INRA96 at 3, 4 or 5×10^6 sperm per insemination dose

The aim of this experiment was to assess the effect of bull sperm concentration on CR following AI with liquid semen. Liquid semen (3, 4 and 5×10^6 sperm per 0.25 mL dose) on Days 1 and 2 post collection and frozen-thawed semen (15×10^6 sperm per 0.25 mL dose) were used for insemination (supplementary material Fig. 1). Semen was collected from Holstein Friesian bulls ($n = 6$; denoted A–F) at a commercial AI centre during May 2016. There were 7 collection days in total, with 3 bulls used per collection day (total of 21 ejaculates). The raw ejaculate was collected, partially diluted, transported, assessed and processed as described above. Each batch of liquid semen was clearly labelled and distributed for insemination on the day of collection. Liquid semen was stored at ambient temperature [4] and used for up to 2 days post collection on both heifers ($n = 391$) and multiparous ($n = 1884$) dairy cows. Due to logistical constraints, frozen-thawed semen doses were derived from previously collected ejaculates from the same 6 bulls which were processed and frozen using routine procedures ($n = 3467$ inseminations consisting of 1084 heifers and $n = 2383$ multiparous dairy cows). Briefly, upon collection of semen samples for cryopreservation, the raw ejaculate was partially diluted in 10 mL of pre-warmed BullXcell (IMV Technologies) at 37°C and transported to the laboratory. Semen samples were assessed for volume, sperm concentration and total and progressive motility as described above. Only ejaculates achieving a total and gross motility score of $\geq 70\%$ and ≥ 3 were used for cryopreservation. Following *in vitro* assessments, the semen was fully extended with pre-warmed BullXcell to achieve a concentration of 15×10^6 sperm per 0.25 mL insemination dose. Straws were frozen to -140°C as follows: -5°C per min from $+4^\circ\text{C}$ to -10°C , -40°C per min from -10°C to -100°C and thereafter -20°C per min from -100°C to -140°C in a programmable freezer (Digitcool; IMV Technologies), followed by submersion and storage in liquid nitrogen at -196°C until use.

Inseminations (liquid and frozen-thawed semen) were carried out in May 2016 (coinciding with the peak dairy breeding season) in Irish dairy herds ($n = 750$). The majority of inseminations were in Holstein Friesian cows ($n = 5476$) but small numbers of cows of other breeds were represented: Jersey ($n = 158$), Montbeliarde ($n = 40$), Norwegian Red ($n = 34$) and other ($n = 34$; includes Normande, Rotbunte, Swedish Red, Danish Red and Red Poll). Technicians ($n = 61$) were grouped into geographical areas and treatments were rotated on each collection day to ensure that technicians, who were blinded to treatments, received different diluent treatments from each of three bulls on each day (supplementary material Fig. 2). For each insemination the AI

technician recorded the bull code, cow tag number and the straw code on a handheld electronic device.

Cow characteristics such as parity, days in milk (DIM) and fertility sub-index were also assessed. Fertility sub index, a key component of the Economic Breeding Index (EBI) comprises ~35% of the total EBI and is based on calving interval and cow survival [25]. It was set up to combat a decline in reproductive performance by providing farmers with a profit index enabling the selection of elite sires to breed replacement heifers with increased milk yield, reproductive performance and improved health traits [26].

2.3.1. Capturing of calving rate data

Calving rate data were captured using the Irish Cattle Breeding Federation (ICBF; Bandon, Co Cork) database by cross-referencing the technician name with the bull code and semen type used on each date within the trial period. Obvious errors were extracted from the dataset and data were then interrogated to remove animals based on the following criteria: cows which were not at first AI, cows which received two inseminations from two different bulls or sperm concentration treatments, or cows which were not of a dairy breed. However, if a cow received two inseminations from the same bull with the same sperm concentration treatment within five days of each other, the record was kept and the second date was assumed to be correct. Post editing, a total of 5742 inseminations remained. Calving rate was measured using a cut-off value of 275 and 290 days from date of insemination to calving date.

2.4. Statistical analysis

Data from Experiments 1 and 2a were examined for normality of distribution, homogeneity of variance and analysed using the general linear model (GLM) repeated-measures procedure with a compound symmetry covariance structure in Statistical Package for Social Science (SPSS, Version 22.0; IBM, Chicago, USA). The final model for Experiment 1 included the main effects of N_2 bubbling, day, temperature and their interaction. The final model for Experiment 2a included the main effects of sperm concentration treatment, day and their interaction. In Experiment 2b, CR data were compared using Pearson's chi-squared procedures in SPSS. Data were cross checked using an analysis of variance (ANOVA) model. The dependent variable in the analysis was CR (1 = calved, 0 = not calved). In addition, using a general linear model for binomial data, CR data and correlations were investigated with a number of fixed effects, namely sperm concentration, bull, parity number, cow breed, cow fertility sub-index, days in milk (DIM), herd and technician. Each fixed effect was assessed for an interaction with sperm concentration. All post-hoc tests were carried out using Bonferroni test and results are reported as the mean \pm the standard error of the mean (s.e.m) in Experiments 1 and 2a and the estimated marginal means in Experiment 2b, to adjust for the imbalance between the number of inseminations in each comparison/treatment. Data were considered to differ significantly at $P < 0.05$.

3. Results

3.1. Experiment 1: Effect of diluting liquid semen in INRA96 with/without nitrogen at 5 or 15°C on total and progressive sperm motility

There was no effect of N_2 bubbling on total and progressive motility of liquid semen (Fig. 1). However, there was an effect of temperature, day and a day \times treatment interaction on total and progressive motility ($P < 0.01$). As expected, total and progressive motility declined with increased duration of storage ($P < 0.01$). Semen held at 15°C had a higher total and progressive motility

