Title

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

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Abstract

Equilibration times 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. In Experiment 1, the effect of holding semen at 4 ^{o}C for one of four different equilibration times (6, 24, 48 and 72 h post dilution) before freezing on subsequent post-thaw total and progressive motility was assessed subjectively. In Experiment 2, the field fertility (CR) of frozen-thawed semen held for 6, 24, 48 and 72 h before freezing was assessed (total of n = 1,640 inseminations). In Experiment 1, equilibration for 24 h resulted in higher total and progressive motility than equilibration for 6, 48 or 72 h ($P < 0.01$), however, there was no difference in motility between equilibration for 6, 48 or 72 h ($P > 0.05$). In Experiment 2, 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 thus, providing semen processing centres with flexibility in the time which semen can be held prior to freezing.

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] and thus 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. A number of investigative avenues have been undertaken in order to improve the freeze-thaw process including; modifying freezing rates [3], identifying the best thawing procedures [4], altering equilibration periods [5], comparing semen diluents [6] and optimising sperm concentration [7]. However, the process of cryopreservation is detrimental to a high proportion of sperm [8] as, due to thermal and osmotic effects and the damage sustained by the plasma membrane, approximately 50% of sperm are compromised during the freeze-thaw process [9].

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 [10]. The components within egg-yolk, in particular low-density lipoproteins, bind to the sperm membrane during the freeze-thaw process, thus increasing chilling tolerance and preventing loss of membrane phospholipids [11]. Typically, frozen-thawed bull semen extenders differ in composition from fresh semen extenders as greater emphasis is placed on stabilising the cell membrane, thereby combating the damage sustained by sperm during cryopreservation and subsequent thawing. The addition of cryoprotectants, which are classified as either penetrating or non-penetrating [12], such as egg yolk, which contains low density lipoproteins and cholesterol, as well as 3 - 6% glycerol, minimises the physical and chemical stresses associated with cryopreservation, thus protecting the sperm membrane during cooling and reducing membrane damage during

freezing [13]. A non-penetrating cryoprotectant, such as egg-yolk, as the name suggests, cannot cross the sperm membrane and thus only acts extracellularly to modify the sperm membrane [14]. 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 [15].

Semen cryopreservation involves several steps including cooling, equilibration, freezing and subsequent thawing [16]. Typically, freezing protocols for bull semen generally include cooling to 4 - 5 ^{o}C followed by a variable duration of equilibration (0 - 24 h) at this temperature prior to freezing [17]. This allows sperm to adapt to cooler temperatures [11], 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 decreasing damage sustained by ice crystal formation during the freeze-thaw process [10]. Osmotic stress is one of the most common factors contributing to sperm damage during cryopreservation as ice crystal formation increases the concentration of solutes in the extender, creating an osmotic gradient [18]. Changes in the osmotic pressure within the extender exerts osmotic stress on sperm resulting in irreversible damage to sperm membrane integrity [19]. Different extenders with varying glycerol concentrations, cooling conditions and equilibration times have been developed and found to be effective for bull sperm cryopreservation.

Equilibration time was believed to be important in allowing glycerol sufficient time to penetrate the sperm membrane (see review [17]), however, a study conducted by Berndtson and Foote [20] 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 [11] as it enables the movement of water across the membrane and reduces the damage sustained by sperm during the freeze-thaw process. A number of investigations aimed at identifying the optimum equilibration time have been conducted on semen from a number of species such as ovine [21], caprine [22] and bovine [23]. Michel, Chaigneau and Guyonnet [24] reported that increasing the equilibration time from 8 to 18 h significantly increased the quality of bull semen assessed *in vitro*, such as sperm motility and viability, but found no effect *in vivo*. Similarly, Fleisch et al. [23] reported that extension of equilibration time from 4 - 72 h improved motility and viability post-thaw of bull sperm and had no effect on field fertility. However, although the majority of cryopreservation protocols for bovine semen involve an equilibration period of 4 h, the optimum duration of equilibration time varies as a wide range of equilibration times exist within the literature: 0 h [17], 1- 4 h [25], 18 - 24 h [26] and 24 - 72 h [23, 27].

