# Optimising reproductive efficiency in dairy cows by improving sperm quality for artificial insemination

**Background:** SLF granted funding for two years (2014-2015) for a project to improve sperm quality in bull semen doses for artificial insemination (AI), in an effort to reverse the trend towards declining fertility seen in dairy cattle worldwide. An extension was granted in 2015 to enable us to finish work on the project. The proposed plan was to measure the content of reactive oxygen species (ROS) in semen samples from bulls of high and low fertility, to determine if production of ROS plays a role in determining fertility, or whether ROS levels could be used as a marker of fertility. In addition, the effect of selecting robust spermatozoa by colloid centrifugation on their ROS-content would be investigated. Finally, the potential beneficial effect of adding heat shock proteins to bull spermatozoa on their survival during freezing and cryostorage would be assessed. These proteins are considered to be involved in sperm binding to oviductal epithelial cells and therefore may be associated with fertility.

Reactive oxygen species (ROS) are produced as byproducts of metabolism; the more metabolically active the spermatozoa are, the more ROS they produce. However, if high levels of ROS are found in the vicinity of spermatozoa they can damage membranes and DNA. Thus high levels of ROS may indicate high metabolic activity but could also indicate potential sources of harm to the spermatozoa. Differences in bull fertility may be due to differences in ROS production, or to differences in antioxidants in the seminal plasma. Therefore, investigating ROS-production in bull spermatozoa may help to unravel some of the intricacies of bull fertility.

**Sector relevance**: an alarming trend in reduced fertility has been observed in dairy cattle worldwide in recent decades, with high yielding breeds apparently being more affected than lower yielding ones. Although there does not seem to have been a concomitant decline in sperm quality using conventional methods of evaluation, there is a high rate of early embryonic loss *in vivo* that may be linked to sperm factors. Sperm preparation techniques, such as colloid centrifugation, that select the best quality spermatozoa could help to reduce harmful ROS-production and aid cryosurvival, thus contributing to improved cow fertility.

## Methods and Results

### 1. ROS production in high and low fertility bulls

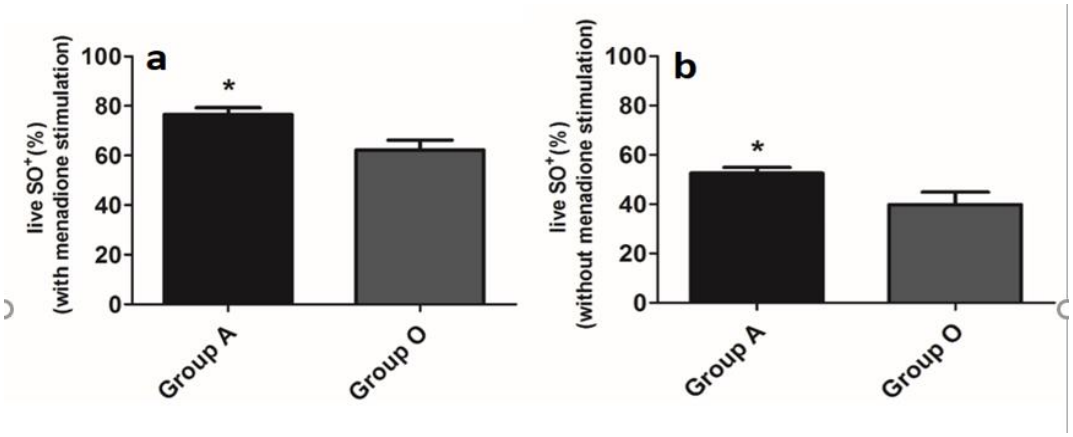
Commercial frozen semen was available from bulls of known fertility. After thawing, the sperm samples were analysed for motility (computer assisted sperm analysis), membrane integrity, chromatin integrity, acrosome status, mitochondrial activity and reactive oxygen species. A fertility index score based on the adjusted 56-day non-return rate for >1000 inseminations was available for each bull.

Our initial results indicated that there were no differences in ROS production between high and low fertility bulls (Table 1) but individual variation existed which may have masked a fertility effect. Therefore additional studies were performed to investigate some of the effects that could contribute to individual variation in ROS, such as type of bull (dairy or beef), breed, extender, season of semen collection. Using split ejaculates, the effects of the extenders Andromed and Optixcell on sperm quality was investigated; superoxide production was significantly different between the two extenders (Figure 1; Lima-Verde et al. submitted). Semen from Holstein bulls in northern Spain was collected in three seasons, frozen and later evaluated for ROS-production and other characteristics of sperm quality. Superoxide production was different depending on season of collection (Table 2; Sabes-Alsina et al, 2017).

