Testicular biometry and hemodynamic of the testicular artery by Doppler ultrasonography in Braford bulls from 12 to 24 months of age

L A Claus[1], G Pereira[1], F Barca Jr[1], P Favaro[1], I Dias[1], C H Bofinger[1], E L Ribeiro[2], C Koetz Jr[1]
[1]*Universidade Norte do Paraná - UNOPAR, Arapongas, PR, Brazil,* [2]*Universidade Estadual de Londrina - UEL, Londrina, PR, Brazil*
celsokoetzjr@yahoo.com.br

Application Evaluation of the testicular hemodynamics can significantly contribute to understand the scrotal thermoregulatory mechanisms in domestic animals.

Introduction The production of viable spermatozoa depends on the physiological testicular mechanisms, especially thermoregulation, where the temperature of the scrotum must be maintained from 4 to 6 ^{o}C below of body temperature (Brito *et al.*, 2004; Kastelic, 2014). The uses of Doppler ultrasonography to evaluation changes in bull reproductive tract evaluation have not been used as a routine technique. In addition to the oxygen and nutrient supply function, blood flow plays an important role in the control of testicular temperature maintenance (Herzog & Bollwein, 2007). The aim of this study was to compare the biometric testicular characteristics and the hemodynamics of the testicular artery of 12 and 24-month-old Braford bulls by Doppler ultrasonography.

Material and methods All procedures with animals were approved by the Animal Ethical Committee (CEUA/UEL #18656/2014/58). The study was conducted with 48 Braford bulls (3/8 Nellore - 5/8 Hereford) in Southern Brazil (latitude 23°30'10"S and longitude 51°14'10''W), where the climate in the region is considered Cfa (humid subtropical climate) according to Köppen classification. Animals were divided into 2 groups according to age, 12 months (n=29) and 24 months (n=19). Scrotal circumference and biometry were measured to calculate the testicular volume. Measurements of testicular biometry were performed using a pachymeter and only the glandular portion of the testis was considered, excluding the tail of the epididymis. Testicular shape was classified into 5 types, as follow: long, long/moderate, long/oval, oval/spherical and spherical. Doppler ultrasonography was used to obtain the mean velocity (MV), pulsatility index (PI) and resistivity index (RI). Data were subjected to normality assessment and variance analysis was performed using the statistical package R. In addition, Tukey's mean test and Pearson's linear correlation were used to evaluate VM, PI, and RI variables.

Results There was no difference between the right and left testicles when compared all animals together or separated according to their ages. Scrotal circumference was lower in animal with 12 months (25.5±2.2) compared to 24 months (34.0±2.1) of age ($P<0.05$). We observed a tendency in blood velocity ejection between animals with 12 (16.3±3.84 cm/s) and 24 (19.0±6.0 cm/s) months ($P=0.06$). In diastole, MV was lower in animals of 12 (7.98±3.83) compared to 24 (11.37±4.15) months ($P<0.05$). The 12-month-old animals had higher PI and RI values compared to 24-month-old animals (0.49±0.02 vs. 0.32±0.16 and 0.51±0.20 vs. 0.40±0.15, respectively; $P<0.05$). The moderate/oval (n=28) and long/moderate (n=10) shape classifications were used to evaluate the final velocity in the diastole. Then, we observed that elongated testicles showed smaller (7.31±2.91) compared to animals with spherical testes (11.48±4.33; $P<0.05$). Animals with elongated testes presented higher values of PI and RI (0.51±0.05 and 0.55±0.04, respectively) than animals with spherical shape (0.29±0.20 and 0.55±0.04, respectively; $P<0.05$). The highest positive correlation was found between PI vs. IR (0.966). In addition, negative correlations were observed between MV vs. IR (-0.795) and MV vs. PI (-0.746).

Conclusion The supra testicular artery patterns values measured by Doppler ultrasonography showed the resistance and pulsatility indexes parameters higher in younger animals. The blood flow mean velocity of the supra testicular artery was higher in the animals of 24 months that presented a better capacity of thermoregulation. The higher resistance and pulsatility indexes in animals with elongated testicles suggests that a higher vascular resistance imposed to the blood flow results in lower vascular velocities, indicating a better heat dissipation in this format.

Acknowledgements This study was supported by CNPq/Universal, Brazil (Grant #456724/2014-1) and CAPES, Brazil (Grant CAPES/PNPD). The authors thank Boa Sorte Farm/PR for providing the animals and technical assistance for this study.

