Interpretive Summary

Optimising Storage Temperature of Liquid Bovine Semen Diluted in INRA96

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This study examined the effects of storage temperature on *in vitro* sperm motility and kinematic parameters and the *in vivo* fertility of liquid bovine semen with the aim of optimising the storage temperature of liquid bovine semen. This novel study provides data on the *in vitro* analysis of semen diluted in INRA96 stored at three different storage temperatures and critically, the *in vitro* results are supported by a large-scale field trial.

Optimising Storage Temperature of Liquid Bovine Semen Diluted in INRA96

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Sperm, storage temperature, liquid semen, bovine, artificial insemination, non-return rate

ABSTRACT

Temperature regulation of liquid bovine semen can be difficult in field situations. Two experiments were carried out to assess the effect of storage temperature on *in vitro* sperm characteristics and 60-day non-return rate (NRR) following artificial insemination (AI) of liquid bovine semen. In Experiment 1, 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 ^{o}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 2, the field fertility of liquid semen stored at a constant 5 or 15 ^{o}C or in an unregulated manner was assessed and compared to frozen-thawed semen (total of n=106,738 inseminations). In Experiment 1, there was a linear decrease in motility with increased duration of storage ($P < 0.01$). Semen stored at a constant 15 ^{o}C or fluctuated between 5 and 15 ^{o}C had greater total motility than semen held at 5 ^{o}C ($P < 0.05$), 28 and fluctuated between 5 and 28 ^{o}C ($P < 0.01$); however, 15 ^{o}C or fluctuated between 5 and 15 ^{o}C did not differ from each other. Semen held at 5, 15 or fluctuated 5 and 15 ^{o}C, although not differing from each other, had higher progressive motility scores than storage at 28 and or fluctuated between 5 and 28 ^{o}C ($P < 0.01$). Semen stored at a constant 28 ^{o}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 2, semen stored at a constant 5 ^{o}C resulted in a lower 60-day NRR (62.5%) than storage at constant 15 ^{o}C, unregulated or frozen-thawed semen (73.6, 74.6 and 74.4%, respectively; $P < 0.01$). In conclusion, sperm stored in IRNA96 are quite tolerant in terms of storage temperature, retaining acceptable motility between temperatures of 5 and 15 ^{o}C. Storing semen at a

constant 15 ºC resulted in greater *in vitro* sperm motility and higher NRR rates than storage at 5 ºC 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.

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 ºC while the minimum and maximum temperatures recorded in one study were 6.4 and 27.9 ºC, 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 ºC are optimal for bovine semen when semen is purged with nitrogen gas (N_2; Vishwanath and Shannon 2000), while storage at 5 ºC 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 N_2 gassing (Shannon 1965). While storage of liquid semen at 5 ^{o}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 ^{o}C) were devised (Shannon and Curson 1984). Although storing semen at 15-20 ^{o}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 ^{o}C in an egg-yolk based diluent (Caprogen) had greater total and progressive motility than semen stored at 5, 22, 32 ^{o}C or fluctuating (day to night-time) between these temperatures. That study also reported that sperm were tolerant to temperatures between 5 and 22 ^{o}C. Milk-based extenders, such as INRA96, are widely used in the dilution and storage of equine semen cooled to between 4-8

105 °C (Batellier et al. 2001). However, Batellier et al. (1997) demonstrated that the survival of
106 equine sperm stored in INRA96 at 15 °C was better than that stored in milk-based extenders
107 at 4 °C and others have demonstrated improved pregnancy rates when equine semen was
108 stored at 15 °C (Batellier et al. 2001, Cuervo-Arango et al. 2014). Although liquid bull semen
109 has traditionally been stored at ambient temperature in the egg-yolk based diluent, Caprogen,
110 we have recently demonstrated that INRA96 is effective for the preservation of bovine semen
111 stored at ambient temperature, resulting in similar calving rates to semen diluted in Caprogen
112 (Murphy et al. 2017). Furthermore, as the preparation of Caprogen is complex and time
113 consuming, the use of INRA96 is a suitable alternative for the dilution and storage of bovine
114 semen and is more convenient for the busy working schedule of an AI centre as it can be used
115 directly off the shelf, thus, reducing time constraints within the laboratory. Therefore, the
116 objectives of this study were to investigate if temperature regulation could improve the *in*
117 *vitro* sperm motility and kinematic parameters and *in vivo* fertility of liquid bovine semen
118 stored in INRA96.

