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Interpretive Summary

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This study examined the effect of a wide range of semen diluents on both the *in vitro* and *in vivo* fertility of liquid bull semen stored for up to 3 days post collection. The semen diluents BioXcell, INRA96 and Caprogen maintained sperm motility best when stored at a constant temperature, however, when used in the field, at an unregulated temperature, BioXcell performed poorly. This study clearly illustrates the importance of *in vivo* fertility data in assessing semen diluents.

SEMEN DILUENTS FOR LIQUID BULL SEMEN

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

Edel M. Murphy *†, Craig Murphy‡, Ciara O'Meara†, Gemma Dunne†, Bernard Eivers†, Patrick Lonergan§ and Sean Fair*[1]**.**

* Laboratory of Animal Reproduction, Department of Life Sciences, Faculty of Science and Engineering, University of Limerick, Castletroy, Limerick, V94 T9PX, Ireland.

† National Cattle Breeding Centre, Naas, Co Kildare, W91 WF59, Ireland.

‡ Animal & Grassland Research and Innovation Centre, Teagasc, Moorepark, Fermoy, Co. Cork, P61 C996, Ireland.

§ School of Agriculture and Food Science, University College Dublin, Belfield, Dublin 4, D04 N2E5, Ireland.

[1]**Corresponding Author**: Dr Sean Fair, Laboratory of Animal Reproduction, Department of Life Sciences, Faculty of Science and Engineering, University of Limerick, Limerick, Ireland.

Tel: + 353 61 202548, Fax: + 353 61 331490, E-mail sean.fair@ul.ie

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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 (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 (homemade)), OptiXcell, BioXcell, BullXcell, INRA96, NutriXcell or AndroMed (all commercially available) 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 ^{o}C and 18 ^{o}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 CR ($P < 0.01$) 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 ^{o}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.

INTRODUCTION

Artificial insemination (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). 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 in order 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 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. 2002).

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 in order to reduce the effects of the natural day to night time temperature fluctuations. Murphy et al. (2015) reported that although fluctuating storage temperature between 5 and 32 ^{o}C had no effect on viability, total progressive motility was greatest for liquid semen stored at 15 ^{o}C compared to other temperatures assessed. In addition to dilution of the semen, diluents provide protective compounds such as bovine serum albumin, antioxidants and antibiotics to maintain sperm function (van den Berg et al. 2014, Rehmana et al. 2013). 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. 2015, 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 ^{o}C (Saha et al. 2014), reducing sperm concentration (Murphy et al. 2013), altering the pH (Ferdinand et al. 2014) as well as N_2 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 bull semen (Stradaioli et al. 2007). Although the benefits of using lecithin-based extenders on the cryopreservation of bull 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 ^{o}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 bull 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 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. 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 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 (i) liquid semen diluent on total and progressive sperm motility (ii) liquid semen diluent on CR and (iii) 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: 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 artificial insemination (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 ^{o}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 $\geq$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 x 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 ^oC. 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.

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 ^oC) 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.

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 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 directly to a final concentration of 5 x 10^6 sperm per 0.25 mL insemination dose in one of three different diluents: namely Caprogen, BioXcell or INRA96 (Figure 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 ^oC). 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 $\geq$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 10^6 sperm per 0.25 mL insemination dose. Straws were frozen to -140 ^{o}C as follows: -5 ^{o}C per min from +4 ^{o}C to -10 ^{o}C, -40 ^{o}C per min from -10 ^{o}C to -100 ^{o}C and thereafter -20 ^{o}C per min from -100 ^{o}C to -140 ^{o}C in a programmable freezer (IMV Technologies), followed by submersion and storage in liquid nitrogen at -196 ^{o}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 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 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 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 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. CR was measured using a cut-off value of 275 and 290 days from date of insemination to calving date.

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, 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 ^{o}C during the day and gradually brought to 4 ^{o}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).

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). 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 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 was evaluated and correlations were investigated with a number of 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 ± the standard error of the mean (s.e.m) 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: 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 ($P > 0.05$). 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 ± 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).

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 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%, $P < 0.01$; Figure 4). 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 5).

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 ($P < 0.01$) represented by bulls B and D having a higher CR on Day 1 than liquid semen on Day 2 ($P < 0.05$) but did not differ from Day 3 ($P > 0.05$). Bull F had a higher CR for liquid semen on Day 1 than liquid semen on Day 3 ($P < 0.05$) but did not differ from liquid semen inseminated on Day 2 ($P > 0.05$). 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 1). 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 1) but this was primarily a result of the poor CR recorded for BioXcell.