**Table 1: Least Squares Mean ± S.E. proportions of bull spermatozoa characterized for their reactive oxygen species status, grouped according to fertility index score (lowest 10%; n = 8) versus highest 10%; n = 13).**

| Fertility Index | Living SO negative (%) | Living SO positive (%) | Dead SO positive (%) | Living H2O2 negative (%) | Living H2O2 positive (%) | Dead H2O2 negative (%) | Dead H2O2 positive (%) |
|---|---|---|---|---|---|---|---|
| Lowest 10% (≤93) | 30±4 | 19±2 | 51±4 | 48±4 | 1.4±2.1 | 51±4 | 0.2±0.7 |
| Highest 10% (≥103) | 39±3 | 20±2 | 41±3 | 55±3 | 3.8±1.7 | 39±3 | 1.7±0.5 |

**Figure 1: Effect of extender on Reactive Oxygen Species production by bull spermatozoa**




Note: A and O refer to extenders Andromed and Optixcell, respectively.

**Table 2. Season effect on reactive oxygen species in frozen-thawed bull spermatozoa.**

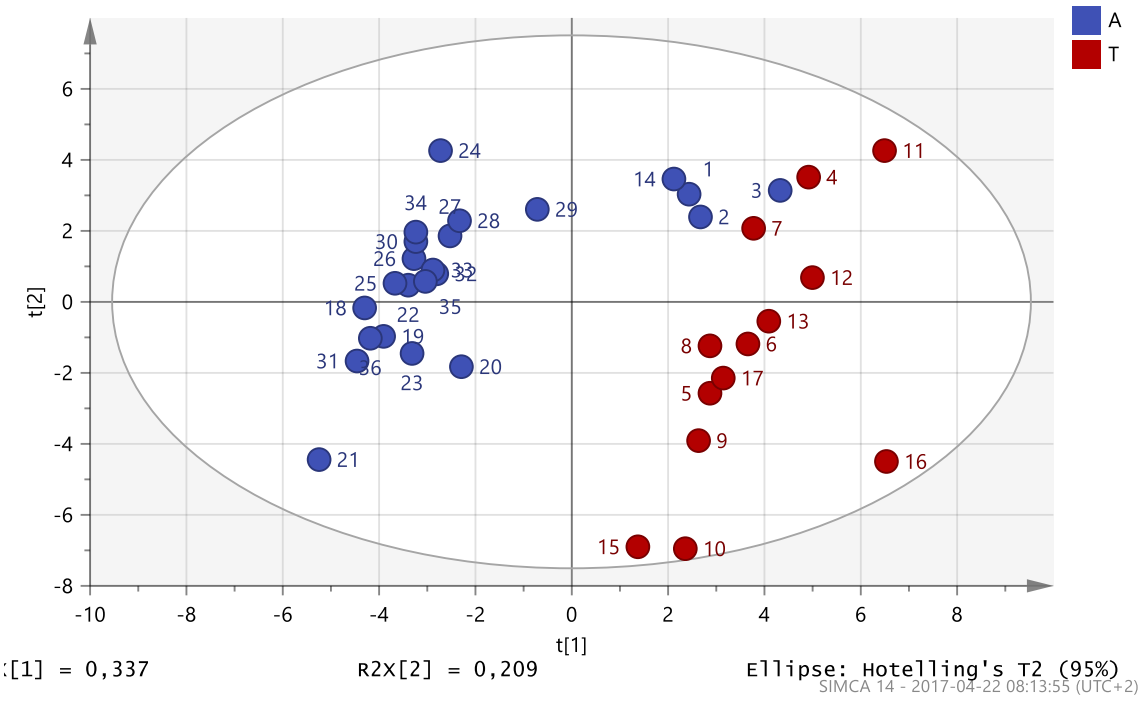
| Parameters | Winter | Spring | Summer |
|---|---|---|---|
| Live, Superoxide anion negative | 39.83 ± 7.67[b] | 49.29 ± 5.54[ab] | 44.88 ± 12.07[b] |
| Live, Superoxide anion positive | 24.48 ± 8.13[a] | 19.90 ± 8.39 | 19.73 ± 6.90[b] |
| Dead, Superoxide anion | 35.93 ± 8.23 | 30.33 ± 8.65 | 35.63 ± 12.89 |
| Live, Hydrogen peroxide negative | 63.22 ± 9.2[a] | 69.47 ± 9.57[a] | 63.42 ± 14.18 |
| Live, Hydrogen peroxide positive | 0.92 ± 0.70 | 0.52 ± 0.30 | 1.14 ± 1.24 |
| Dead, Hydrogen peroxide negative | 35.74 ± 8.67 | 30.09 ± 9.17 | 35.34 ± 13.09 |
| Dead, Hydrogen peroxide positive | 0.11 ± 0.32 | 0.04 ± 0.10 | 0.04 ± 0.07 |

Values are least-square mean ± standard deviation. Means within row, with one letter in common are significantly different (P > 0.05).