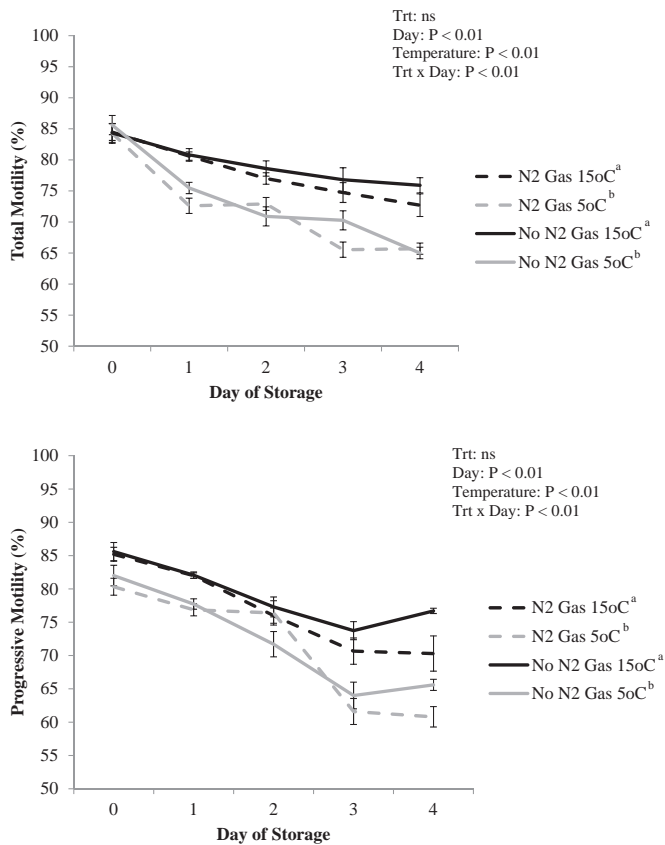


Fig. 1. The effect of nitrogen bubbling on total (upper panel) and progressive (lower panel) motility of liquid semen on Days 0, 1, 2, 3 and 4 post collection ($n = 5$ bulls; 3 ejaculates/replicates per bull; Experiment 1). Vertical bars represent \pm s.e.m. ^{ab}Treatments with different superscripts differ significantly ($P < 0.01$). Trt = treatment, ns = non-significant.

score throughout the duration of storage compared to semen held at 5°C regardless of N₂ treatment (N₂ bubbled or non-bubbled INRA96; $P < 0.01$). There was no effect of bull, bull \times day and or bull \times temperature interaction on total and progressive motility.

3.2. Experiment 2a: Effect of storing liquid semen in INRA96 at varying sperm concentrations on total and progressive sperm motility

Although total and progressive motility declined linearly with increased duration of storage ($P < 0.01$), there was no effect of sperm concentration on both total and progressive motility. Semen diluted to a concentration of 3, 4 and 5 $\times 10^6$ sperm per dose maintained acceptable total and progressive motility scores throughout the duration of storage with a decline in total (81.8–74.2, 81.6 to 73.3 and 81.9 to 75.3%, respectively) and progressive motility (83.3–74.4, 84.3 to 74.4 and 84.2 to 75.6%, respectively) on Days 0, 1 and 2 of storage. There was also no sperm concentration by day interaction.

3.3. Experiment 2b: The field fertility of liquid semen diluted in INRA96 at 3, 4 or 5 $\times 10^6$ sperm per insemination dose

Overall, inseminations with liquid semen on Day 1 post collection resulted in similar CR (52.1%) in comparison to frozen-thawed semen (55.2%). Insemination with liquid semen on Day 2 of storage resulted in a lower CR (45.6%; $P < 0.01$) compared to semen used on Day 1 or frozen-thawed semen (52.1 and 55.2%, respectively;

$P < 0.01$). There was an effect of sperm concentration on CR following AI as liquid semen diluted to 3 or 4 $\times 10^6$ sperm per dose resulted in a lower CR on Day 2 of storage (41.1 and 44.7%, respectively; $P < 0.05$) in comparison to frozen-thawed semen ($P < 0.05$) but did not differ to semen diluted to 5 $\times 10^6$ on Day 2 of storage (Fig. 2). There was no effect of bull on CR with the average CR for bulls used in the trial varying between 50.7 and 54.9%. There was a bull \times sperm concentration interaction ($P < 0.05$), as Bulls D and E in the 3 $\times 10^6$ treatment had a lower CR (47.6 and 43.7%, respectively) in comparison to frozen-thawed semen (58.3 and 61.7%, respectively). There was a bull \times day interaction as Bulls B and E had a lower CR on Days 1 and 2 (Bull B; 42.2 and 42.3% vs Bull E; 48.8 and 40.2% for Day 1 and 2, respectively) in comparison to frozen-thawed semen (58.7 and 61.7%, respectively; $P < 0.05$) while Bulls C and D had a reduced CR on Day 2 (44.0 and 47.5%, respectively), in comparison to frozen-thawed semen (54.8 and 58.3%, respectively; $P < 0.05$).

There was an effect of parity, cow fertility sub-index and DIM on CR ($P < 0.01$). Maiden heifers had a higher CR (57.9%) than primiparous (52.7%; $P < 0.05$) and multiparous dairy cows (50.6%; $P < 0.01$). Cows with a fertility sub-index of greater than €110 recorded a higher CR (61.0%) in comparison to cows with a fertility sub-index of <€50, €50–70, €70–90 and €90–110 at 45.6, 51.0, 53.8 and 55.4%, respectively. Cows greater than 60 DIM at the time of AI recorded a higher CR (55.4%) than cows with a DIM of <20, 20–40 and 40–60 (CR of 22.7, 36.3 and 41.2%, respectively). As expected, CR varied between individual herds and technicians, for herds and technicians with greater than 40 and 45 recorded inseminations, respectively ($P < 0.01$). There was no effect of breed, or a breed, cow fertility sub-index, DIM, herd or technician by sperm concentration interaction.

4. Discussion

The main findings of this study were (i) storage of bull semen in INRA96 at 15°C is superior to semen stored at 5°C as assessed *in vitro* (ii) N₂ bubbling of INRA96 or reduced sperm concentration (within the range 3–5 $\times 10^6$ sperm per dose) had no effect on total

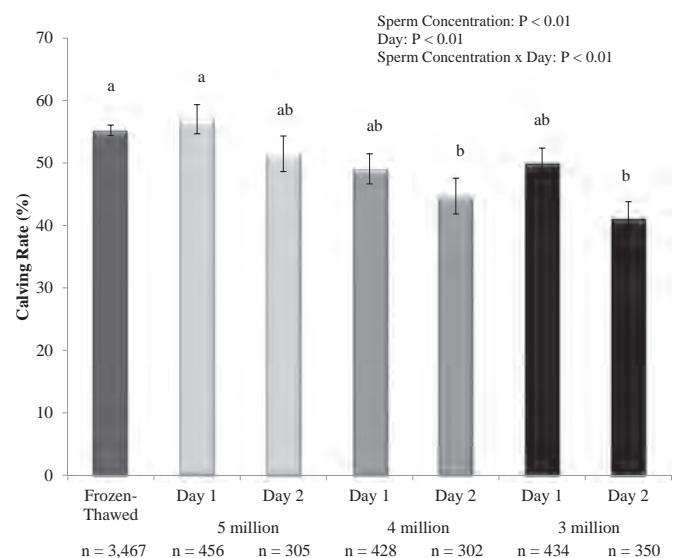


Fig. 2. The effect of sperm concentration and day of storage on calving rate in dairy cows and heifers ($n = 6$ bulls; 3 to 4 ejaculates/replicates per bull; Experiment 2b). ^{ab}Differing superscripts differ significantly between treatments ($P < 0.01$). Vertical bars represent 95% confidence intervals. n = the total number of inseminations per treatment per day.

and progressive motility *in vitro*, and (iii) insemination with liquid semen stored at 3 and 4×10^6 sperm per dose resulted in a reduced CR following 2 days of storage in comparison with frozen-thawed semen but did not differ from semen diluted to 5×10^6 sperm per dose on Day 2 of storage. As each treatment was prepared from the same ejaculate, any potential confounding effects of day of collection were removed, thus providing clear and reliable *in vitro* and *in vivo* data on the use of liquid bull semen.