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 implementing an equilibration time of 24 h resulted in an increase in semen quality and therefore this prolonged period of equilibration 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 sperm.

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) at a commercial AI centre (National Cattle Breeding Centre, Enfield, Co Meath, Ireland) and immediately after collection, the raw ejaculate was partially diluted in 10 mL pre-warmed BullXcell (37 ^{o}C; IMV Technologies, Normandy, France) and transported in a temperature-regulated cooler box at 18 ^{o}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 = < 20% motile sperm; 5 = 81 – 100% 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 then fully extended in BullXcell to achieve a concentration of 15 x 10^{6} sperm per 0.25 mL insemination dose. Semen straws (IMV Technologies) were filled as per routine procedures, placed in an insulated box (to slow the temperature drop) in a fridge at 4 ^{o}C and 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 ^{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 [28] in a programmable freezer (Digitcool, IMV Technologies),

followed by submersion and storage in liquid nitrogen at -196 ^{o}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 microscopic techniques 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 Assessment of sperm motility.

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 ^{o}C for 30 sec. A droplet of diluted semen (5 µL) was placed on a pre-warmed glass slide, covered with a pre-warmed coverslip (37 ^{o}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 2: Field fertility of frozen-thawed semen frozen at 6, 24, 48 and 72 h post dilution.

The aim of this experiment was to assess the effect of equilibration time of frozen-thawed semen (15 x 10^6 sperm per 0.25 mL insemination dose) on CR following AI. Semen was collected from Holstein Friesian bulls (n = 5; denoted A - E) at a commercial AI centre from early February to the end of March 2015. There were 7 collection days in total, with five bulls collected per 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 and distributed for insemination after 30 days of quarantine.

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 time treatments 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 [29].

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 [40] and 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 [30].

2.3 Sperm functional assessments

2.3.1 Computer assisted sperm analysis

Motility of frozen-thawed sperm samples was assessed using IVOS-II CASA system driven by software version 14 (Hamilton Thorne Inc, Beverly, USA). Straws (n=5 per ejaculate) were thawed at 37 ^{o}C for 30 sec 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 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 (%), 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) [31]. Regarding analysis settings the CASA was set to standard

209 factory settings for bull semen; sperm with straightness of >80% and VAP >50 μm/s were
210 considered progressively motile.
211
212 *2.3.2 In vitro* flow cytometric analysis of frozen-thawed semen
213 Before flow cytometric analysis, semen samples were diluted to a final working
214 concentration of 300 x 10^5 sperm/mL in Beltsville Thawing Solution (BTS; 37 ^oC). Samples
215 were analysed on a flow cytometer (Guava easyCyte 6HT-2L, Merck Millipore, Billerica,
216 MA, USA) equipped with both a Krypton (642 nm) and an Argon laser (488 nm).
217 Appropriate single colour controls were prepared to establish the respective fluorescent peaks
218 of the individual stains. These were used in conjunction with the forward scatter (FSC) and
219 side scatter (SSC) signals to discriminate sperm from debris (P0.1 Population). Fluorescent
220 events were recorded using GuavaSoft (Version 2.7; Merck Millipore) and all variables were
221 assessed using logarithmic amplification. In each sample, a minimum of 10,000 gated events
222 were captured.
223
224 2.3.2.1 Assessment of viability
225 Viability was assessed using the fluorescent probes; SYTO 16 (Ex/Em: 488/518; Life
226 Technologies, Carlsbad, CA USA) which is a cell-permeant probe which fluoresces upon
227 binding to nucleic acids and propidium iodide (PI; Ex/Em: 535/617; Life Technologies)
228 which is selectively taken up by membrane compromised cells, thus indicating a loss of
229 viability [29]. SYTO 16 (5 μL) was added to diluted sperm (300 x 10^5 sperm/mL) to give a
230 final concentration of 100 nM and incubated at 37 ^oC in the dark for 10 min. Subsequently, PI
231 was added at a final concentration of 15 μM and incubated for a further 5 min. SYTO 16
232 emission was detected via the Green photomultiplier (PMT; 525/30 nm BP filter) and PI was
233 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.