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

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 7). 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 ^{o}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 ± 10.0% and 54.4 ± 18.7 to 35.4 ± 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 ± 5.02%, respectively) and progressive motility (52.5 ± 3.58% and 59.6 ± 5.04%, respectively) up to 3 days 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: i) liquid semen diluted in Caprogen or INRA96 resulted in a similar CR to frozen-thawed semen; ii) liquid semen stored in BioXcell resulted in a reduced

CR in comparison to semen diluted in Caprogen or INRA96 and frozen-thawed semen; and (iii) temperature fluctuation between 4 and 18 ^{o}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 bull semen diluent, although initially developed for the dilution of semen at 5 ^{o}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 ^{o}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 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 ^{o}C. O'Hara et al. (2010) reported that storing ram semen in INRA96 at a constant 15 ^{o}C resulted in significantly reduced sperm motility but when stored at a constant 5 ^{o}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 ^{o}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, Chaudhari et al, 2015). Both Sharafi et al. (2009) and Gill 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 ^{o}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. 1998). 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, Lusignan et al, 2011). These low density lipoproteins are also believed to be important in protecting sperm cells during the lipid phase transition (Holt et al. 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 source) is used in animal-protein free media 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 soya-lecithin based extender, has been reported to result in better post-thaw sperm quality than milk-based extenders in the goat (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 non-return rates (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 ^{o}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 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 a number of species, there is limited published data 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 to 1-2 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 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 (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 maximise reproductive efficiency within dairy herds, contributing to reducing costs associated with reproductive inefficiencies 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).

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 ^{o}C and 18 ^{o}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 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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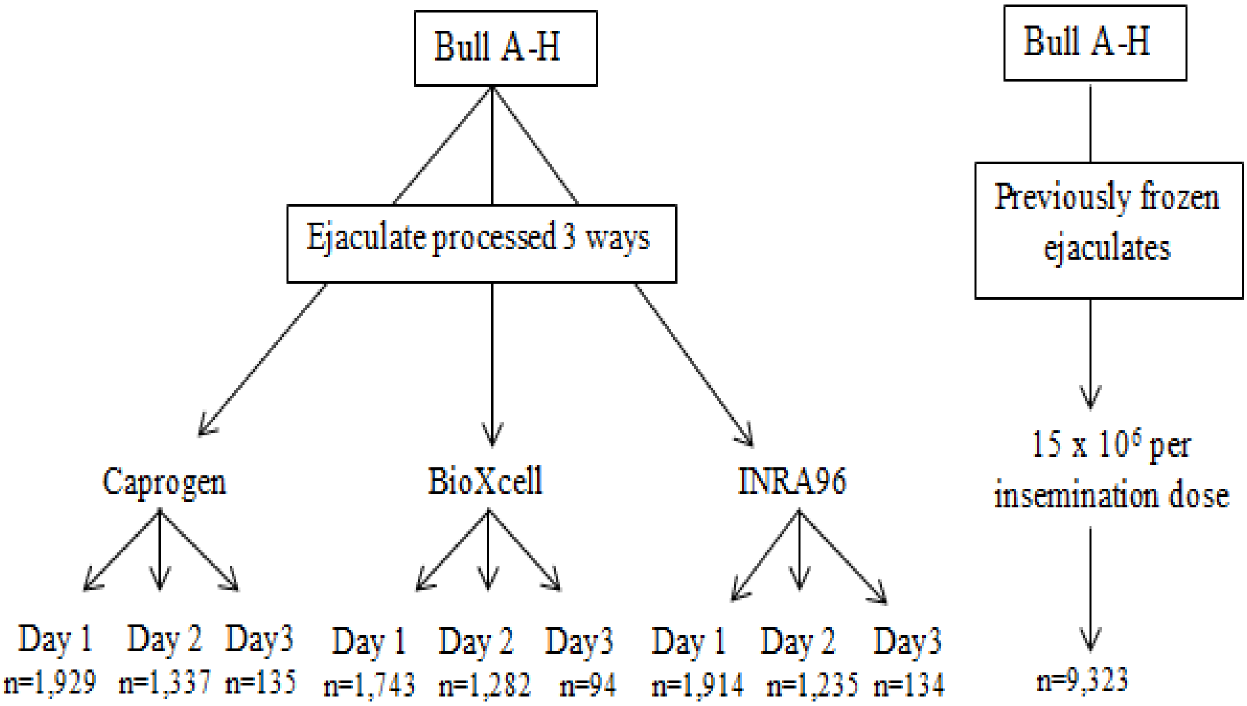


Figure 1: Experimental design for experiment 2. Ejaculates were split for the liquid semen treatments and previously frozen ejaculates from the same bull were sourced for the frozen-thawed treatment (n = the total number of inseminations per diluent per day).