References

Brito L F, Silva A E, Barbosa R T, Kastelic, J P 2004. Theriogenology. 61, 511-528.
Herzog K, Bollwein H 2007. Reproduction in Domestic Animals. 42, 51-58.
Kastelic, J P 2014. Theriogenology. 81, 18-23.

The use of collar accelerometers to investigate the activity of dairy natural service bulls

R Waite[1,2], C Dwyer[2], D Beggs[1], P Mansell[1], M Stevenson[1], J Hills[3], M Pyman[1]
[1]*University of Melbourne, Victoria, Australia,* [2]*Smithton Veterinary Service, Smithton, Tasmania, Australia,* [3]*University of Tasmania, Burnie, Tasmania, Australia*
waiter@student.unimelb.edu.au

Application Knowing more about how natural service sires partition their time and when to rest them will assist decision makers.

Introduction Natural service sires contribute to the origin of 32.4% of the Australian herd recorded cows in 2016 (National Herd Improvement Association and Australian Dairy Herd Improvement Scheme, 2016). But despite this significant contribution, relatively little is known about how bulls partition their time. This information is important, since it can be used to plan periods of rest, or to rotate bull teams into and out of the herd. Provision of an optimal bull-to-cow ratio and ensuring that those bulls that are sound are adequately rested are important first steps to achieving herd reproductive targets (Morton and Larcombe 2008). The aim of this study was to quantify working bull activity.

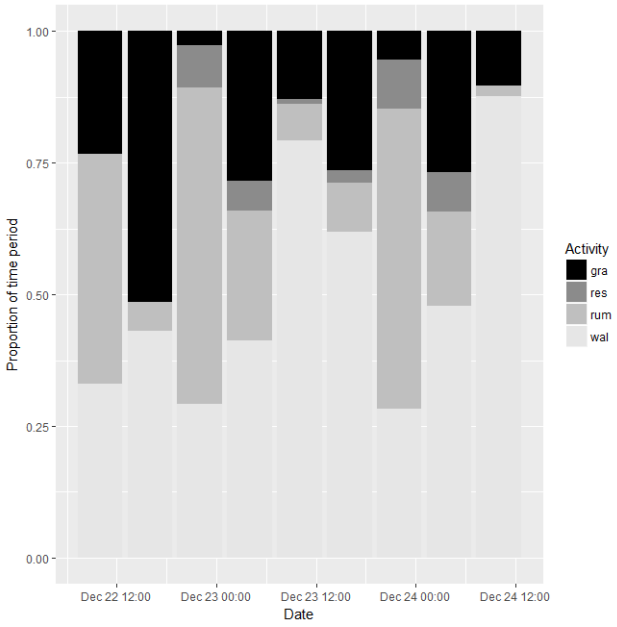


Figure 1 Proportion of a 6-hour period spent grazing (gra), resting (res), ruminating (rum), or walking (wal) for Bull 1 over three days

Material and methods: This was an observational study on an 800-cow dairy farm (run as two separate herds) near Smithton in north-western Tasmania, Australia. At the start of the study on the 22nd of December 2016, collars containing a triaxial accelerometer and a global positioning system (GPS) receiver chip (obtained via the Tasmanian Institute of Agriculture (TIA) and the Commonwealth Science and Industrial Research Organisation (CSIRO)) were deployed on ten working bulls. The collars were kept on the bulls for 14 days, unless they stopped recording or were broken, in which case they were removed. Collared bulls were part of a 30-bull team used on the farm and the herd manager ran each of the collared bulls with the milking herd as he saw fit. Throughout the 14-day follow-up period, direct observations of bull behaviour in the paddock were made by trained observers. Monitored behaviours included grazing, ruminating, resting and walking. Each bull was continuously observed for periods of four hours each day and a record of the start time, end time and details of the type of behaviour expressed recorded using a microcomputer tablet application. Details of bull location and movement were recorded on a secure digital card housed within the collar applied to each bull. After the follow-up period, records of bull activities (from the tablet application) and the accelerometer readings were analysed by the CSIRO, Data61 Computational Intelligence Group, Hobart. Learning algorithms were then applied to the data to associate accelerometer readings from the sensor data with the observed movement categories.

Results: The learning algorithm was better able to predict periods of grazing (F = 0.86) and walking (F = 0.91) compared with ruminating (F = 0.19) and resting (F = 0.38). Estimated behaviours were calculated as a proportion of time spent over 6-hour periods for each bull see Figure 1 for Bull 1.