2.3.2.2 Assessment of acrosomal integrity

Acrosomal integrity was assessed by incubating sperm with SYTO 16 and PI, as described above, followed by incubation with Alexa Flour 647 (AF647; Ex/Em: 650/668; Life Technologies) to identify live sperm which had undergone the acrosome reaction [29]. Alexa Fluor 647 fluoresces in the presence of the enzyme acrosin, which is exposed upon the loss of the acrosomal cap. Alexa Flour 647 (3 µL) was added to diluted sperm (300 x 10^5 sperm/mL) to a final concentration of 4.6 µM and incubated in the dark for 15 min at 37 ^{o}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 Flour 647 and positive for SYTO 16 as part of the total gated sample, P0.1 Population.

2.4 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 assessed using Pearson's chi-squared procedures in SPSS following which they 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; 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 Bonferroni test and results are reported as the mean ± the standard error of the mean (s.e.m) in Experiment 1 and as the estimated marginal means in Experiment 2, to adjust for the fixed effects. Data were considered to differ significantly at $P < 0.05$.

3. Results

3.1 *In vitro* pre-freeze and post-thaw analysis of frozen-thawed semen

There was no effect of equilibration time on pre-freeze total and progressive motility ($P > 0.05$). There was an effect of equilibration time on post-thaw total and progressive motility ($P < 0.01$), viability ($P < 0.01$) and acrosomal integrity ($P < 0.01$). Equilibration for 24 h resulted in the highest total and progressive post-thaw motility score (53.7 and 42.7%, respectively) in comparison to equilibration for 6 (45.1 and 35.1, respectively) or 72 h (48.7 and 36.0, respectively; $P < 0.01$) but did not differ from an equilibration period of 48 h (50.7 and 38.4%, respectively; $P > 0.05$; Figure 1). Critically, semen assessed post-thaw from all 4 equilibration periods passed quality control analysis. Equilibration for 24 and 48 h resulted in better post-thaw total motility than 6 and 72 h equilibration ($P < 0.01$). Equilibration for 6 h resulted in a lower post-thaw viability (53.4%) in comparison to 24, 48 and 72 h (60.5, 61.3 and 61.3%, respectively; $P < 0.01$; Figure 2), however, viability between equilibration periods of 24, 48 and 72 h did not differ ($P > 0.05$). The percentage of live sperm with intact acrosomes post-thawing declined significantly in the 48 and 72 h equilibration treatments (Figure 2). There was an effect of equilibration time on ALH, BCF, LIN, STR, VAP, VSL and WOB ($P < 0.01$) but there was no effect on VCL or on the percentage of sperm with

proximal and distal droplets (P > 0.05; Table 1). Semen equilibrated for 48 and 72 h exhibited inferior kinematic motility parameters compared to an equilibration time of 6 and 24 h (P < 0.01) with the exception of VCL (P > 0.05). All CASA motility characteristics, with the exception of ALH and VCL deteriorated when equilibration time was increased from 6 to 72 h.