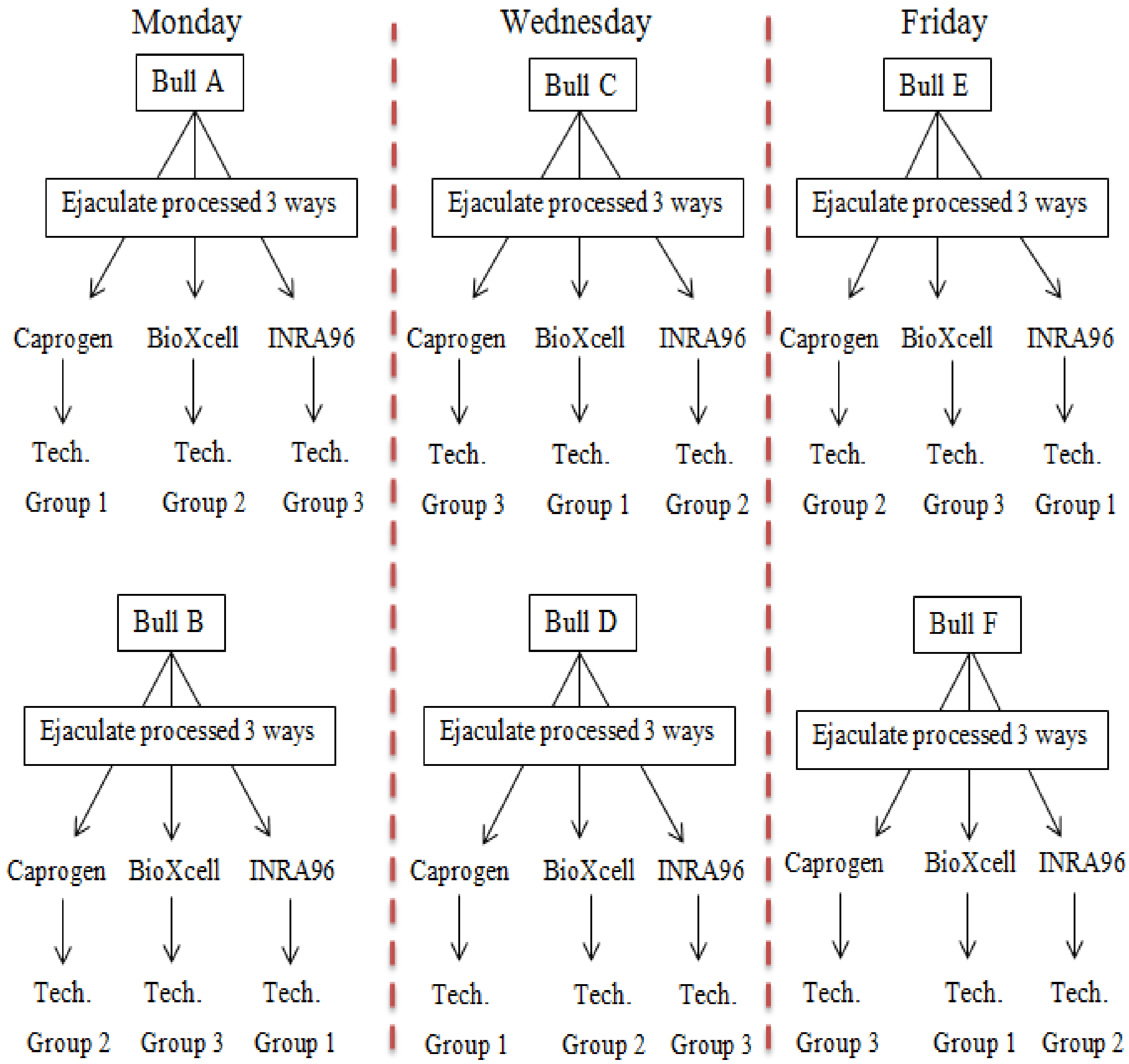
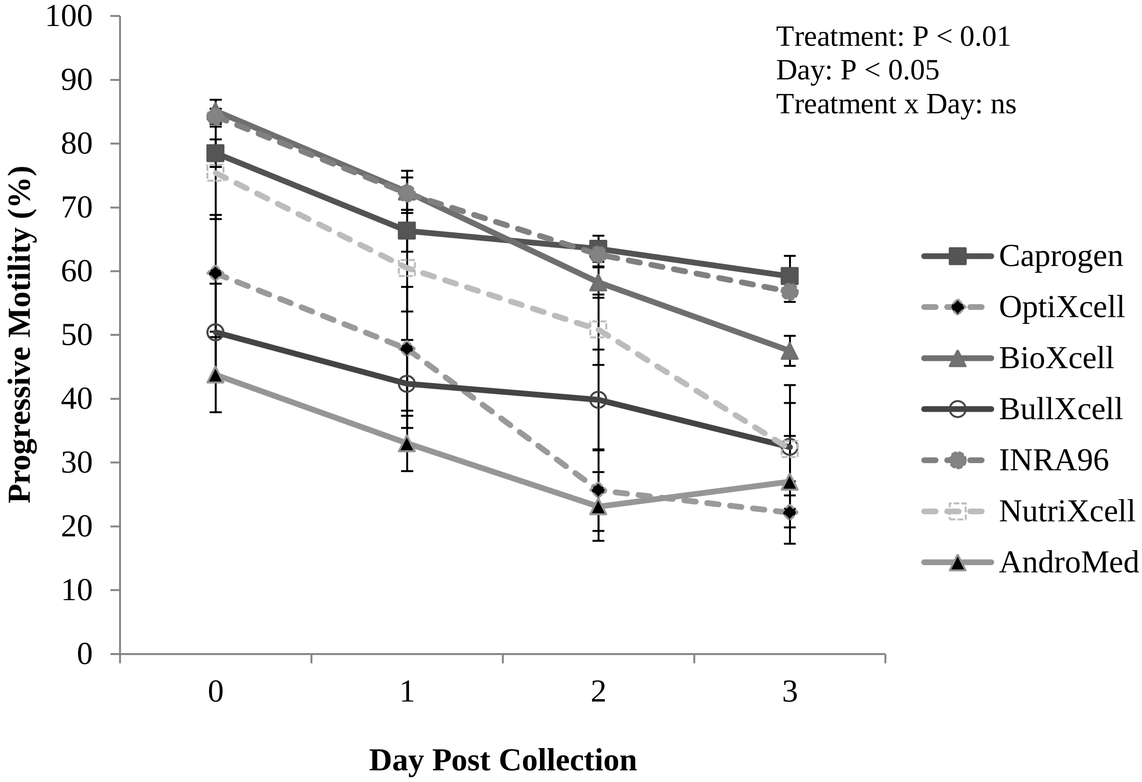