Conclusion: This study represents the first analysis of the daily activity of a commercial pasture based natural service sire and provides insight into the proportion of time spent in various activities. A better knowledge of how natural service sires behave in the paddock can provide herd managers and veterinarians with a better understanding of how bulls might be used, rotated and rested to optimise reproductive performance.

Acknowledgements: The authors gratefully acknowledge funding and support from Dairy Australia, DairyTas, TIA, University of Tasmania, CSIRO Data61 Computational Intelligence Group, Smithton Veterinary Service and the University of Melbourne.

References

Morton J, Larcombe M L S 2008. Bulls: power up. *In* In Calf. Dairy Australia.
National Herd Improvement Association, and Australian Dairy Herd Improvement Scheme. 2016. Australian Dairy Herd Improvement Report.

https://doi.org/10.1017/S2040470018000109 Published online by Cambridge University Press

Bull sperm sncRNAs: A new source for potential fertility biomarkers?

E Sellem[1], S Marthey[2], H Kiefer[2], C Le Danvic[1], A Allais-Bonnet[1], L Jouneau[2], A Rau[2], H Jammes[2], L Schibler[1]
[1]*Allice, Paris, France,* [2]*INRA, Jouy-en-Josas, France*
eli.sellem@allice.fr

Application Ensuring an optimal quality of semen is a major concern for breeding companies, yet semen field fertility prediction remains challenging. Several quality parameters have been proposed to guarantee semen fertility, but they fail to reach high predictive value and high correlation levels. Our work aims to explore the small non coding RNA (sncRNA) content of sperm as a new source of potential semen fertility biomarkers.

Introduction With the advent of Genomic Selection, marketed semen is now produced by younger or barely mature bulls, without any prior data on their field fertility or progeny performance as was the case previously with progeny testing. Efficient quality control procedures are thus crucial for the cattle breeding sector since sub-fertile bulls lead to delayed conception, a prolonged calving season, and increased culling rates, thereby resulting in economic losses. Several quality control procedures and biomarkers have been proposed in the last decades to guarantee semen fertility including flow cytometry to assess functional key parameters. However, the relevance of most of these biomarkers in routine QC procedure has yet to be ascertained and their predictive value is usually too low (Sellem *et al*., 2015). Parameters included in these analyses may represent necessary but not sufficient factors to ensure pregnancy and some other essential sperm attributes should probably be added to the procedure to improve the prediction accuracy. In this respect, attention has been paid in recent years to sncRNAs, especially miRNA and piRNA, which have been shown to play a role in fertilization as well as zygotes and 2 cells-embryo development (Hosken and Hodgson 2014; Yuan *et al*. 2016). Our study aimed to characterise the sncRNA content of frozen bull sperm cells and identify miRNA associated with field fertility.

Material and Methods Total RNA was extracted from 30 Montbeliard ejaculates with contrasting fertility (3 adjusted fertility groups based on NNR282 days: in mean +4.6 / +1.8 / -13; N=10 per groups; balanced according to age at production), using an improved protocol. Quality control was ensured by total RNA quantification (Qubit technology) and quantitation of a reference miRNA (miR125) by RT-qPCR (miRCURY assay, Exiqon). NGS sequencing libraries were prepared using small RNA (<200 nucleotides) and sequenced at 40 million 50bp single reads (Illumina HiSeq; Exiqon). Identification and quantification of miRNAs was performed using the miRDeep2 software and miRNA associated with fertility were identified using both differential analysis using the DESeq2 package and discriminant analyses (BCA and Ro-PLS).

Results 67% of reads were annotated as miRNA (16%), rRNA (13%), tRNA (7.5%), long noncoding RNA (7.2%), mitochondrial RNA (6.5%) and mRNA (17%). The lack of *Bos Taurus* databases referencing piRNA hampered identification of these sncRNA in our dataset. A total of 3196 miRNAs (583 known and 2613 putative miRNAs) were identified. The 20 most abundant miRNAs account for the majority of miRNAs reads (75%). The most expressed miRNA, bta-miR-100, accounted for nearly 33% of all reads. An in depth study of miR sequence content showed a great diversity of Isomirs sequences (85±143 Isomirs per miR, min=2 / max=1678). Surprisingly, the canonical sequence as defined by cross-species comparative genomics were frequently not the most expressed (49%) or even not expressed at all (8%). Differential and discriminant analyses were thus performed at the Isomir level. Deseq2 highlighted 91 significant Isomirs (adjusted p value <0.05) between fertility groups, while BCA/Ro-PLS identified a signature effect of 72 isomirs discriminating between the three fertility groups. 16 isomirs are in common between the two statistical approaches, including isomirs from bta-miR-100, bta-miR-148, bta-miR-21, bta-miR-26, bta-miR-335 and bta-miR-130.