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120 **MATERIAL AND METHODS**

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

123 The aim of this experiment was to establish the optimum storage temperature range for liquid
124 bovine semen stored in INRA96. The effect of five different storage temperature conditions
125 (5, 15, 28, fluctuating 5-15 or fluctuating 5-28 °C) on total and progressive motility and
126 kinematic parameters of liquid bovine semen for up to four days post collection was assessed.
127 Semen was collected from Holstein Friesian bulls (n = 7) at a commercial AI centre on three
128 different occasions (occasion = replicate). The raw ejaculate was partially diluted in 10 mL

pre-warmed INRA96 (IMV Technologies, Normandy, France) at 37 ^{o}C and transported in a temperature-regulated cooler box at 18 ^{o}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 $\geq$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 x 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 ^{o}C. Fluctuating temperatures were designed to mimic day to night time fluctuations; semen was held at 5 ^{o}C at night (in a fridge) and at either 15 (in temperature-regulated box) or 28 ^{o}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 ^{o}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.

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 35 ^{o}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 ^{o}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 ^{o}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 as average path velocity (VAP above 10 μm/s), straight line velocity (VSL), curvilinear velocity (VCL), linearity (LIN), straightness (STR), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF). 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.

Experiment 2: 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 ^{o}C) and the third was the industry standard which involves storage of straws in an unregulated temperature flask. Semen was collected from Holstein Friesian bulls (n = 16; denoted A-P) at a commercial AI centre from mid-April to 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 x 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 ^{o}C and distributed to technicians where it was then stored at either a constant 5 ^{o}C, 15 ^{o}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 ^{o}C with minimum and maximum temperatures of 0.3 and 21.6 ^{o}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 which require that frozen semen is held for 30 days before use (Irish Statue Book, 2004), frozen-thawed semen doses (15 x 10^6 sperm per dose) were derived from previously collected ejaculates from the same 16 bulls which were processed and frozen using routine procedures (n = 58,533 inseminations consisting of 10,440 heifers and 48,093 multiparous dairy cows) as described by Murphy et al. (2017).

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 ^{o}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 non-return rate (NRR) 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 (n = 10,917) 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 inseminations remained for Experiment 2.

Cow characteristics such as parity, days in milk (DIM) and fertility sub-index were included in the model. Fertility sub index is a key component of the Economic Breeding Index (EBI) comprising ~35% of the total EBI (ICBF, 2017). 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).

Statistical Analysis

Data from Experiment 1 were examined for 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 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 ± the standard error of the mean (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 $P < 0.05$.

RESULTS

Experiment 1: 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 1; $P < 0.01$), 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 ^{o}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 ^{o}C ($P < 0.05$); however, this did not differ from semen fluctuated between 5 and 15 ^{o}C ($P > 0.05$). Semen held at 5, 15 and fluctuated between 5 and 15 ^{o}C had a higher progressive motility score than semen held at 28 and fluctuated between 5 and 28 ^{o}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 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 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 ($P > 0.05$).

Experiment 2: 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 2); 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 ($P < 0.01$). There was an effect of bull on NRR

(P < 0.01) with NRR for individual bulls varying from 69.9 to 78.7% (Table 2). There was a bull by treatment interaction as all bulls had a lower NRR for semen stored in 5 ^{o}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; Table 2). 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). 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). 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 in comparison to cows with a fertility sub-index of less than €70 (77.9 vs 73.3%, respectively; 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 cow breed, nor was there a breed, parity, cow fertility sub-index, DIM, herd or technician by storage temperature interaction (P > 0.05).

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 ^{o}C resulted in greater sperm quality than semen stored at 5, 28 or fluctuating between 5 and 28 ^{o}C and (ii) semen stored at a constant 15 ^{o}C resulted in greater NRR on Days 1 and 2 of storage in comparison to semen stored at 5 ^{o}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. 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 ^{o}C recorded higher progressive motion values than any other storage temperature, however, storing semen at extreme high temperatures of 28 ^{o}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 ^{o}C, while storing semen at a constant 15 ^{o}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 ^{o}C had greater motility compared to semen stored at 5, 22, 32 ^{o}C or fluctuating temperatures between 5 and 15, 5 and 22 and 5 and 32 ^{o}C.

Although milk-based extenders are more widely used at storage conditions of between 4-8 ^{o}C, INRA96 has also been shown to be beneficial in the preservation of equine sperm stored at 15 ^{o}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 ^{o}C or unregulated temperature compared to storage at a constant 5 ^{o}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 i*n 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 ^{o}C, respectively.