The effect of season on sperm quality was most pronounced in Sweden compared to bulls in Spain and in Thailand. This finding tends to suggest that bulls can adapt to climatic conditions such as warm temperatures or high humidity, but that sperm quality is more likely to be affected by extreme changes in climate occurring over a few months, as between winter and summer temperatures in Sweden, than between the different season in Spain or in Thailand. An IVF study with semen from the same bulls collected in difference seasons revealed significant differences in fertilization rates ($p<0.05$) (Wallgren et al., in preparation) and also in the number of cells in the blastocysts (an indicator of speed of development of the embryo) (Sabes-Alsina et al, in preparation). In this study, a new protocol for sperm preparation for IVF was developed, enabling spermatozoa to be separated from cryoextender and seminal plasma without selecting the best spermatozoa, so that the effect of treatment (in this case season) was not masked by selecting only the most functional spermatozoa (Wallgren et al. in preparation).

In another experiment, sperm quality was evaluated in beef and dairy bulls. Beef bulls produced less superoxide than dairy bulls but more hydrogen peroxide. Beef bull spermatozoa were less metabolically active than dairy bull spermatozoa and showed more DNA damage; however, there were more live spermatozoa with unreacted acrosomes in semen from beef bulls (Morrell et al., in preparation). Although initially it was thought that these differences might have been attributable to the different extenders used, a scatter plot of semen quality indicated that beef bulls tended to cluster together regardless of the type of extender (Figure 2).

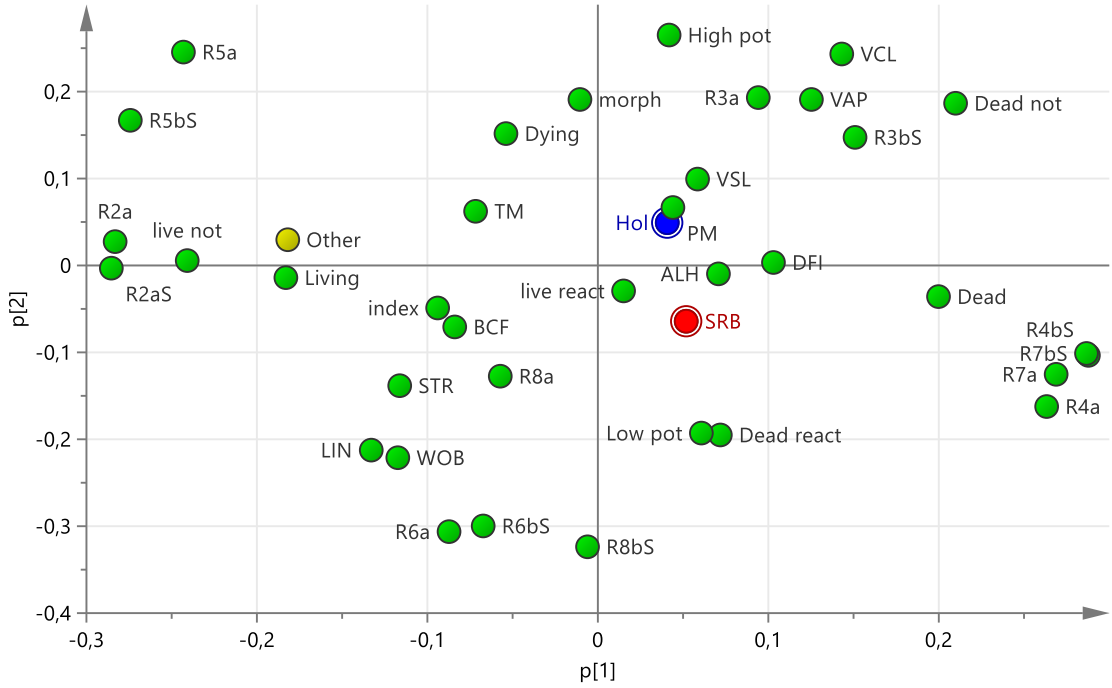
**Figure 2: Scatter plot of beef and dairy bulls according to sperm quality (n=37).**




Note: numbers 1-17 are beef bulls, 18-37 are dairy bulls. Blue circles represent semen extended in Andromed and red circles represent semen extended in Triladyl. The dairy bulls cluster together on the left hand side of the diagram and the beef bulls on the right, regardless of which extender was used.