Conclusion Work is ongoing to confirm the relevance of miRNA signatures on a larger experimental design comprising 300 bulls. In the meantime, longitudinal studies will explore whether the sperm sncRNA pattern evolves along a bull's lifetime. The final objective is to develop fertility prediction tools based on miRNA expression that could be used as routine quality control by the breeding industry.

Acknowledgements This work is done within tle SeQuaMol Labcom, which has been funded by the French National Agency for Research (ANR) and APIS-GENE.

References

Hosken D J, Hodgson D J. 2014. Trends in Ecology & Evolution 29, 451-455.
Sellem E, Broekhuijse ML, Chevrier L, Camugli S, Schmitt E, Schibler L, Koenen EP. 2015. Theriogenology 84, 1447-1454 e1445.
Yuan, S, Schuster A, Tang C, Yu T, Ortogero N, Bao J, Zheng H and Yan W 2016. Development 143(4) 635-647.

https://doi.org/10.1017/S2040470018000109 Published online by Cambridge University Press

Effects of copper and zinc supplementation on standard and novel measures of fertility in peripubertal beef bulls

T Geary[1], R Waterman[1], M Van Emon[2], A Zezeski[1], J Heldt[3], J Spears[3]
[1]*USDA-ARS, Fort Keogh, Miles City, MT, USA,* [2]*Montana State University, Bozeman, MT, USA,* [3]*Micronutrients USA LLC, Indianapolis, IN, USA*
tom.geary@ars.usda.gov

Application National Research Council (NRC) recommendations of dietary copper and zinc are beneficial for yearling bull gain, but may exceed that needed for fertility.

Introduction Considerable variation exists in the percentage of beef bulls that reach puberty and pass a breeding soundness exam (BSE) by 14 months of age. Understanding the variables that enhance puberty and improve fertility of bulls would allow management of bulls for optimal fertility. Supplementation of minerals in diets of peripubertal bulls have not consistently improved sperm motility or morphology. Effects of mineral supplements on novel measures of sperm fertility using flow cytometry have not been evaluated previously. Our objective was to determine the effects of copper and zinc supplementation on novel measures of sperm fertility in the peripubertal bull measured with flow cytometry.

Material and methods Angus-Hereford bull calves (n = 80) weaned at approximately 178 days of age and maintained on a growing diet without mineral supplementation until 307 days of age were blocked by sire and stratified by body weight to 1 of 20 pens equipped with Calan gate individual feed bunks. Bulls were randomly assigned within pen to one of four trace mineral treatments: 1) zinc with no copper (**ZN**, 299 mg/d; n = 20), 2) copper with no zinc (**CU**, 131 mg/d; n = 20), 3) ZN + CU (**ZNCU**; n = 20), and 4) no copper or zinc (**CON**; n = 20) as supplements provided daily to a corn silage/alfalfa hay/corn/wheat growing diet. Mineral treatments were supplied as basic copper chloride and zinc hydroxychloride (Micronutrients USA LLC, Indianapolis, IN, USA) formulated so that the total mixed ration would meet or exceed NRC requirements for copper and zinc with the assumption that only 50% of the mineral in the basal diet was bio-available. Diets were fed 84 d with liver biopsies collected on d 0 and 85, and weight, scrotal circumference (SC), semen, and blood collected every 28 d. Two ejaculates were collected from each bull on d 0, 28, 56, and 84 for evaluation of sperm concentration, motility, and morphology as part of a standard BSE, as well as energy potential, acrosome integrity, viability, antioxidant capacity, and DNA integrity using flow cytometry. Liver biopsies (d 0 & 85) and semen samples (d 0 & 84) were evaluated for trace mineral concentration using inductively coupled plasma mass spectrometry. Bulls were classified as pubertal (50 x 10^6 spermatozoa in the ejaculate with > 10% progressive motility) and assigned a BSE score of Pass (prog. motility ≥ 30% + SC ≥ 30 cm + normal morph. ≥ 70%), Pass-High (Pass with prog. motility ≥ 60%), or Fail. Linear data were analysed using mixed model with d 0 measures as covariates, whereas categorical data were analysed using Chi Square. Pearson correlation coefficients were analysed using a multivariate analysis.