It has previously been reported that sperm are quite versatile in relation to sperm quality between storage temperatures of 5–22°C as they were found to retained acceptable *in vitro* standards [4], with semen stored at 15°C having greater motility compared to semen stored at 5, 22, 32°C or fluctuating temperatures between 5 and 15, 5–22 and 5–32°C. This is in agreement with the results of the current study as although semen held at both a constant 15 or 5°C recorded acceptable total and progressive motility values up to 4 days of storage, storing semen at 15°C resulted in better total and progressive motility throughout the duration of storage compared to semen stored at 5°C. In the current study N₂ bubbling had no effect on sperm motility regardless of storage temperature. This is in agreement with Krzyzosiak et al. [27] who reported no significant difference in the percentage of motile bull sperm up to Day 3 of storage or on the *in vitro* fertility of sperm stored under aerobic, N₂ gassed and anaerobic conditions (N₂ gassed and placed in an anaerobic chamber overnight with 5% Hydrogen and 95% N₂). Although not assessed in the current study, plasma membrane integrity deteriorated quicker when stored in aerobic conditions compared to anaerobic or N₂ gassed conditions [27], however, motility on Day 7 was significantly lower after storage under anaerobic conditions compared to aerobic or N₂ gassed storage conditions [27]. Therefore, it may be possible that diluents provides sufficient support for sperm motility over prolonged periods (up to 3 days of storage) regardless of the oxygenated state of the media and that the benefits of N₂ gassing are not observed by assessing motility alone.

As expected, in the current study, total and progressive motility declined linearly in all treatments with increased duration of storage regardless of storage temperature, N₂ gassing or sperm concentration; however, although all sperm concentration treatments maintained acceptable total and progressive motility on Day 2 of storage with scores above 74% respectively, semen diluted to 3 and 4×10^6 sperm per dose resulted in a reduced CR on Day 2 of storage (41.1 and 44.7%, respectively) compared to frozen-thawed semen (55.2%). A study conducted by Vishwanath and Shannon [16] reported that bull sperm have gradual decreasing motility scores for up to 4 weeks when stored in Caprogen diluent; however, there was a sharp decline in NRR after 3–5 days ($69.9 \pm 1.2\%$) compared to after 10 days ($41.5 \pm 3.7\%$). This suggests that additional factors relating to fertility other than sperm motility are essential in achieving high pregnancy rates.

Murphy et al. [4] previously reported that some bulls are more susceptible to sperm aging resulting in a decrease in their fertility due to the cumulative generation of reactive oxygen species with increased duration of storage. However, the results of the current study would suggest that some bulls may be more suitable for use in frozen-thawed semen rather than liquid semen programs regardless of the duration of storage. Three bulls in particular performed relatively poorly, in relation to CR, following AI on Day 1 and 2 of storage of liquid semen compared to frozen-thawed semen with these bulls combined having an average CR of 45.9% and 42.4% on Day 1 and 2, respectively, compared to 58.4% for frozen-thawed semen. Notwithstanding the limitations of the relatively modest number of inseminations per bull [28] a possible explanation for poor fertility performance of liquid semen may be due to the inability of the sperm to adapt to temperature variation

associated with storage of liquid semen at ambient temperature. A previous study by our group found that semen stored at ambient temperatures in unregulated temperature control boxes in the trunk of a car (similar storage conditions to liquid semen in Ireland) where subjected to day to night time temperature variations with minimum and maximum temperature values of 6.4 and 27.9°C, respectively, [4]. Although Murphy et al. [6] reported that storing diluted semen at constant or fluctuating temperatures between 4 and 18°C had no impact on motility when stored in INRA96, unpublished data by our group shows that while sperm are quite versatile in terms of storage temperature, fluctuating temperatures between 4 and 28°C (night-time to daytime over 4 days) resulted in a significant loss of motility (unpublished). Exposure to such daytime/night-time temperature fluctuations may result in a decline in membrane integrity as a consequence of morphological membrane changes which are consistent with the lipid phase transition [29], thus resulting in a reduction in sperm quality and fertility [30]. Therefore, further investigation should be undertaken with a strict temperature regulation regime in place so that liquid semen is maintained at a constant temperature.

In the current study, CR of liquid semen stored at ambient temperature and diluted in INRA96 to 3 and 4×10^6 sperm per dose on Day 2 of storage decreased significantly compared to frozen-thawed semen but did not differ from semen diluted to 5×10^6 sperm per dose after 2 days of storage. Additionally, CR of semen diluted to 3, 4 or 5×10^6 sperm per dose on Day 1 of storage did not differ from frozen-thawed semen. This supports the findings of Murphy et al. [4] as semen diluted in Caprogen had a reduced NRR on Day 2 of storage at lower concentrations compared to frozen-thawed semen. However, in the current study, the overall CR of liquid semen following AI on Day 1 of storage was comparable to the previous study reported by Murphy et al. [6] who compared semen diluents at the higher concentration of 5×10^6 sperm per dose. Consequently, it could be recommended to increase sperm numbers for targeted use on subsequent days after collection; namely 4 and 5×10^6 sperm per dose for insemination on Day 1 and 2 post collection, respectively. This would ensure that the number of insemination doses per ejaculate is maximised, thus resulting in an increase in the use of individual sires. In contrast to the results of this study, previous studies have shown that similar conception rates can be achieved with liquid and frozen-thawed semen [31] and that NRRs of liquid and frozen-thawed semen diluted to 2.5×10^6 and 20×10^6 sperm, respectively, do not differ up to Day 2 of storage [32]. However, over-compensation of sperm numbers typically occurs in the preparation of frozen-thawed semen, resulting in a sperm concentration which considerably exceeds the number of sperm necessary for maximum fertility, thus, masking the ‘true fertility’ potential of a bull [33].

It is well established that cow characteristics such as parity, fertility sub-index and DIM play a role in fertility [4,6]. Cow fertility declines with increased age as stresses associated with calving, lactation and clinical abnormalities at parturition or postpartum have a negative effect on fertility [34,35]. Previous studies have found that heifers had a higher NRR compared to multiparous dairy cows [36,37]. The results of the current study show that, while numerically greater, CR of heifers did not differ to primiparous dairy cows but was ~13% higher than multiparous dairy cows with a parity of greater than 5. Furthermore, there was no treatment by parity interaction highlighting that no particular semen type has an advantage in negating the effects of parity. In the current study, a positive linear relationship between fertility sub-index and CR was observed with animals in the highest fertility sub-index (>€110) having a higher CR than any other sub-index. Therefore, the results clearly highlight the importance of the EBI and its contribution to herd fertility. In this study, the number of DIM affected CR with

cows of greater than 60 DIM having a higher CR than those of less than 60 DIM. Therefore, this study demonstrates that increasing the number of DIM increases fertility; consequently, allowing a DIM of 60 days can be beneficial to the overall production of a dairy herd. However, in the case of late calving cows, some confidence can also be taken from inseminating these animals at a shortened DIM interval, with reasonable success, in order to bring forward the calving date of this cohort of cows.