The identification of breed as a factor contributing to sperm quality means that it may not be possible to use the same sperm quality parameters as a marker of sperm quality in all breeds. Additional statistical analysis was carried out to determine which parameters of sperm quality were most closely linked to fertility for Holstein and Swedish Red bulls (Figure 4: Morrell et al., submitted). Interestingly, some categories of ROS production clustered together with a particular breed, and also some sperm kinematics, indicating that different factors are linked with sperm quality for each breed. This finding is of considerable relevance since most semen collection stations use the same assays to evaluate all bull semen regardless of the type or breed of bull. Our results suggest that such an approach may not be optimal in terms of fertility indicators.

**Figure 4: Partial Least Squares loadings scatter plot showing the distribution of sperm quality variables and their relationship to breed of bull (n=60).**

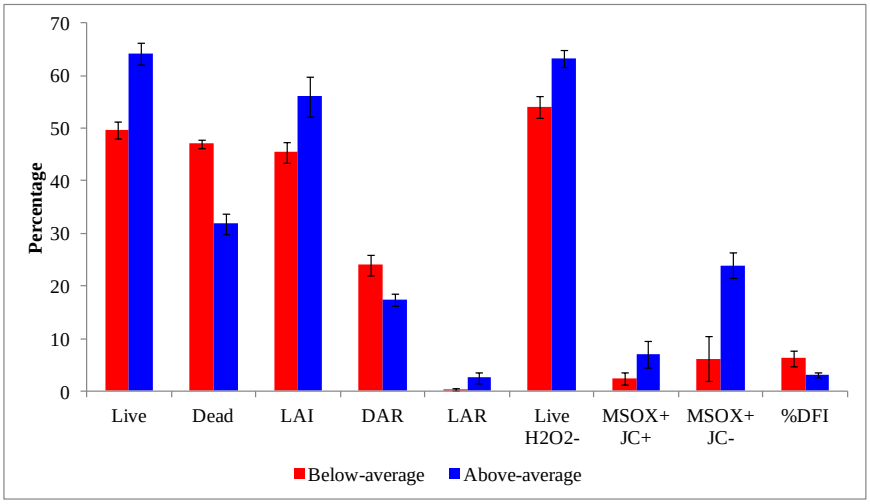



Note: Holstein (Hol, blue circle) is in the upper right quadrant, SRB (red circle) is in the bottom right quadrant, Other breeds (yellow circle) is in the upper left quadrant. Parameters of sperm quality in the top left quadrant: R2a = living superoxide negative; R2aS = menadione stimulated living superoxide negative; live not = live not acrosome reacted; R5a = living hydrogen peroxide negative; R5bs = menadione stimulated, living hydrogen peroxide negative; TM = total motility; Dying = membrane damaged, not yet dead; morph = normal morphology; live not = live not acrosome reacted. Top right quadrant: PM = progressive motility; VSL= straight line velocity; high pot = high mitochondrial membrane potential; R3a = living superoxide positive; VCL = curvilinear velocity; VAP = average path velocity; R3bS = menadione stimulated, living superoxide positive; dead not = dead not acrosome reacted; DFI = DNA fragmentation index. Bottom left quadrant: Living = membrane intact; index = fertility index score; BCF = beat cross frequency; STR = Straightness; R8a = Dead hydrogen peroxide positive; LIN = linearity; WOB = wobble; R6a = living, hydrogen peroxide positive; R6bS = menadione stimulated, living hydrogen peroxide positive; R8bS = menadione stimulated, dead hydrogen peroxide positive. Bottom right quadrant: live reacted = live acrosome reacted; Dead = membrane damaged, non-living; ALH = amplitude of lateral head deviation; low potential = low mitochondrial membrane potential; dead reacted = dead acrosome reacted; R4a = living superoxide positive; R4bS = menadione stimulated, living superoxide positive; R7a = dead superoxide negative; R7bS = menadione stimulated dead superoxide negative.