3.2 Field fertility of frozen-thawed semen frozen at 6, 24, 48 and 72 h post dilution.

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; Figure 3) this was not significantly different (P > 0.05). There was no bull, parity, cow fertility sub-index, DIM, herd or technician by treatment interaction on CR (P > 0.05). There was an effect of bull on CR (P < 0.01) with the CR for individual bulls varying from 42.7 to 56.8%. There was a positive linear increase in CR with increase 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 with cows which were greater than 40 days in milk prior to insemination. Maiden heifers had the highest CR (59.2%) in comparison to primiparous (52.5%) and multiparous dairy cows (49.4%) but this was not significant (P > 0.05). As expected, CR varied between individual herds and technicians; for herds and technicians with greater than 15 and 20 recorded inseminations, respectively (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 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 [32]. Therefore, motility assessment constitutes an integral part of semen quality analysis with the use of CASA systems allowing an objective, accurate and high repeatability assessment of sperm motility kinematics. A number of studies have correlated motility kinematics of bull sperm with field fertility [32-35]; however, many of these studies are conflicting in terms of the relative importance of different motility characteristics. Oliveira et al. [36] reported a correlation between a combination of CASA parameters and bull fertility such as total and progressive motility, ALH and BCF. In contrast, Amann and Waberski [37] 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. In the current study, semen quality declined gradually *in vitro* with increasing equilibration time although 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 ^{o}C before freezing in order to attain optimal post-thaw semen quality. This is in agreement with Fleisch et al. [23] and Rickenbacher [38] 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 [26] and Anzar, Kroetsch and Boswall [39] 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 [40] 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 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 significant effect of on CR. This finding is supported by Fleisch et al. [23] 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 [26]. This indicates that semen frozen on days 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 [29, 41]. 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

356 characteristics (<€50 and <40 DIM, respectively) had lower CR than animals of greater than
357 €90 and 40 DIM, respectively. While this study demonstrates that a higher CR can be
358 achieved through increasing the number of DIM before insemination, it also illustrates that
359 late calving cows can be inseminated with reasonable success.

360

361 **5. Conclusion**

362 In conclusion, increasing equilibration time up to 72 h post collection did not negatively
363 affect field fertility with an equilibration period of 24 h resulting in the greatest total and
364 progressive motility scores. Therefore, implementing an equilibration period of 24 h may be
365 more suitable to the working environment and collection schedules of AI centres, while
366 confidence can also be gained knowing that in circumstances where it is required (e.g.,
367 mechanical failure, avoidance of weekends), allowing an equilibration time of 3 days will not
368 negatively impact on field fertility.

369

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376 **Figure captions**

377 **Figure 1:** The effect of equilibration time on post-thaw total motility (upper panel) and
378 progressive motility (lower panel) of bull semen frozen at 6, 24, 48 and 72 h post dilution as
379 assessed by computer assisted sperm analysis (Experiment 1). Vertical bars represent s.e.m.
380 [abc]Values with different superscripts differ significantly.

381

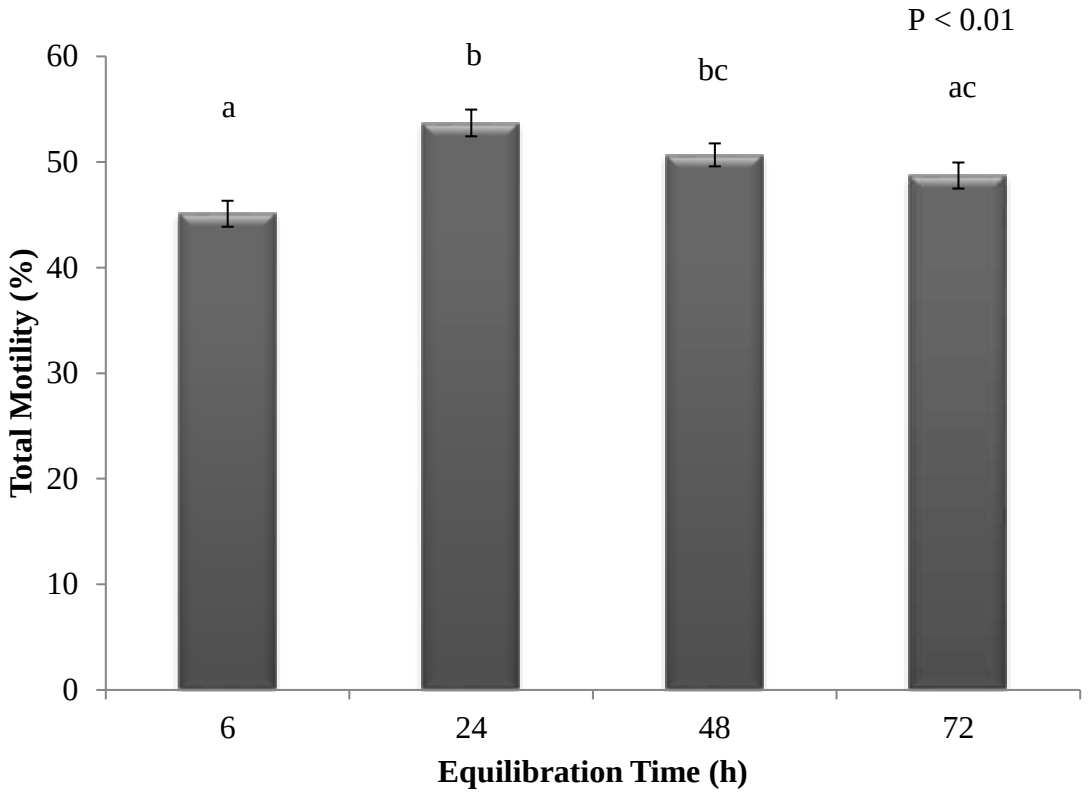
382 **Figure 2:** The effect of equilibration time on viability (upper panel) and acrosomal integrity
383 (lower panel) of frozen-thawed bull semen frozen at 6, 24, 48 and 72 h post dilution as
384 assessed by flow cytometer (Experiment 1). Vertical bars represent s.e.m. [abc]Values with
385 different superscripts differ significantly.