Figure 2: Experimental design for experiment 2, highlighting the distribution of different liquid semen treatments to technicians within a week (Displaying Bulls A-F only). Each technician received two different treatments each day. Frozen-thawed semen was also inseminated throughout the period.



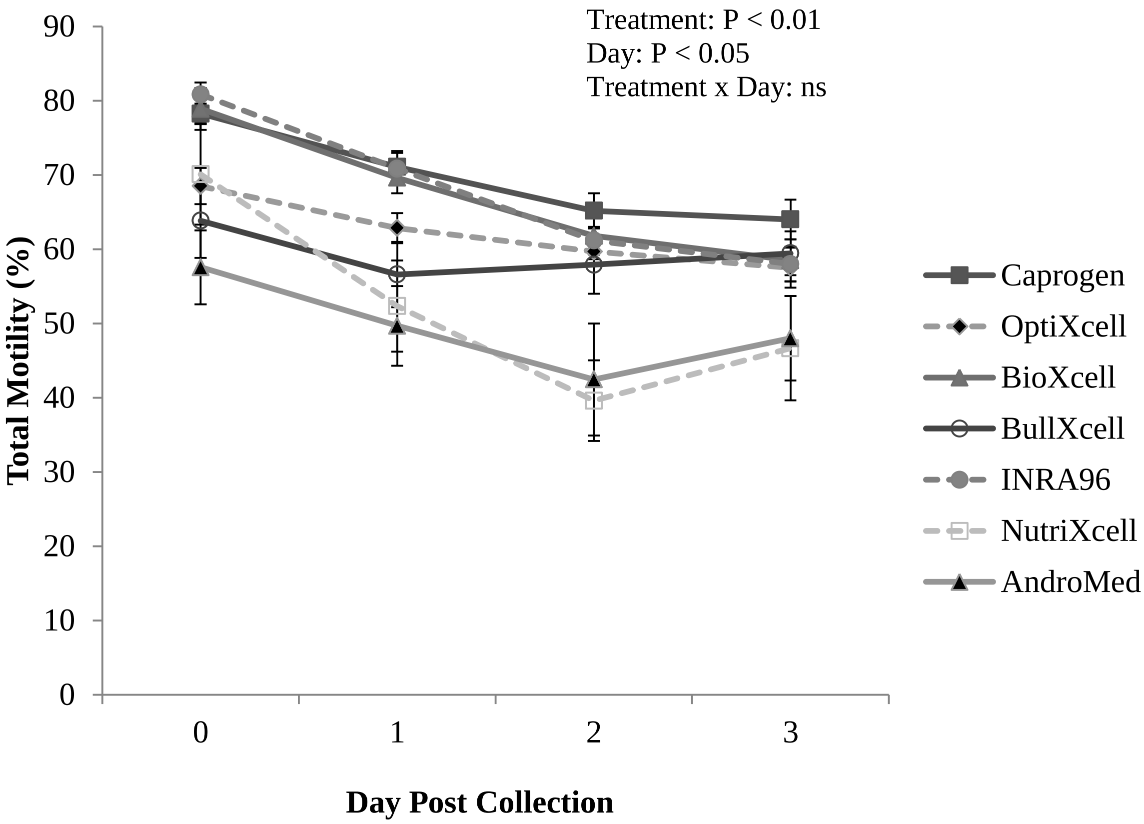


Figure 3: The effect of diluent on progressive motility (upper panel) and total motility

(lower panel) of liquid bull semen on Days 0, 1, 2 and 3 post-collection (Experiment 1). Vertical bars represent s.e.m. ns = not significant