Thus the conclusion from this series of studies was that more factors influence sperm quality in insemination doses than previously realized. Therefore, additional assays, or new ones, may be required to provide more reliable indicators of fertility than those used at present.

The accurate prediction of bull fertility is of major economic importance in the dairy breeding industry. A study was carried out on 20 bulls of known fertility, to determine which combination of assays could be useful in predicting fertility, introducing an assay for free thiols and also protamination of DNA to the array of assays performed previously. These assays provide additional information about the condensation (packaging) of DNA in the sperm nucleus and thus the likelihood of damage to the DNA occurring following attack by ROS, or even the possibilities for epigenetic changes occurring. A combination of sperm attributes was identified that could be used to discriminate below-average fertility dairy bulls from above-average fertility dairy bulls. A model was developed for predicting bull fertility with a high degree of accuracy (Figure 5; Kumaresan et al., 2017). (Note: this article was selected as the Editor´s Choice for Journal of Dairy Science in June 2017).

**Figure 5: Differences in the sperm functional attributes between below average (n=5) and above average (n=6) fertility bulls. All the parameters indicated in the graph differed significantly between the two groups of bulls.**




Note: LAI = live acrosome intact; DAR = dead acrosome reacted; LAR =live acrosome reacted; live H2O2- = living spermatozoa not producing hydrogen peroxide; MSOX+JC1+ = spermatozoa that are metabolically active and producing superoxide; MSOX+JC1- = spermatozoa that have low metabolic activity and are producing superoxide; %DFI = DNA fragmentation index.

## Seminal plasma

Fertility-associated proteins are present in bull seminal plasma; their respective concentrations may be correlated with fertility. Proteins can be separated according to their binding characteristics, e.g. by Fast Protein Liquid Chromatography (FPLC). A pilot study was carried out using FPLC to characterize the seminal plasma of high and low fertility bulls. Seminal plasma was obtained from 3 Holstein and 3 Swedish Red mature bulls of high, average and low fertility. Separation of SP resulted in three groups of proteins i.e. non-heparin binding, phosphorylcholine binding and heparin-binding proteins. Phosphorylcholine-binding proteins (F3) were present in the highest amounts (67.8 – 80.5 %) and heparin-binding proteins (F4) in the lowest amounts (2.8 – 6.5 %). The ratio percentage of area for F3/percentage of area for F4 was calculated for each bull (a low ratio indicates that the proportion of heparin-binding proteins is increased, and vice versa). There were significant differences ($P < 0.05$) in this ratio between Holstein bulls, being lowest in the high fertility bull and highest in the low fertility bull ($11.32 \pm 0.32$ vs. $19.87 \pm 0.31$). In the case of the Swedish Red bulls, however, there were no significant differences in this ratio. Our results suggest that FPLC quantification of SP proteins may provide an indicator of a bull´s potential

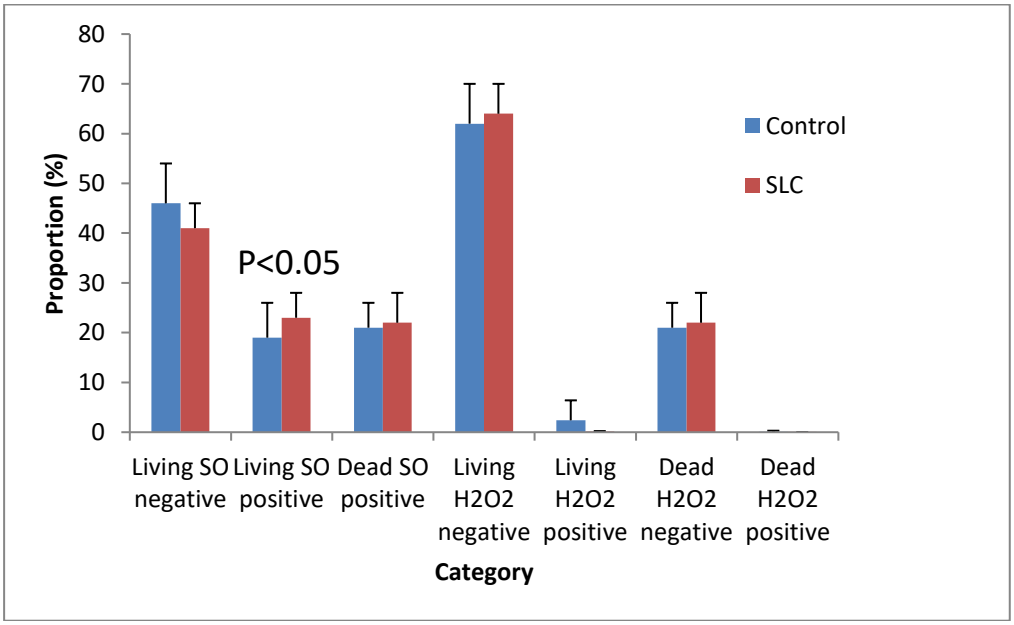
fertility, at least for Holstein bulls (Morrell et al., 2015). However, additional research with a larger number of bulls is needed to establish whether this type of analysis could be useful for bull fertility evaluation on commercial bull semen stations.