386

387 **Figure 3:** The effect of equilibration time prior to freezing bull semen on calving rate in dairy
388 cows and heifers (Experiment 2).

389 Murphy et al. Figure 1.

390



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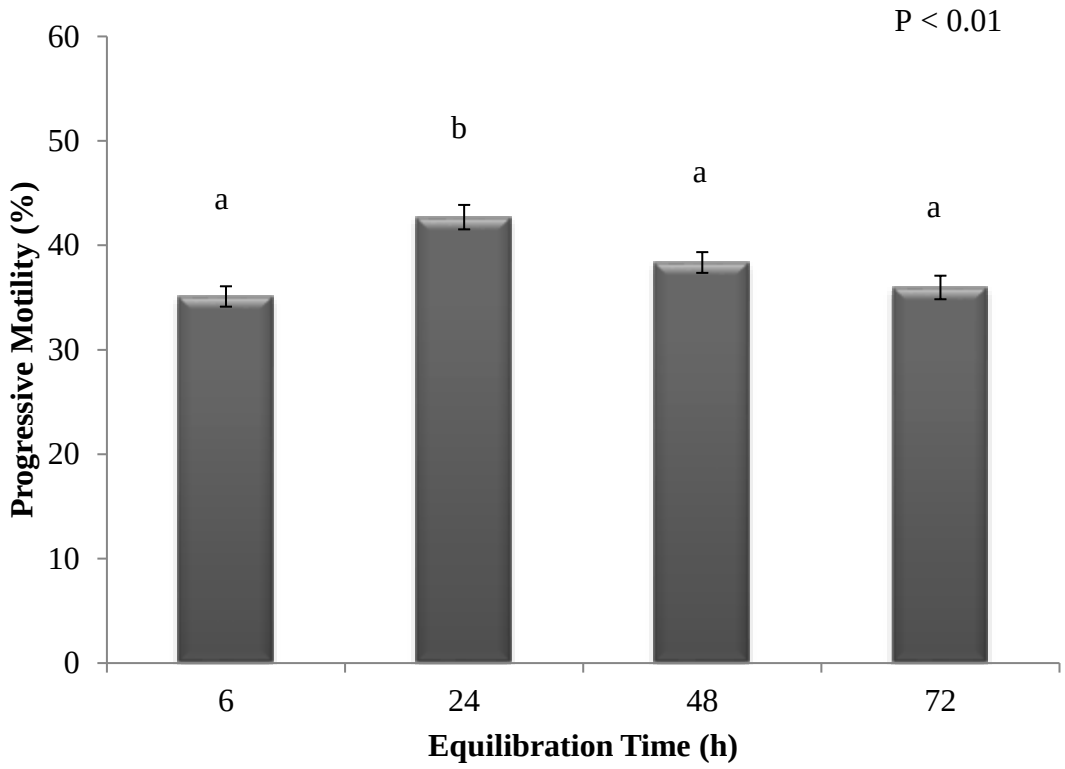

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393 Murphy et al. Figure 2.