5. Conclusion

In conclusion, storing semen at 15°C resulted in superior total and progressive motility values compared to semen stored at 5°C, while N₂ gassing and sperm concentration (3, 4 or 5 × 10⁶ sperm per dose) had no effect on sperm motility. On Day 1 of storage there was no difference in CR between liquid and frozen-thawed semen; however, on Day 2 of storage insemination with 3 or 4 × 10⁶ sperm per dose resulted in a lower CR in comparison to frozen-thawed semen but did not differ from 5 × 10⁶ sperm per dose. Thus, given that reducing sperm concentration per dose results in an increased number of doses per ejaculate, therefore increasing the utilisation of superior sires, it may be justifiable to increase sperm numbers for targeted use on subsequent days after collection. Furthermore, additional consideration should be given to the storage temperature of liquid semen with an aim to storing liquid semen at a constant temperature.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.theriogenology.2017.12.012>.

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Effect of increasing equilibration time of diluted bull semen up to 72 h prior to freezing on sperm quality parameters and calving rate following artificial insemination

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ABSTRACT

An equilibration period of approximately 3–4 h prior to semen cryopreservation is standard practice for maintaining membrane integrity and motility of bull sperm. However, a number of studies indicate that an overnight equilibration period prior to freezing results in improved post-thaw semen quality thus optimising pregnancy rates. The aim of this study was to assess the effect of increasing the equilibration time of bull semen up to 72 h before freezing on sperm quality parameters and calving rate (CR) following artificial insemination (AI) with frozen-thawed semen. The effect of holding semen at 4 °C for 6, 24, 48 or 72 h post dilution before freezing on subsequent post-thaw total and progressive motility (Experiment 1) and field fertility (n = 1640 inseminations, Experiment 2) of frozen-thawed semen was assessed. Equilibration time did not affect post-thaw total and progressive motility (P > 0.05). In addition, there was no effect (P > 0.05) of equilibration time on field fertility with a CR of 53.3, 50.5, 51.3 and 47.3 for the 6, 24, 48 and 72 h treatments, respectively. In conclusion, increasing the equilibration time of diluted bull semen from 6 to 72 h had no significant effect on CR, within the expected range of fertility outcomes, thus providing semen processing centres with flexibility in the time which semen can be held prior to freezing.

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1. Introduction

The use of artificial insemination (AI) facilitates the rapid dissemination of genetic material from a relatively small number of superior sires to a large number of females [1]. Thus, AI is considered to be the single most important technique devised to facilitate the genetic improvement of animals [2]. The widespread use of frozen-thawed semen in cattle is partly attributed to the development of suitable cryopreservation protocols and a number of investigative avenues have been undertaken in order to improve the freeze-thaw process [3,4]. Bull semen used for cryopreservation is typically diluted in an egg yolk-based extender as egg yolk is known to be one of the best cryoprotectant components for the preservation of post-thaw sperm function and subsequent fertility

[4]. The components within egg-yolk, in particular low-density lipoproteins (LDLs), bind to the sperm membrane during the freeze-thaw process, increasing chilling tolerance and preventing loss of membrane phospholipids [5]. Typically, extenders used for the cryopreservation of bull semen differ in composition from fresh semen extenders as greater emphasis is placed on stabilising the cell membrane. The addition of cryoprotectants, which are classified as either penetrating (glycerol; 3–6%) or non-penetrating (egg yolk; containing LDLs and cholesterol) [6], minimises the physical and chemical stresses associated with cryopreservation, thus reducing membrane damage during freezing [7]. As the name suggests, a non-penetrating cryoprotectant, such as egg-yolk, cannot cross the sperm membrane and thus only acts extracellularly to modify the sperm membrane [8]. On the other hand, penetrating cryoprotectants, the most common of which is glycerol, are membrane permeable and so act both intra- and extracellularly causing dehydration of the sperm and membrane lipid and protein rearrangement resulting in increased membrane

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fluidity and a decrease in the freezing point of the cell [3].

Semen cryopreservation involves several steps including cooling, equilibration, freezing and subsequent thawing [9]. Typically, freezing protocols for bull semen include cooling to 4–5 °C followed by a variable duration of equilibration (0–24 h) at this temperature prior to freezing [10]. Equilibration allows sperm to adapt to cooler temperatures [5], facilitating the movement of the cryoprotectant across the cell membrane (in the case of penetrating cryoprotectants) and enables the movement of water out of the cell, thus minimizing damage sustained by ice crystal formation during the freeze-thaw process [5]. Equilibration time was first believed to be important in allowing glycerol sufficient time to penetrate the sperm membrane [10]. However, Berndtson and Foote [11] reported that glycerol penetration in bull sperm is rapid, taking no more than 5 min; therefore, it is now suggested that a period of equilibration is necessary to allow sperm membranes sufficient time to adapt to cooler temperatures [5]. Studies aimed at identifying the optimum equilibration time have been conducted on semen from a number of species including sheep [12], goats [13] and cattle [14]. Although the majority of cryopreservation protocols for bovine semen involve an equilibration period of 4 h, a wide range of equilibration times have been reported: 0 h [10], 1–4 h [15], 18–24 h [16] and 24–72 h [14,17]. Increasing equilibration time from 8 to 18 h [18] or from 4 to 72 h [14] increased the quality of bull sperm motility and viability, and did not affect field fertility.

Implementing a shorter equilibration time such as 4–6 h can result in processing difficulties within a commercial AI centre, as semen has to be frozen on the same day of collection. A number of studies have found that using an equilibration time of 24 h resulted in an increase in semen quality [14,16] and therefore may be more convenient for the working schedule in AI centres which involves the collection of semen from a large number of bulls on a daily basis or where semen has to be transported over long distances to a central processing laboratory. In particular, to provide flexibility over weekends, it would be useful if semen collected on a Friday or Saturday could be processed the following Monday. Therefore, the aim of this study was to assess the effects of increasing the equilibration time up to 72 h post dilution on sperm motility *in vitro* and on *in vivo* fertility following artificial insemination.