It is possible that seasonal differences in sperm quality may, in fact, be due to differences in seminal plasma composition. If this is the case, removing the seminal plasma from insemination doses e.g. by colloid centrifugation, may improve bull sperm quality, as has been shown for other species e.g. stallion.

## 2. Effect of SLC on ROS-production

Previous studies in other species indicated that sperm preparation by colloid centrifugation, especially Single Layer Centrifugation (SLC), results in improved sperm quality in the selected sperm samples compared to controls. These selected sperm samples survive cryopreservation better than controls and show better fertilizing capacity (stallions and boars), but also show a different pattern of ROS production. In the present study, the effect of SLC on bull sperm quality was investigated, with particular reference to ROS production. The proportion of spermatozoa producing superoxide was increased in the SLC-selected sperm samples compared to the unselected controls (Figure 6; Nongbua et al., 2017). These findings are similar to previous experiments with stallion spermatozoa where the proportion of hydrogen peroxide producing spermatozoa was found to be significantly decreased as well.

**Figure 6: Reactive oxygen species in control and SLC-selected bull sperm samples, means ±SD (n=8).**




Other aspects of sperm quality were improved by SLC, e.g. the proportion of spermatozoa with high mitochondrial membrane potential was increased (Figure 7), and chromatin damage was less in some SLC samples (Figure 8; Nongbua et al., 2017). Good chromatin integrity has been related to fertility since spermatozoa with damaged chromatin may still be able to fertilize oocytes but subsequent development of the embryo is compromised at some stage. Thus conception occurs, or even implantation, but the pregnancy is not maintained.

To further explore the effects of SLC on bull sperm samples, an experiment was carried out at a commercial freezing station in which 54 ejaculates were split and prepared by SLC before

freezing or by the usual sperm preparation method. Sperm quality was evaluated post-thaw. Sperm kinematics and normal morphology were better in the SLC-samples than in the controls ($p<0.05$). Unfortunately it was not possible to evaluate the samples for ROS because the flow cytometer did not have the laser necessary for this measurement (Nongbua et al., submitted). Further studies are underway to determine whether fertility is also improved in the SLC samples since this procedure is practical for field use and could be adopted easily by semen processing stations.

**Figure 7: Mean (±SD) values of mitochondrial membrane potential in control and SLC-selected bull sperm samples (n=8).**

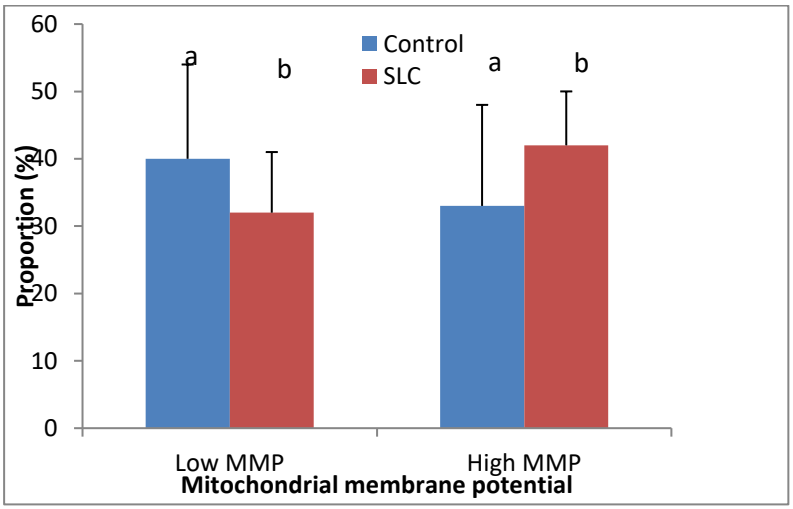
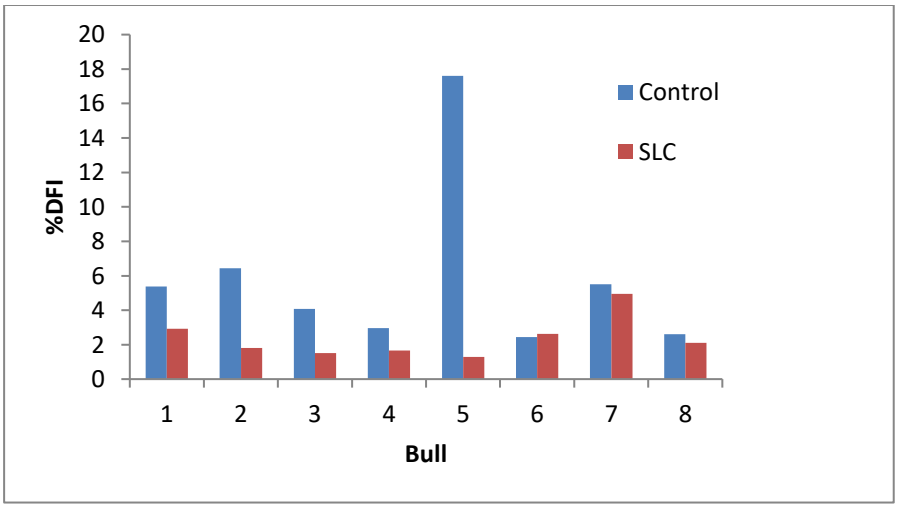