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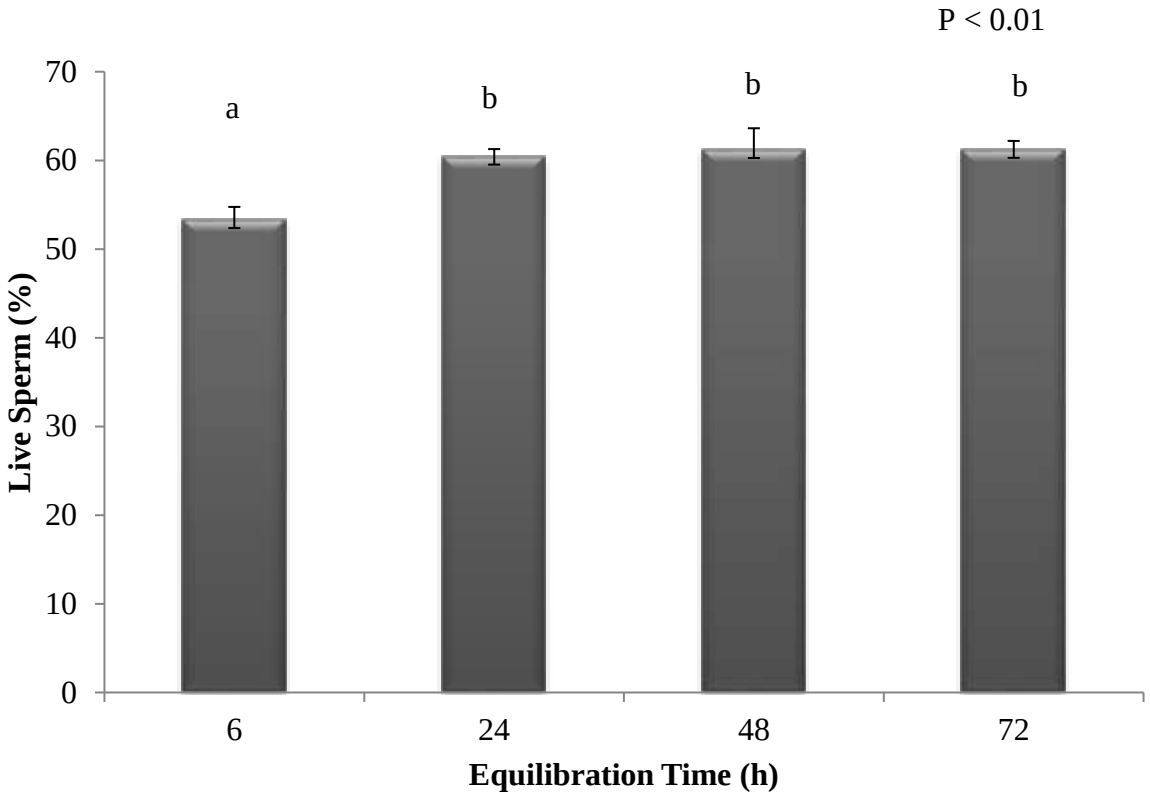