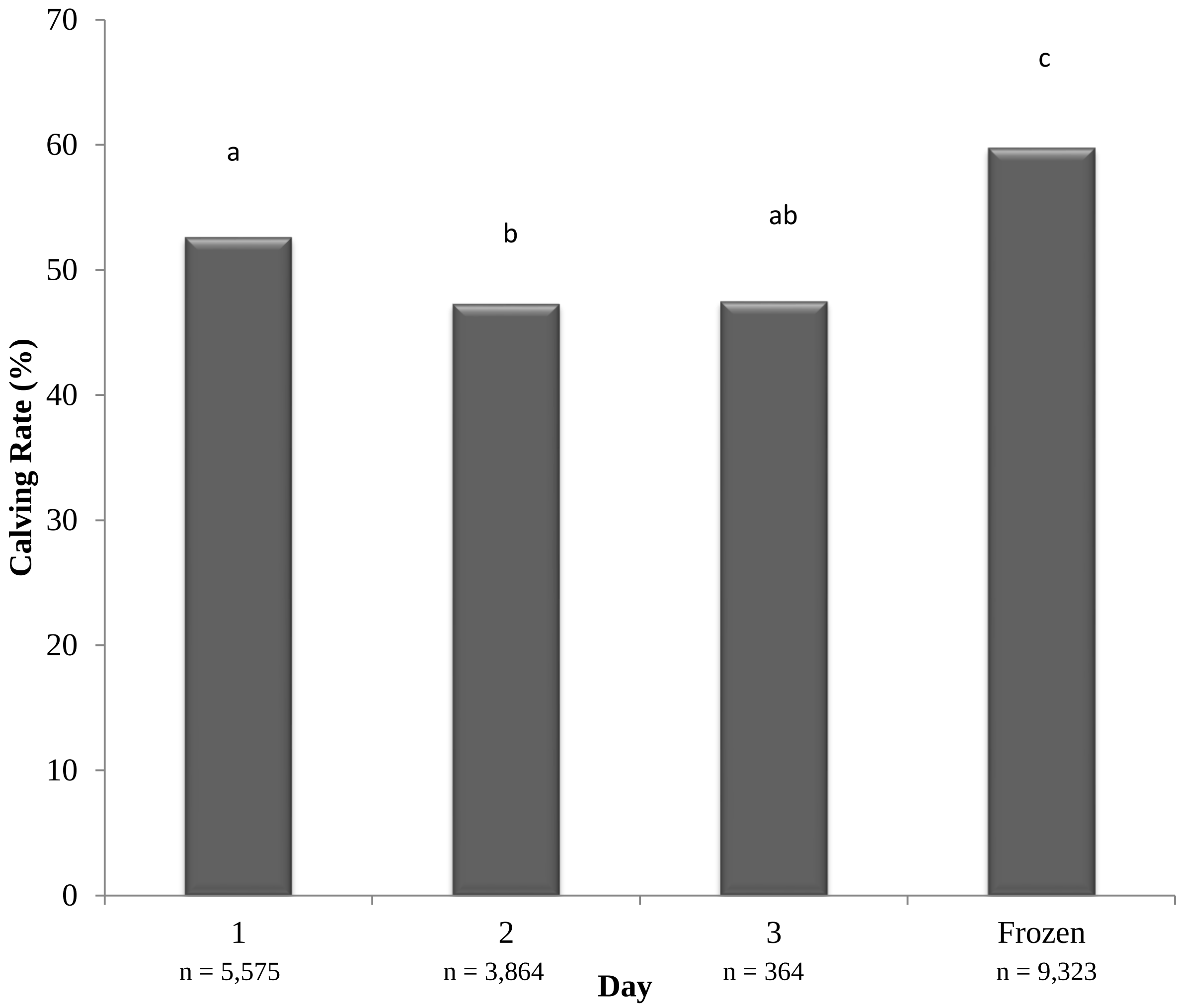


Figure 4: The effect of day of storage of liquid bull semen on calving rate in dairy cows and heifers (Experiment 2). Values with different superscripts differ significantly ($P < 0.01$).