**Figure 8: Chromatin damage (%DFI) in control and SLC-selected bull sperm samples (n=8).**




An additional study was carried out with SLC using bull semen in the liposome-based extender, Optixcell, which has recently become commercially available. However, this extender did not appear to be suitable for use in combination with SLC, since mitochondrial membrane potential (MMP) and membrane integrity (MI) were significantly lower than

controls (MMP: 48.3 ± 3.5% vs. 56.9 ± 3.5%; MI: 48.1 ± 2.8% vs. 57.6 ± 2.8%). Motility was lower than expected in both control and SLC samples. However, DNA fragmentation was less in the SLC group compared to the control group (3.4 ± 0.5 vs. 4.4 ± 0.5) (Lima-Verde et al, in preparation). To determine the effect of the liposome extender, a study was performed to compare sperm quality in bull semen extended in Andromed (a soy lecithin-based extender) and in Optixcell (the liposome-based extender), using split ejaculates. Sperm kinematics were not as good in the liposome-based extender as in the soy lecithin extender, confirming our previous observation (Lima-Verde et al, in preparation). Therefore, we cannot recommend that liposome-based extender is used for samples prepared by SLC.

## 3. Effect of Heat Shock Protein on sperm quality

In contrast to a previous study (Moein-Vaziri et al, 2014), in our hands HSP did not improve bull sperm cryosurvival. We obtained some of the same material from the authors of the previous study as a collaboration but we were still not able to obtain an improvement. This study was subsequently halted so that resources could be devoted to more promising areas of research.

## 4. Summary and Relevance to the Cattle Breeding Industry

The studies undertaken in conjunction with this project have shown that sperm quality is affected by many more extraneous factors than previously reported. Assays of sperm quality, or indicators of fertility, should be adjusted according to the type of bull semen being evaluated instead of using generic thresholds, as is frequently done at present. The effects of season should be considered when collecting semen, or measures taken to mediate extreme climatic conditions. Importantly, it is possible to improve sperm quality by colloid centrifugation, especially using Single Layer Centrifugation. This procedure is simple and effective, and is practical for use on semen collecting stations. However, not all extenders are suitable for use with SLC-selected samples. Therefore, the recommended protocol for SLC must be followed exactly to give good results.

## 5. Peer-reviewed articles arising from this project

Sabes-Alsina M, Johannisson A, Lundeheim N, Morrell JM (2017) Effects of season on bull sperm quality in thawed samples in northern Spain. Veterinary Record 180, 251. doi:10.1136/vr.103897

Kumaresan A, Johannisson A, Al-Essawe E, Morrell JM. (2017) Sperm Viability, Reactive Oxygen Species and DNA Fragmentation Index combined can discriminate between above- and below-average fertility bulls. Journal Dairy Science 100, 5824–5836.