Surprisingly, in the current study, semen stored at a constant 5 ^{o}C 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 ^{o}C compared to any other storage conditions. A possible explanation for the poor fertility performance of liquid semen stored at 5 ^{o}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 these samples *in vitro*. In addition, the *in vitro* results of the current study highlight that fluctuating storage conditions between 5 and 28 ^{o}C resulted in a significant loss of sperm motility, thus, suggesting 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 ^{o}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 ~20-25%, despite semen meeting minimum routine quality control standards (Kastelic and Thundathil 2008, Holden et al. 2017). The results of the current study 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 aging effects when stored in liquid semen. The production of reactive oxygen species, 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.

CONCLUSION

In conclusion, bovine semen held at a constant 15 ^{o}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 ^{o}C. Semen held at a constant 15 ^{o}C resulted in similar NRR to semen stored in unregulated storage conditions but NRR was significantly reduced at storage of 5 ^{o}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 ^{o}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.

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Figure captions

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

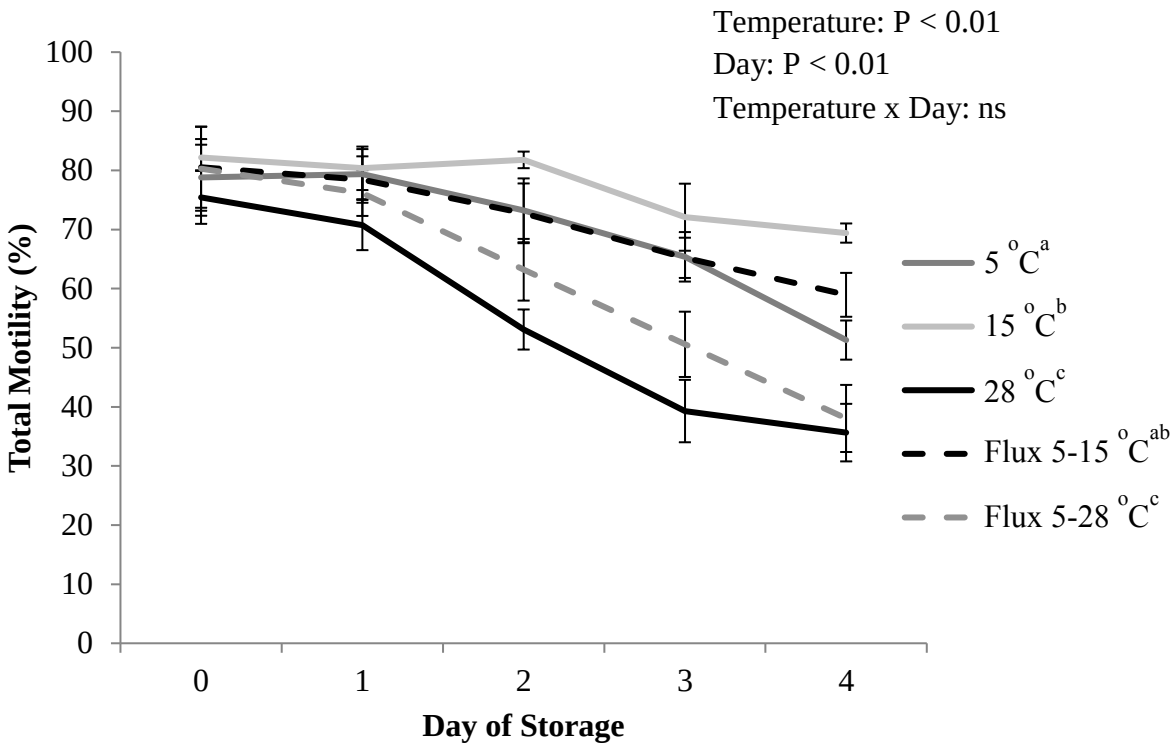
Figure 2: The effect of storage temperature and day of storage on 60 Day non-return rate in dairy cows and heifers (Experiment 2). Vertical bars represent 95% confidence intervals. n = number of inseminations. [ab]Values with different superscripts differ significantly ($P < 0.01$).