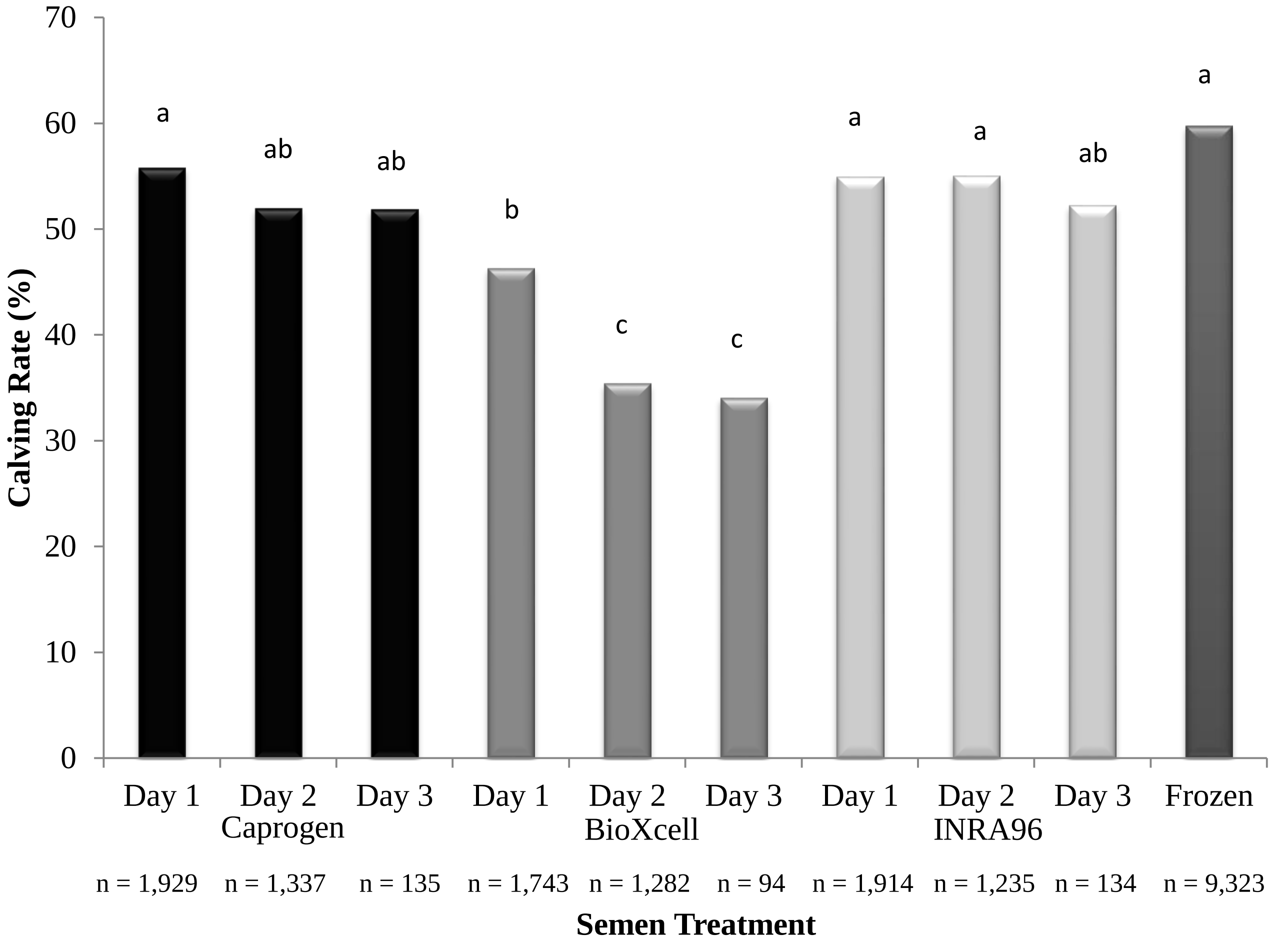


Figure 5: The effect of liquid semen diluent and day of storage on calving rate in dairy cows and heifers (Experiment 2). Values with different superscripts differ significantly ($P < 0.01$).