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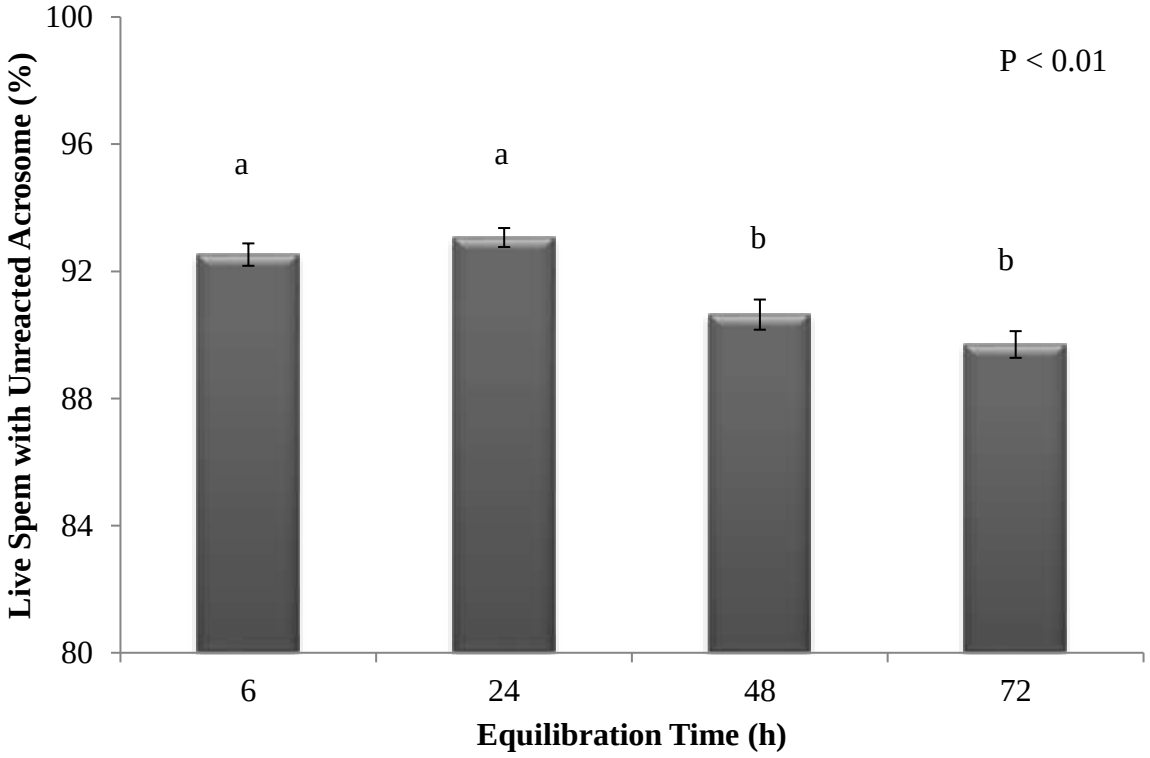

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397 Murphy et al. Figure 3.