2. Materials and methods

2.1. Experiment 1: the effect of equilibration time on the *in vitro* quality of frozen-thawed semen

The aim of this experiment was to assess the effects of holding time (6, 24, 48 and 72 h post dilution) on total and progressive motility of bull sperm pre-freeze and post-thawing. Semen was collected from Holstein Friesian bulls ($n = 8$) via artificial vagina at a commercial AI centre (National Cattle Breeding Centre, Enfield, Co Meath, Ireland). The raw ejaculate was placed into a 15 mL tube and was partially diluted immediately after collection in 10 mL pre-warmed BullXcell (37 °C; IMV Technologies, Normandy, France) by slowly pouring the extender into the 15 mL tube containing the raw ejaculate. Typically this dilution rate was 2:1 diluent to raw ejaculate ratio. The samples were transported in a temperature-regulated cooler box at 18 °C to the laboratory (within 3 h). Upon arrival, the ejaculate was assessed for sperm concentration using a coulter counter (Z Series, Beckman Coulter, Clare, Ireland), as well as an initial score of total motility (%) and gross motility on a 5-point subjective scale (1 = twitching/no forward progressive motility; 5 = excellent forward progressive motile sperm) to ensure all semen samples were of a commercial standard (results not shown). Initial quality control cut-off values were a total and gross motility of <70% and a score of <3, respectively, and any ejaculates

failing to meet these criteria were rejected. Three ejaculates (replicates) were assessed per bull and semen from each ejaculate was kept separate throughout processing.

Following initial *in vitro* assessment, the ejaculate was fully extended in BullXcell, based on initial sperm concentration, to achieve a concentration of 15×10^6 sperm per 0.25 mL insemination dose. Semen straws (IMV Technologies) were filled as per routine procedures using the IS4 machine (IMV Technologies), placed in an insulated box (to slow the temperature drop) and stored in a fridge at 4 °C. Straws from each ejaculate ($n = 20$ straws per equilibration time point) were frozen at 6, 24, 48 or 72 h post dilution as follows: 5 °C per min from +4 °C to –10 °C, –40 °C per min from –10 °C to –100 °C and thereafter –20 °C per min from –100 °C to –140 °C [19] in a programmable freezer (Digitcool, IMV Technologies), followed by submersion and storage in liquid nitrogen at –196 °C until use. Each ejaculate was split so that each bull was represented in each treatment in each replicate. Samples from the different treatments were assessed in a randomised sequence to remove bias as a result of sampling order. The evaluator was blinded to the treatment. Total and progressive motility was assessed *in vitro* just prior to freezing at 6, 24, 48 and 72 h post dilution as well as immediately post-thawing via standard phase-contrast microscopy (described below) and computer-assisted sperm analyser (CASA; Hamilton Thorne IVOSII, IMV). In addition, sperm viability and acrosomal integrity were assessed using flow cytometry. Within each replicate, at each assessment time (both pre- and post-freezing), five straws from each bull for each of the equilibration times were assessed.

2.1.1. Standard microscopic analysis of frozen-thawed semen frozen at 6, 24, 48 and 72 h post dilution

Sperm motility (total and progressive) was assessed subjectively pre-freeze and post-thaw using a phase contrast microscope (CX31; Olympus, Centre Valley, PA, USA) at a magnification of 400 \times . Frozen straws were thawed at 35 °C for 30 s. Each straw was dried fully, to remove any excess water, cut at the sealed end and separately placed into a pre-warmed eppendorf. The plug end of each straw was then cut to expel the contents of the straw into the eppendorf and the semen sample was mixed thoroughly to ensure homogeneity. A droplet of diluted semen (5 μ L) was placed on a pre-warmed glass slide, covered with a pre-warmed coverslip (37 °C) and assessed by counting a minimum of 100 sperm, over at least five different fields of view, for each treatment. Total motility was expressed as a percentage of the total sperm population (motile and non-motile). A sperm was deemed to display progressive motility if it moved in a linear fashion (ie; movement of sperm in a straight or curvilinear motion); progressive motility was expressed as the percentage of live and progressively motile sperm in the total sperm population.

2.1.2. Computer assisted sperm analysis of frozen-thawed semen frozen at 6, 24, 48 and 72 h post dilution

Motility of frozen-thawed sperm samples was assessed using the IVOS-II CASA system driven by software version 14 (Hamilton Thorne Inc, Beverly, USA). Straws ($n = 5$ per ejaculate) were thawed at 37 °C for 30 s and a drop (3 μ L) of diluted semen was placed in a pre-warmed chamber (37 °C; Leja counting chambers, depth 20 μ m; Microptics, Barcelona, Spain) and analysed for sperm motion and kinematic characteristics immediately post-thaw. A minimum of 1000 sperm were analysed in at least eight microscopic fields with 30 frames acquired per field at a frame rate of 60 Hz. Objects incorrectly identified as sperm were edited out using the playback function. The CASA-derived motility and kinematic characteristics assessed were total motility (%), progressive motility (%), proximal and distal droplets (%), as well as average path velocity

(VAP above 10 $\mu\text{m/s}$), straight line velocity (VSL), curvilinear velocity (VCL), linearity (LIN), straightness (STR), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF) [20]. Regarding analysis settings, the CASA was set to standard factory settings for bull semen; sperm with straightness of >80% and VAP >50 $\mu\text{m/s}$ were considered progressively motile.

2.1.3. Flow cytometric analysis of frozen-thawed semen frozen at 6, 24, 48 and 72 h post dilution

Before flow cytometric analysis, semen samples were diluted to a final working concentration of 300×10^5 sperm/mL in Beltsville Thawing Solution (BTS; 37 °C). Samples were analysed on a flow cytometer (Guava easyCyte 6HT-2L, Merck Millipore, Billerica, MA, USA) equipped with both a Krypton (642 nm) and an Argon laser (488 nm). Appropriate single colour controls were prepared to establish the respective fluorescent peaks of the individual stains. These were used in conjunction with the forward scatter (FSC) and side scatter (SSC) signals to discriminate sperm from debris (P0.1 Population). Fluorescent events were recorded using GuavaSoft (Version 2.7; Merck Millipore) and all variables were assessed using logarithmic amplification. In each sample, a minimum of 10,000 gated events were captured.

2.1.3.1. Assessment of viability. Viability was assessed using fluorescent probes. SYTO 16 (Ex/Em: 488/518; Life Technologies, Carlsbad, CA USA) is a cell-permeant probe which fluoresces upon binding to nucleic acids and propidium iodide (PI; Ex/Em: 535/617; Life Technologies) is selectively taken up by membrane-compromised cells, thus indicating a loss of viability [21]. SYTO 16 (5 μL) was added to diluted sperm (300×10^5 sperm/mL) at a final concentration of 100 nM and incubated at 37 °C in the dark for 10 min. Subsequently, PI was added at a final concentration of 15 μM and incubated for a further 5 min. SYTO 16 emission was detected via the Green photomultiplier (PMT; 525/30 nm BP filter) and PI was read with the Red1 PMT (690/50 nm BP filter); no compensation was required. Viability was defined as the percentage of sperm positive for SYTO 16 but negative for PI and all percentages were calculated as part of the total gated sample, P0.1 Population.