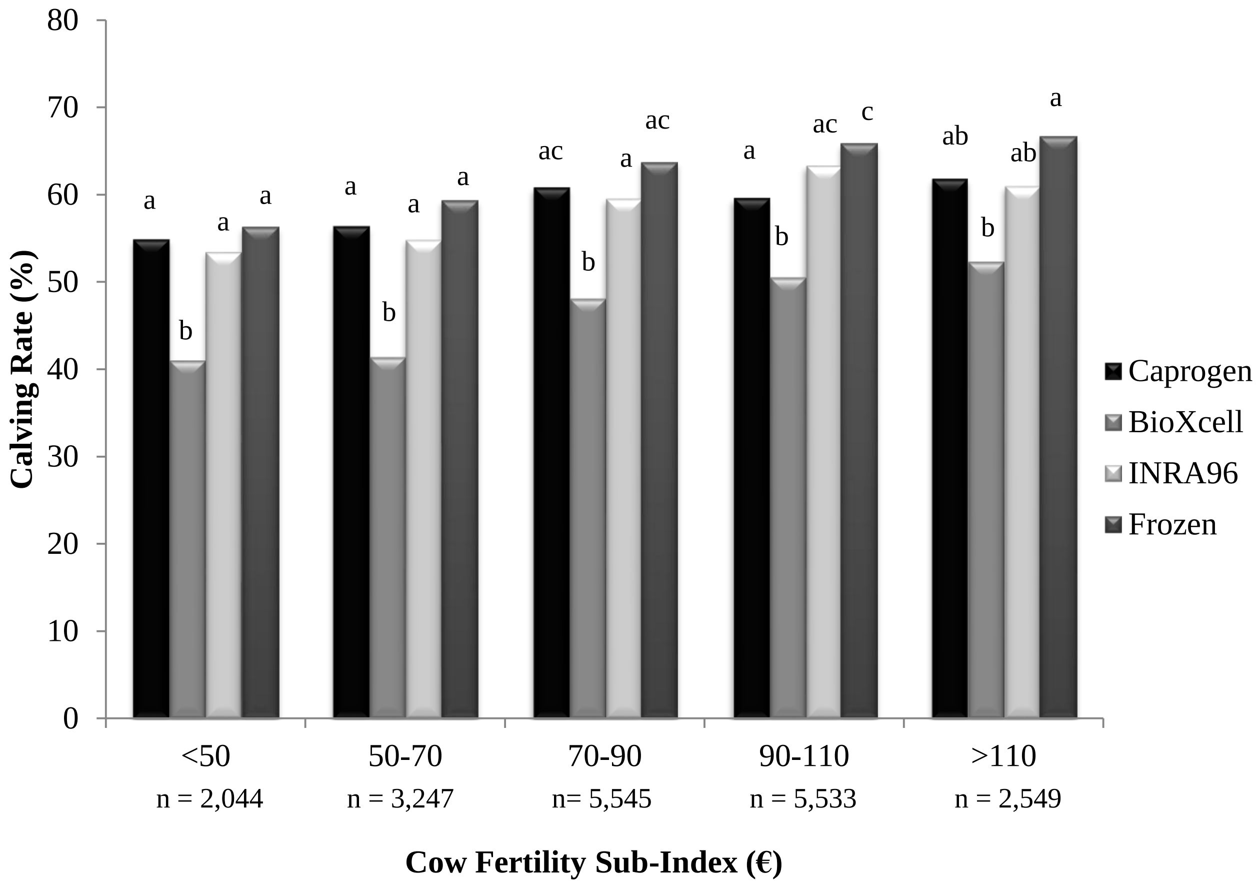


Figure 6: The effect of cow fertility sub-index by treatment interaction on calving rate in dairy cows and heifers (Experiment 2). Values with different superscripts differ significantly within fertility sub-indices ($P < 0.01$).