Table captions

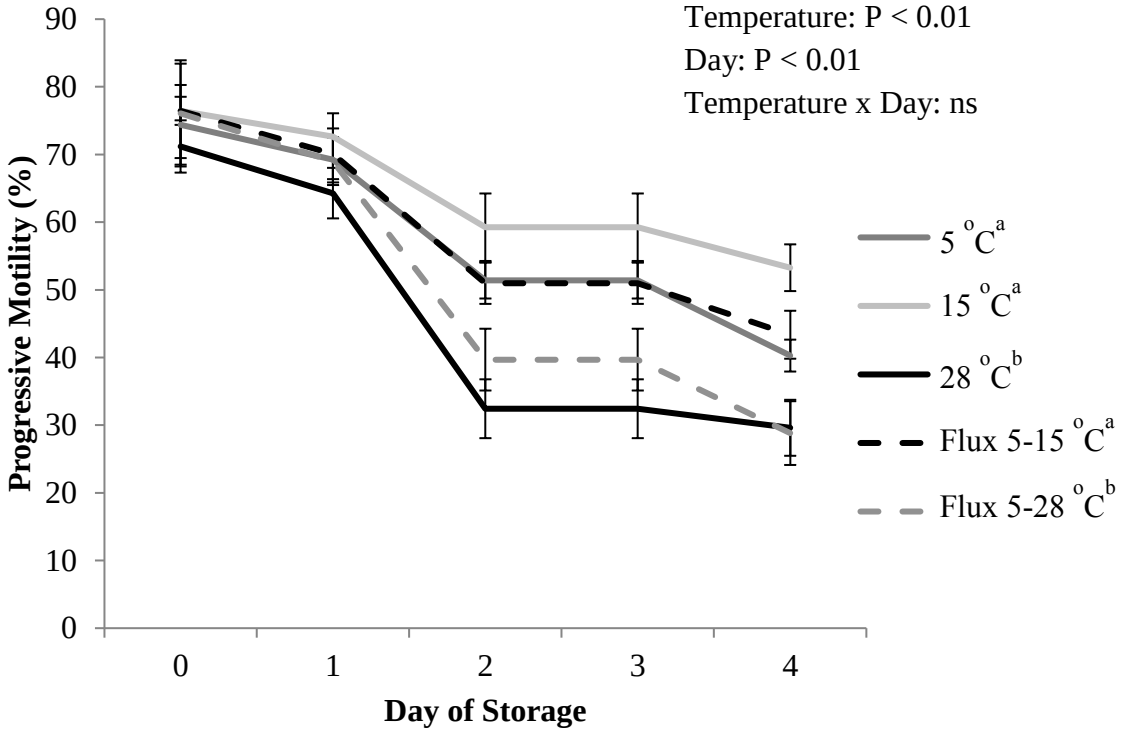
Table 1: The overall effect of storage temperature on total and progressive motility and kinematic parameters as assessed using computer assisted sperm analysis in bovine semen stored at 5, 15, 28, fluctuated between 5 and 15 or fluctuated between 5 and 28 ^{o}C (Experiment 1). ^{abc}Values with different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

Table 2: The effect of liquid bovine semen inseminated on Day 1 or Day 2 post collection and frozen-thawed semen on 60 Day non-return rate in dairy cows and heifers (Experiment 2). ^{ab}Values in the same row with different superscripts differ significantly ($P < 0.01$). n = the total number of inseminations per treatment per day.