2.1.3.2. Assessment of acrosomal integrity. Acrosomal integrity was assessed by incubating sperm with SYTO 16 and PI, as described above, followed by incubation with Alexa Fluor 647 (AF647; Ex/Em: 650/668; Life Technologies) to identify live sperm which had undergone the acrosome reaction [21]. Acrosomal integrity can be verified cytometrically using glycoprotein markers such as lectin [22]. Pisum sativum agglutinin (PSA) is an agglutinin which can bind to glycoconjugates from the acrosome and has an affinity for terminal residues of glycoproteins, binding specifically to α -mannosidase [23]. PSA is routinely tagged to either FITC or Alexa Fluor probes and fluoresces upon contact with acrosome reacted sperm [24]. Alexa Fluor 647 (3 μL) was added to diluted sperm (300×10^5 sperm/mL) to a final concentration of 4.6 μM and incubated in the dark for 15 min at 37 °C. AF647 positive events were read on the Red2 PMT (661/19 nm BP filter) and no compensation was required. The percentage of acrosome-intact sperm in the live population was expressed as a percentage of the sperm negative for Alexa Fluor 647 and positive for SYTO 16 as part of the total gated sample, P0.1 Population.

2.2. Experiment 2: the effect of equilibration time on field fertility of frozen-thawed semen

The aim of this experiment was to assess the effect of equilibration time of frozen-thawed semen (15×10^6 sperm per 0.25 mL

insemination dose) on CR following AI. Semen was collected from Holstein Friesian bulls ($n = 5$) at a commercial AI centre from early February to the end of March 2015. There were 7 collection days in total, with all five bulls collected on each collection day (total of 35 ejaculates). Following initial assessment for volume, concentration and motility (as described in Experiment 1), each acceptable ejaculate was diluted and filled in straws which were then printed and sealed as per Experiment 1. Straws were stored for 6, 24, 48 or 72 h prior to freezing. Each batch of semen was clearly labelled, assessed post-thaw to ensure samples were of a commercial standard, and distributed for insemination after 30 days of quarantine as per European regulations [25].

Inseminations were carried out in May 2016 (coinciding with the peak dairy breeding season) in Irish dairy herds ($n = 284$). The majority of inseminations were in Holstein Friesian animals ($n = 1582$) but a small number of other breeds were represented including Jersey ($n = 20$), Montbeliarde ($n = 12$), Norwegian Red ($n = 9$) and Others ($n = 17$; includes Ayrshire and Shorthorn). Technicians ($n = 24$) were blind to treatments and received equal numbers of straws from each of the four equilibration times from each bull. For each insemination, the technician recorded the bull code, cow tag number and the straw code on an electronic handheld device. Inseminations and CR data were captured using the Irish Cattle Breeding Federation (ICBF; Bandon, Co. Cork, Ireland) database by cross-referencing the technician name with the bull code and semen type used on each date within the trial period. Obvious errors were extracted from the dataset and data were then interrogated to remove animals ($n = 382$) based on the following criteria: cows which were not at first AI, cows which received two inseminations from two different bulls or equilibration time treatments, or cows which were not of a dairy breed. However, if a dairy cow received two inseminations from the same bull with the same time treatment within five days of each other, the record was kept and the second date was assumed to be correct. Post editing, a total of 1640 inseminations (1537 cows and 103 heifers) remained. Calving rate was measured using a cut-off value of 275 and 290 days from date of insemination to calving date [21].

Cow characteristics such as parity, days in milk (DIM) and fertility sub-index were also assessed. Fertility sub index is a key component of the Economic Breeding Index (EBI) comprising ~35% of the total EBI [26]. The EBI is an estimate of the economic value of an animal's genetic merit. It was established to combat a decline in reproductive performance by providing farmers with a profit index enabling the selection of elite sires to breed replacement heifers with increased milk yield, reproductive performance and improved health traits [27].

2.3. Statistical analysis

Data from Experiment 1 were examined for normality of distribution, homogeneity of variance and analysed using the general linear model (GLM) repeated-measures procedure with a compound symmetry covariance structure in Statistical Package for the Social Sciences (SPSS, Version 22.0; IBM, Chicago, USA). In Experiment 2, CR data were compared using Pearson's chi-squared procedures in SPSS. The dependent variable in the analysis was CR (1 = calved, 0 = not calved). In addition, a GLM for binomial data was used to assess a number of fixed effects on CR, including equilibration time, bull, parity, breed, fertility sub-index, DIM, herd and technician. Each fixed effect was assessed for an interaction with equilibration time treatment. All post-hoc tests were carried out using the Bonferroni test. Results are reported as the mean \pm the standard error of the mean (s.e.m) in Experiment 1 and as the estimated marginal means in Experiment 2, to adjust for imbalance between numbers of inseminations in each treatment.

Values were considered to differ significantly at $P < 0.05$.

3. Results

3.1. Experiment 1: the effect of equilibration time on the *in vitro* quality of frozen-thawed semen

Equilibration time had no effect on pre-freeze total and progressive motility ($P > 0.05$) as subjectively assessed via standard microscopic techniques or objectively using CASA (Table 1). There was an effect of equilibration time on viability ($P < 0.01$) and acrosomal integrity ($P < 0.01$; Table 1). The percentage of live sperm post-thaw increased from 6 to 72 h ($P < 0.01$) while the percentage of live sperm with intact acrosomes post-thawing declined in the 72 h equilibration treatment ($P < 0.01$; Table 1). Semen equilibrated for 72 h exhibited inferior kinematic motility parameters compared to an equilibration time of 6 and 24 h ($P < 0.01$) with the exception of ALH and VCL ($P > 0.05$; Table 1). All CASA motility characteristics, with the exception of ALH and VCL deteriorated when equilibration time was increased from 6 to 72 h (106.3 ± 3.56 versus $97.3 \pm 1.16 \mu\text{m/s}^{-1}$, 31.1 ± 0.77 versus 24.9 ± 0.26 Hz, 41.6 ± 0.45 versus $35.4 \pm 0.83\%$, 76.6 ± 0.98 versus $70.2 \pm 1.14\%$, 81.7 ± 3.28 versus $67.9 \pm 0.72 \mu\text{m/s}^{-1}$ and 53.3 ± 0.48 versus $49.6 \pm 0.39\%$ for VAP ($P < 0.05$), BCF, LIN, STR, VSL and WOB, respectively; $P < 0.01$).

3.2. Experiment 2: the effect of equilibration time on field fertility of frozen-thawed semen

While CR declined numerically with increased equilibration time (53.3, 50.5, 51.3 and 48.3% for 6, 24, 48 and 72 h, respectively), the difference between treatments was not significantly different ($P > 0.05$; Table 2). There was no bull, parity, cow fertility sub-index, DIM, herd or technician by treatment interaction on CR ($P > 0.05$). Calving rate varied from 42.7 to 56.8% for individual bulls ($P < 0.01$). There was a positive linear increase in CR with increasing cow fertility sub-index and DIM ($P < 0.01$). Cows with a fertility sub-index greater than €90 had a higher CR in comparison with cows with a sub-index of less than €90 ($P < 0.05$). Cows which were less than 40 DIM had a reduced CR (27.8%) in comparison to cows which were greater than 40 days in milk prior to insemination. Maiden heifers had a numerically higher CR (59.2%) than primiparous (52.5%) and multiparous cows (49.4%) but this was not significant

($P > 0.05$). For herds and technicians with greater than 15 and 20 recorded inseminations, CR varied between individual herds and technicians ($P < 0.01$).