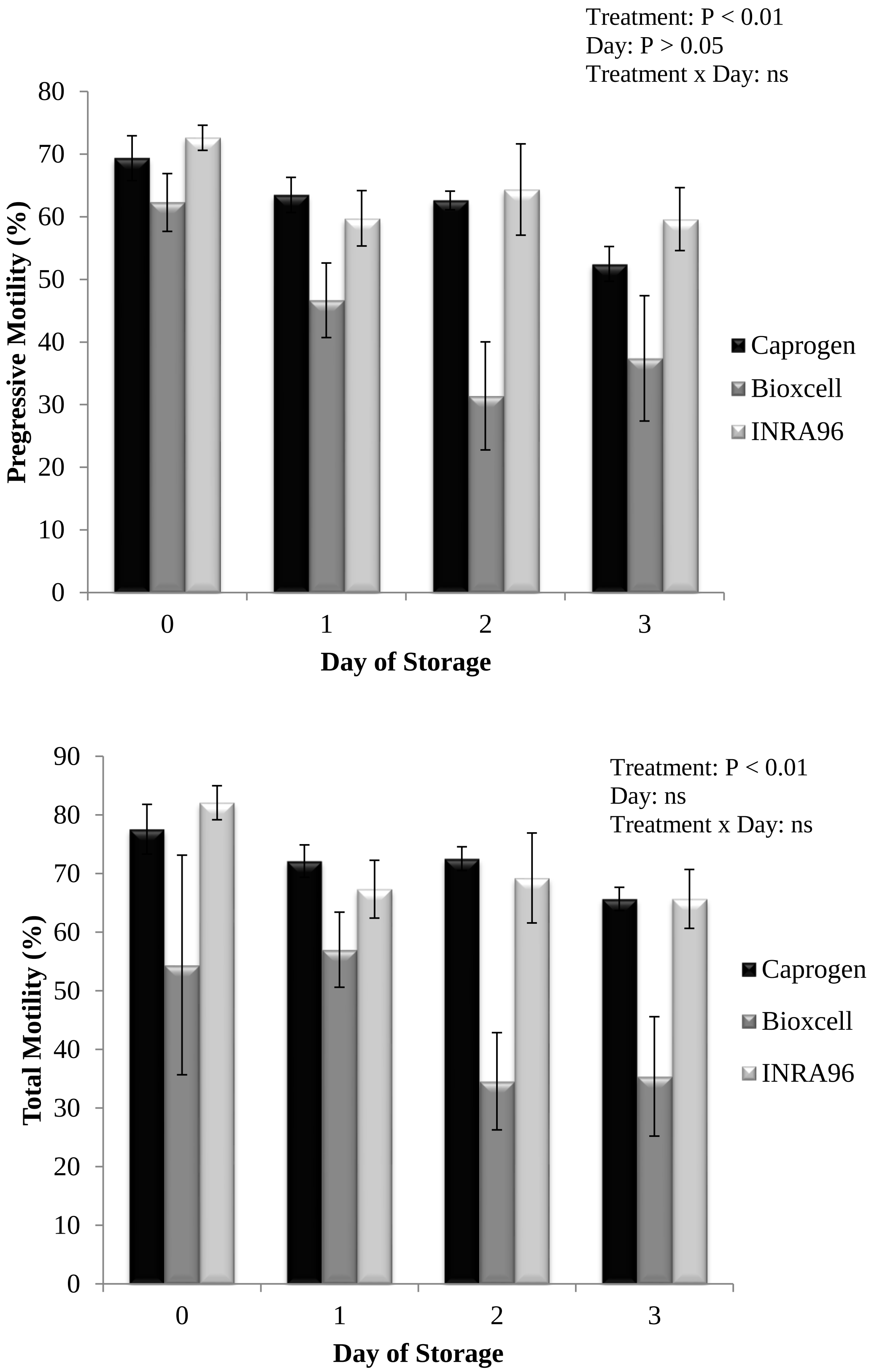


Figure 7: The effect of temperature fluctuation on progressive motility (upper panel) and total motility (lower panel) of liquid bull semen stored in three diluents and assessed on Days

0, 1, 2 and 3 post collection (Experiment 3). Vertical bars represent s.e.m. ns = not significant.

Table 1: The effect of liquid bull 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 ^{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)

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