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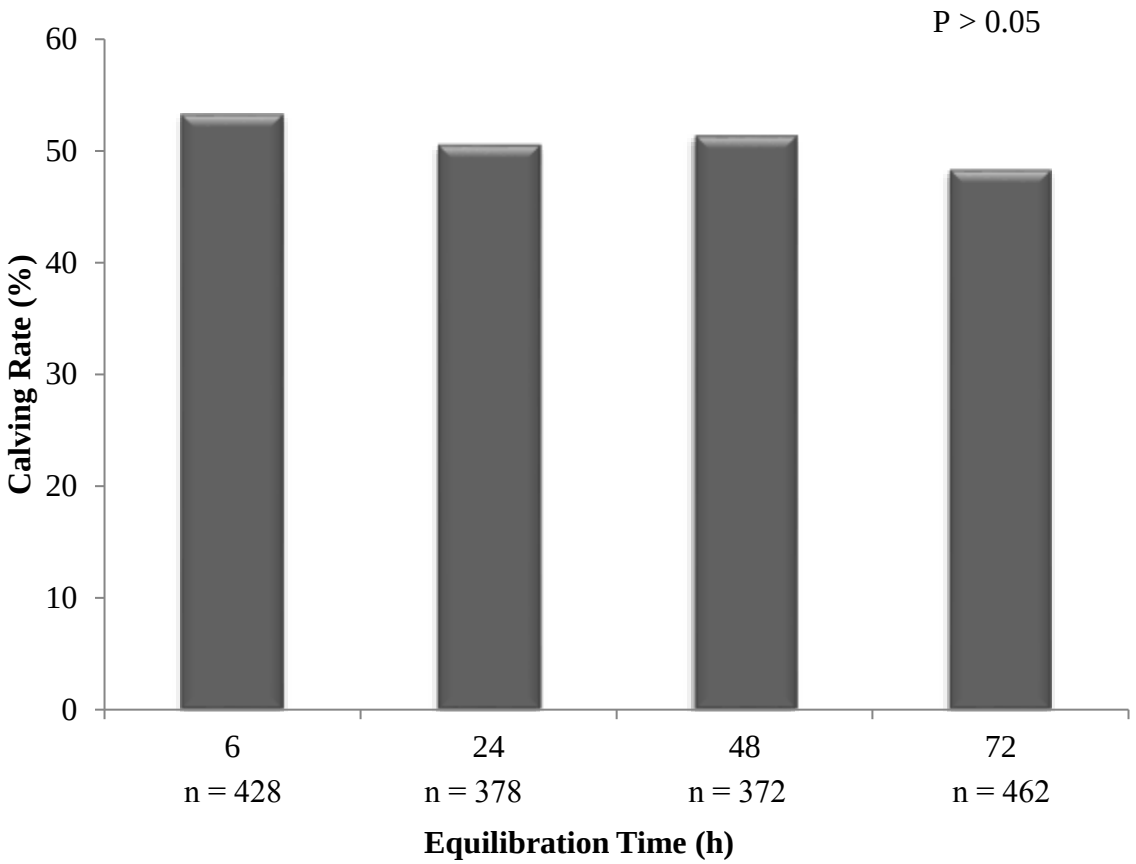

399

400 **Table captions**

401 **Table 1:** The effect of equilibration time on computer assisted sperm analyser post-thaw
402 kinematic parameters in bull semen extended for 6, 24, 48 and 72 h post dilution prior to
403 freezing (Experiment 1). [abc]Values with different superscripts differ significantly within row
404 ($P < 0.01$; values are mean ± s.e.m)

405 Murphy et al. Table 1.

406

Kinematic Parameters	Equilibration Time (h) mean ± s.e.m				P value
	6	24	48	72	Effect of Treatment
ALH (μm)	8.67 ± 0.10[a]	9.33 ± 0.10[b]	9.76 ± 0.11[c]	9.78 ± 0.16[c]	P < 0.01
BCF (Hz)	31.2 ± 0.39[a]	28.9 ± 0.27[b]	26.0 ± 0.19[c]	24.9 ± 0.21[c]	P < 0.01
LIN (%)	41.5 ± 0.40[a]	39.3 ± 0.32[b]	36.4 ± 0.38[c]	35.4 ± 0.54[c]	P < 0.01
STR (%)	76.3 ± 0.51[a]	75.5 ± 0.45[a]	71.3 ± 0.57[b]	69.9 ± 0.77[b]	P < 0.01
VAP (μm/s^{-1})	106.1 ± 1.44[a]	106.0 ± 1.18[a]	101.9 ± 1.06[ab]	97.5 ± 1.27[b]	P < 0.01
VCL (μm/s^{-1})	201.7 ± 2.77	209.6 ± 2.22	207.7 ± 2.48	201.8 ± 3.32	ns
VSL (μm/s^{-1})	81.2 ± 1.19[a]	80.4 ± 0.99[a]	72.4 ± 0.85[b]	67.7 ± 0.87[b]	P < 0.01
WOB (%)	53.3 ± 0.22[a]	51.3 ± 0.18[b]	50.1 ± 0.21[c]	49.7 ± 0.24[c]	P < 0.01
Proximal Droplets (%)	3.7 ± 0.67	3.8 ± 0.71	4.0 ± 0.83	3.9 ± 0.97	ns
Distal Droplets (%)	4.6 ± 0.24	3.9 ± 0.26	4.3 ± 0.28	4.3 ± 0.25	ns

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

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