4. Discussion

The main findings of this study were that: (i) increasing equilibration time from 6 to 72 h does not detrimentally affect sperm quality *in vitro* with 24 h equilibration resulting in the highest numerical post-thaw total and progressive motility scores, (ii) although there was a numerical decline in CR with increased equilibration time, equilibration up to 72 h did not significantly affect CR, (iii) cows with a higher fertility sub-index had a higher CR than those with a lower sub-index, and (iv) cows with a greater number of DIM (>40 days) at AI, had a higher CR than those which were inseminated closer to their calving date (<40 days).

Motility is one of the most important characteristics associated with fertilising potential of a sperm as it is indicative of sperm viability and structural integrity [28]. Therefore, motility assessment constitutes an integral part of semen quality analysis with the use of CASA systems allowing an objective accurate assessment of sperm motility kinematics [29]. A number of studies have correlated motility kinematics of bull sperm with field fertility [28,30–32]; however, many of these studies are conflicting in terms of the relative importance of different motility characteristics. Oliveira et al. [33] reported a correlation between a combination of CASA parameters and bull fertility such as total and progressive motility, ALH and BCF, based on 191 inseminations. In contrast, Amann and Waberski [34] suggested that sperm kinematic characteristics are not an accurate predictor of fertilising potential but instead could be used to provide important information relating to the quality assurance of semen. This is supported by Oliveira et al. [35] who concluded that while there was no effect of sperm characteristics on field fertility, sires producing lower semen quality had lower pregnancy rates. In the current study, semen quality initially increased with increasing equilibration time from 6 to 24 h but declined gradually thereafter *in vitro* up to 72 h. Although not significant, post-thaw total and progressive motility scores were higher for 24 h compared with 6, 48 and 72 h. All CASA motility parameters, with the exception of ALH and VCL, deteriorated significantly with increasing equilibration time from 6 to 72 h. Furthermore, the percentage of viable sperm increased when

Table 1
The effect of equilibration time on viability and acrosomal integrity as assessed via flow cytometry as well as computer assisted sperm analyser post-thaw total and progressive motility and kinematic parameters in bull semen extended for 6, 24, 48 and 72 h post dilution prior to freezing (Experiment 1). ^{abc}Values with different superscripts differ significantly within row ($P < 0.01$; values are mean \pm s.e.m).

Parameters	Equilibration Time (h)				P value
	mean \pm s.e.m				
	6	24	48	72	Effect of Treatment
Viability (%)	53.4 ± 2.76^a	60.5 ± 2.16^{ab}	61.3 ± 3.72^b	61.3 ± 2.06^b	$P < 0.01$
Acrosomal Integrity (%)	92.3 ± 0.57^a	93.1 ± 0.54^a	90.6 ± 0.63^{ab}	89.7 ± 0.89^b	$P < 0.01$
Total Motility (%)	44.4 ± 2.64	53.7 ± 2.30	50.1 ± 3.16	48.9 ± 2.80	ns
Progressive Motility (%)	34.7 ± 1.80	42.8 ± 1.90	38.0 ± 2.47	36.4 ± 2.12	ns
ALH (μm)	8.7 ± 0.19^a	9.3 ± 0.18^{ab}	9.7 ± 0.21^b	9.8 ± 0.16^b	$P < 0.01$
BCF (Hz)	31.1 ± 0.77^a	28.9 ± 0.73^a	26.0 ± 0.46^b	24.9 ± 0.26^b	$P < 0.01$
LIN (%)	41.6 ± 0.45^a	39.4 ± 0.79^{ab}	36.5 ± 0.95^{bc}	35.4 ± 0.83^c	$P < 0.01$
STR (%)	76.6 ± 0.98^a	75.6 ± 1.03^{ab}	71.5 ± 1.18^{bc}	70.2 ± 1.14^c	$P < 0.01$
VAP ($\mu\text{m/s}^{-1}$)	106.3 ± 3.56^a	105.9 ± 0.94^a	101.7 ± 0.91^{ab}	97.3 ± 1.16^b	$P < 0.05$
VCL ($\mu\text{m/s}^{-1}$)	202.3 ± 7.04	209.4 ± 1.17	207.0 ± 3.13	201.9 ± 3.17	ns
VSL ($\mu\text{m/s}^{-1}$)	81.7 ± 3.28^a	80.5 ± 1.57^{ab}	72.5 ± 1.11^{bc}	67.9 ± 0.72^c	$P < 0.01$
WOB (%)	53.3 ± 0.48^a	51.3 ± 0.46^{ab}	50.2 ± 0.62^b	49.6 ± 0.39^b	$P < 0.01$
Proximal Droplets (%)	3.8 ± 0.97	3.8 ± 1.04	4.1 ± 1.22	3.9 ± 1.35	ns
Distal Droplets (%)	4.6 ± 0.32	3.9 ± 0.36	4.4 ± 0.38	4.4 ± 0.33	ns

ALH = amplitude of lateral head displacement, BCF = beat cross frequency, LIN = linearity, STR = straightness, VAP = average path velocity, VCL = curvilinear velocity, VSL = straight line velocity, WOB = wobble, ns = not significant.

Table 2

The effect of equilibration time on calving rate following AI of bull semen extended for 6, 24, 48 and 72 h post dilution prior to freezing (Experiment 2) including expected fertility outcomes for each treatment group.

Equilibration Time (h)	Number of Inseminations	Calving Rate %	95% Confidence Interval	
			Lower Bound	Upper Bound
6	428	53.7	48.5	58.0
24	378	50.5	45.5	55.6
48	372	51.3	46.3	56.4
72	462	48.3	43.7	52.8

equilibration time was increased from 6 to 24 h. Thus, this study highlights the importance and beneficial effect of prolonging equilibration of semen at 4 °C before freezing up to 24 h in order to attain optimal post-thaw semen quality. This is in agreement with Fleisch et al. [14] and Rickenbacher [36] who reported higher semen quality values after 24 h equilibration of bovine semen compared to their retrospective shorter equilibration time of 4 and 1.5 h, respectively. Similarly, Foote and Kaproth [16] and Anzar et al. [37] achieved greater motility of bovine semen after 18 and 24 h in comparison to equilibration time of 2 and 4 h, respectively.