437 Murphy et al. Figure 1.

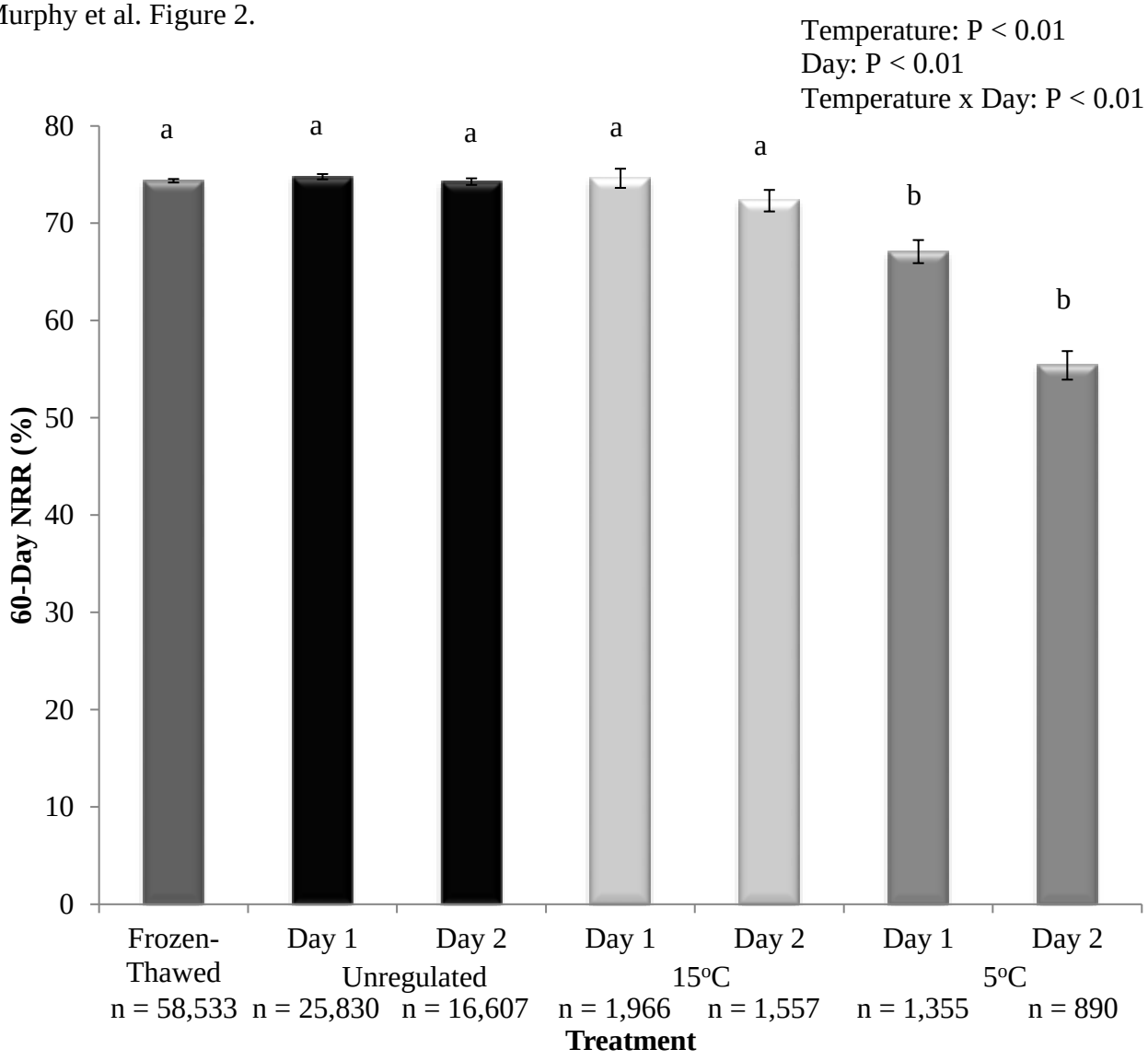


438



439

440 Murphy et al. Figure 2.



441

442

443 Murphy et al. Table 1.

Parameter	Storage Temperature (ºC) mean ± sem					P Value
	5	15	28	Fluctuating 5 and 15	Fluctuating 5 and 28	Effect of treatment
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

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

447

448 Murphy et al. Table 2

Bull	Liquid Day 1 % Non-Return Rate (n)	Liquid Day 2 % Non-Return Rate (n)	Frozen-thawed % Non-Return Rate (n)	P value
A	69.9 [a]	72.2 [ab]	75.4 [b]	P < 0.01
	(1,165)	(759)	(1,772)	
B	77.6	75.0	73.9	ns
	(1,243)	(1,166)	(1,983)	
C	72.5 [ab]	71.5 [b]	78.8 [a]	P < 0.01
	(109)	(747)	(2,322)	
D	69.7	71.0	69.1	ns
	(1,718)	(1,135)	(1,151)	
E	75.8 [ab]	77.2 [b]	73.1 [a]	P < 0.01
	(1,537)	(890)	(6,768)	
F	78.2 [a]	72.5 [b]	79.5 [a]	P < 0.01
	(734)	(541)	(4,612)	
G	74.3 [a]	71.1 [ab]	69.8 [b]	P < 0.01
	(2,111)	(1,117)	(4,689)	
H	78.7 [a]	75.5 [b]	79.5 [a]	P < 0.01
	(4,198)	(2,041)	(5,886)	
I	76.6	73.6	74.0	ns
	(1,710)	(1,011)	(7,846)	
J	73.1	70.9	70.8	ns
	(2,498)	(2,171)	(1,457)	
K	75.4 [ab]	71.9 [b]	77.6 [a]	P < 0.01
	(1,448)	(950)	(1,763)	
L	71.4	75.3	74.9	ns
	(1,224)	(790)	(2,311)	
M	73.2 [ab]	75.0 [b]	70.3 [a]	P < 0.05
	(930)	(721)	(2,546)	
N	73.2 [a]	72.6 [a]	69.7 [b]	P < 0.01
	(4,087)	(2,148)	(4,593)	
O	76.7	72.8	74.9	ns
	(1,111)	(894)	(3,267)	
P	73.7	74.1	74.8	ns
	(3,328)	(1,973)	(5,567)	
Overall	74.4 [a]	73.2 [b]	74.4 [a]	P < 0.01
	(29,151)	(19,054)	(58,533)	

449 ns = non-significant

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