Nongbua T, Johannisson A, Morrell JM. Effects of Single Layer Centrifugation (SLC) on bull spermatozoa prior to freezing on post-thaw semen characteristics. Reprod Domest Anim *In press*

Alonso CAI, Osycka-Salut CE, Castellano L, Cesari A, di Siervi N, Mutto A, Johannisson A, Morrell JM, Davio C. Perez Martinez, S. (2017) Extracellular cAMP activates molecular signalling pathways associated to sperm capacitation in bovines. Molecular Human Reproduction *In press*

Nongbua T, Guo Y, Edman A, Humblot P, Morrell JM. Effect of bovine seminal plasma on bovine endometrial epithelial cells in culture. Reproduction in Domestic Animals (provisionally accepted).

**Articles in preparation**

Lima-Verde IB; Johannisson A, Ntallaris T, Al-Essawe E, Al-Kass Z,Nongbua T, Fernanda Dórea F, Lundeheim N. Kupisiewicz K, Edman A, Morrell JM. Effect of freezing bull semen in two non-egg yolk extenders on post-thaw sperm quality. *Submitted*

JM Morrell, T. Nongbua, S Valeanu, Lima Verde I, K Lundstedt-Enkel, A Edman, A Johannisson. Sperm quality parameters as indicators of bull fertility may be breed dependent. *Submitted*

Nongbua T, Al-Essawe E, Edman A, Johannisson A, Morrell JM. Effect of adding seminal plasma prior to cryopreservation on bull sperm quality after thawing. *Submitted*

Morrell et al. Sperm quality in beef and dairy bull semen used for artificial insemination. *Submitted*

Nongbua et al. Effects of season and Single Layer Centrifugation on bull sperm quality in Thailand *Submitted*

Morrell JM, Kumaresan A, Johannisson A. Practical implications of sperm selection techniques for improving reproductive efficiency. *Submitted*

Morrell JM, Madej M, Madej A, Stålhammar H. Seminal plasma proteins of Swedish Red and Holstein bulls may be related to fertility. *In preparation*

M. Sabés-Alsina, A. Johannisson, N. Lundeheim, M. López-Béjar, JM. Morrell. (2016) Seasonal effects on sperm function in semen from Spanish and Swedish bulls. *In preparation*

Wallgren et al. Seasonal effects on bull sperm fertility in an IVF system. *In preparation*

**Conference presentations**

Essraa Al-Essawe and Jane M. Morrell (2014) Relationship between bull sperm kinematics and fertility. Reprod Domest Anim. 49, Suppl abst.3

Thanapol Nongbua, Lavanya Goodla, Anders Johannisson & Jane M. Morrell (2014) Relationship between 56-day non-return rate and the quality of frozen thawed bull semen. Reprod Domest Anim. 49 Suppl abst. 3.

JM Morrell, M Madej, A Madej, H Stålhammar (2015) Fast Protein Liquid Chromatography of Swedish Red and Holstein bull seminal plasma proteins in relation to bull fertility. Reprod Dom Anim 50 (Suppl. 3), P99

J.M. Morrell, T. Nongbua, S. Valeanu, K. Lundstedt-Enkel, A. Edman, A. Johannisson. (2016) Bull breed affects which parameters of sperm quality are indicative of fertility. Anim Reprod Sci 169, 112-113.

Thanapol Nongbua, Anders Johannisson, Anders Edman, Jane M. Morrell (2016) Effect of bovine seminal plasma on sperm motility and chromatin integrity Anim Reprod Sci 169, 113-114.

I Lima-Verde, A Johannisson, F Dórea, T Ntallaris, E Al-Essawe, Z Al-Kass, A Edman JM Morrell (2016). Effect of soy lecithin and liposome-based extenders on bull sperm quality. Theriogenology 86, 145.

Anders Johannisson, Thanapol Nongbua, Anders Edman, Jane M Morrell (2016). Effects of Single Layer Centrifugation (SLC) on bull spermatozoa prior to freezing on post-thaw semen characteristics. Theriogenology 86,140.

Nongbua Thanapol, Guo Yongzhi, Edman Anders, Humblot Patrice, Morrell Jane (2016). Effect of bovine seminal plasma on bovine endometrial epithelial cells proliferation in culture. Theriogenology 86, 348.

M. Sabés-Alsina, A. Johannisson, N. Lundeheim, M. López-Béjar, JM. Morrell. (2016) Comparative study of seasonal epigenomic effects on sperm cells from Spanish and Swedish bulls. Animal Reproduction 13, 464.

CAI Alonso, CE Osycka-Salut, L Castellano, A Cesari, A Johannisson, JM Morrell, C. Davio and S Perez Martinez (2016) Extracellular cAMP activates molecular signalling pathways associated to sperm capacitation in bovines.