Generally, most bull semen freezing protocols involve an equilibration period of 3–4 h, resulting in semen being frozen on the day of collection. However, an extensive review of bovine fertility trials by Pickett and Berndtson [38] established that a prolonged period of equilibration of 18 h was required in order to obtain maximum fertility. In AI centres, where daily collection schedules involve the collection of a large number of bulls and/or where semen is being transported over long distances for processing, a prolonged period of equilibration (up to 24 h) would be more convenient for the working operations of the centre as semen could be frozen the subsequent day or even after a weekend. The results of the current study indicate that increasing equilibration time from 6 to 72 h has no effect on CR. This finding is supported by Fleisch et al. [14] who reported that increasing equilibration time from 4 to 72 h had no effect on 90-day non-return rate (NRR), while no difference in 56-day NRR was reported when bull semen was equilibrated between 4 and 28 h [16]. This indicates that semen frozen on the day following collection should be of better quality and yield comparable fertility compared to semen frozen on the day of collection, thus, creating greater flexibility within the working environment of a commercial AI centre.

It is widely acknowledged that cow characteristics such as fertility sub-index and DIM play a role in fertility [32,39]. The current study, followed the same trend, whereby, cows with a greater fertility sub-index (≥ 110) and DIM (> 80 days) had a higher CR than cows with lower fertility sub-indices and less DIM. Animals in the lowest categories for both characteristics ($< \leq 50$ and < 40 DIM, respectively) had lower CR than animals of greater than ≥ 90 and 40 DIM, respectively. While this study demonstrates that a higher CR can be achieved through increasing the number of DIM before insemination, it also illustrates that late calving cows can be inseminated with reasonable success.

5. Conclusion

In conclusion, increasing equilibration time up to 72 h post collection, whilst resulting in a numerical decline, did not significantly affect field fertility within the expected range of fertility outcomes. Furthermore, an equilibration period of 24 h resulted in numerically greater post-thaw total and progressive motility scores. Therefore, implementing an equilibration period of 24 h may be more suitable to the working environment and collection schedules of AI centres, while confidence can also be gained

knowing that in circumstances where it is required (e.g., mechanical failure, avoidance of weekends), allowing an equilibration time of 3 days will not negatively impact on field fertility.

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Effect of seminal plasma from high-andlow-fertility bulls on cauda epididymal spermfunction

S. A.Holden, B. Fernandez-FuertesB, E. M.Murphy, P. Lonergan and S.Fair

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The impact of storage temperature and sperm number on the fertility of liquid-stored bull semen

Craig Murphy, Shauna A. Holden, Edel M. Murphy, Andrew R. Cromie, Patrick Lonergan and Sean Fair

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**Effects of Increasing Equilibration Time of Diluted Bovine Semen up to 72 Hours
Before Freezing on In Vitro and In Vivo Fertility**

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The aim of this study was to assess the effect of increasing equilibration time (ET) of bull semen up to 72 h before freezing on 60-day non-return rate (NRR) following artificial insemination (AI) with frozen-thawed semen. Semen was collected from Holstein Friesian bulls (n=5) at a commercial AI centre. Following quality assessment, each ejaculate was diluted to 15×10^6 sperm/0.25 mL straw in BullXcell (IMV Technologies, L'Aigle, France). Straws were filled, printed, sealed and held at 4 °C for four different ETs (6, 24, 48 and 72 h post dilution) prior to freezing. Each batch of semen was clearly labelled and distributed for insemination (total of n=1,640 inseminations) to technicians (n=24) who were blinded to treatment. Each technician received straws from each treatment and each bull, while a subset of straws were retained and assessed *in vitro* for total and progressive motility post-thawing. The NRR data were analysed using ANOVA in SPSS (version 22.0, IBM, USA) with Bonferroni adjustments applied. Equilibration for 24 h resulted in higher total and progressive motility than 6, 48 or 72 h ($P < 0.01$); however, 6, 48 and 72 h did not differ from each other ($P > 0.05$). There was a tendency ($P = 0.51$) for NRR to decline with increasing ET. There was a positive effect of cow fertility sub-index on NRR ($P < 0.05$) but there was no effect of parity or days in milk on NRR ($P > 0.05$). In conclusion, increasing ET of diluted

semen from 6 to 72 h did not affect NRR. This allows significant flexibility in semen production processes and may be more convenient for the working schedule of an AI centre as semen can be frozen up to three days after collection without any negative effects on NRR.

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A Comparison of Semen Diluents on the *In Vivo* Fertility of Liquid Bull Semen

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In countries with seasonal grass based production systems, such as Ireland and New Zealand, the use of fresh semen at lower sperm concentrations is an attractive alternative to frozen-thawed semen due to the short breeding season. The objective of this study was to assess the effect of semen diluents on non-return rate (NRR) following artificial insemination (AI) with fresh (liquid) semen for up to 3 days post collection. Semen was collected from Holstein Friesian bulls (n=8; total of 22 ejaculates) at a commercial AI centre. Following assessment for semen volume, sperm concentration and motility, each ejaculate was split into three equal volumes and diluted to 5×10^6 sperm/0.25 mL insemination dose in one of three different semen diluents, namely (i) Caprogen, the industry standard (homemade) (ii) BioXcell® (IMV, Technologies, Normandy, France) and (iii) INRA96® (IMV). Previously collected ejaculates from the same 8 bulls which were processed and frozen using routine procedures were used as a control (n=16,339 inseminations). Each batch of liquid semen was clearly labelled, stored at ambient temperature, distributed for insemination on the day of collection and used for up to 3 days post collection (n=7,802, 5,340 and 617 inseminations for Day 1, 2

and 3, respectively) on both heifers (n=1,440) and multiparous (n=28,658) dairy cows across 2,490 dairy herds. Insemination technicians (n=108) did not know the semen treatments. The 60-day NRR data were analysed using ANOVA in SPSS (version 22.0, IBM, USA), with Bonferroni adjustments applied. There was an effect of treatment on NRR ($P < 0.01$) as semen diluted in BioXcell had a lower NRR on Day 1, 2 and 3 of storage (67.9, 59.0 and 56.5%, respectively) in comparison with Caprogen (74.6, 74.9 and 83.1%, respectively), INRA96 (74.8, 74.4 and 79.0%, respectively) and frozen-thawed semen (75.9%). There was a treatment x day interaction ($P < 0.01$) as BioXcell on Day 2 resulted in a lower NRR than BioXcell on Day 1 ($P < 0.05$). There was an effect of parity ($P < 0.01$), cow fertility sub-index ($P < 0.01$) and the number of days in milk (DIM; $P < 0.01$) on NRR as primiparous heifers had a higher NRR in comparison with multiparous dairy cows. Cows with a higher fertility sub-index ($>€70$) had an increased NRR than those with a lower fertility sub-index. There was a positive linear relationship between the number of DIM (0-80+ days) and NRR. In conclusion, semen diluted in INRA96 had a similar 60-day NRR to semen diluted in Caprogen and frozen-thawed semen. Given that it can be used directly off-the-shelf, INRA96 may be a suitable alternative to Caprogen for the storage of liquid bull semen.