Results Four bulls were removed from the study for reasons unrelated to treatments so that each treatment was applied to 19 bulls. On d 0, liver copper (30.6 ± 4.0 ppm) was low, but liver copper and zinc (105.0 ± 2.1 ppm) were similar for all bulls. Bulls that received CON and ZN supplements were still low (64.0 ± 6.0 ppm) in liver copper on d 85, whereas CU and ZNCU supplemented bulls were adequate (237. 8 ± 6.0 ppm) in liver copper and greater (P<0.001) than CON and ZN bulls. Supplement type did not affect (P=0.65) liver zinc concentration (118.4 ± 2.1 ppm) on d 85. There was an effect (P=0.02) of treatment on ADG of bulls, with ZNCU bulls (1.53 ± 0.04 kg) gaining faster (P<0.05) than other bulls (1.42 ± 0.04 kg). Fewer (P<0.01) bulls assigned the CU treatment were pubertal on d 0, but by d 28, there were no differences (P=0.55) in pubertal status across treatments. A greater (P=0.04) percentage of CON bulls passed the d 0 BSE than bulls in other treatments, but by d 28, there were no differences (P=0.57) between treatments. Sperm motility, concentration, morphology, energy potential, viability, acrosome integrity, antioxidant capacity, and DNA integrity improved with increased bull age and were improved in the second ejaculate collected each day. Bulls supplemented with zinc (ZN & ZN/CU) produced sperm with greater (P<0.05) mitochondrial energy potential in d 28 ejaculates than CON bulls, and bulls receiving zinc and/or copper supplement produced a greater (P<0.05) proportion of viable sperm, as determined in the reactive oxygen species assay, in their ejaculates than CON bulls throughout the study. Percentage of normal sperm in the ejaculates on d 28 tended (P=0.06) to differ between treatments with ZN treated bulls having fewer abnormalities than CON bulls. Among novel measures of spermatozoa fertility, liver copper concentration was correlated with viable sperm antioxidant capacity (r = 0.34; P=0.03), DNA integrity (r = 0.38; P=0.01), and semen copper (r = 0.34; P=0.03) and zinc (r = 0.45; P=0.003) concentration were correlated with sperm DNA integrity. No other sperm fertility measurements were affected by mineral supplementation.

Conclusion Peripubertal bulls may have lower copper and zinc requirements for fertility than growth and/or are extremely efficient with homeostasis of copper and zinc recycling when provided at low levels in the diet. Sperm abnormalities were decreased by zinc supplementation and both zinc and copper supplementation had short term improvements in sperm viability. Liver copper was correlated with semen copper, sperm antioxidant capacity, and sperm DNA integrity.

https://doi.org/10.1017/S2040470018000109 Published online by Cambridge University Press

Objective analysis of frozen bovine semen using CASA and flow cytometry

M Spilman[1,2], K Burton[1], J Statham[1]
[1]*RAFT Solutions Ltd, North Yorkshire, UK,* [2]*Glasgow University, Scotland, UK*
breeding@raftsolutions.co.uk

Application Independent and reliable assessment of frozen bovine artificial insemination (AI) semen is not routinely performed in the UK. However, using a multi-parametric semen analysis system, our data indicate there is wide variation in the quality of semen currently in use on UK farms.

Introduction Quality of frozen semen for AI is routinely assessed by semen production centres (SPCs) prior to release. Once semen has been released there is generally no further routine quality assessment prior to use and subjective on farm assessments are shown to be relatively inaccurate, imprecise and operator dependant (Vincent *et al* 2012). Incorrect transport and storage of frozen AI straws may result in deterioration of semen quality. Semen may be handled a number of times after the initial quality control procedures at stud and this could result in significant reduction in fertility. Significant differences have already been found between insemination technicians with respect to motility and acrosome integrity of semen from the same ejaculates, indicating that storage and transport protocols can have an impact on semen quality. Currently SemenRate is the only independent service offering computerised semen analysis to cattle farms in the UK. This study aimed to investigate variation in computer assisted semen analysis (CASA) and flow cytometry (FC) parameters for semen stored on Yorkshire cattle farms and establish means and normal ranges for these parameters.

Material and methods Frozen/thawed, bovine AI semen from Yorkshire cattle farms was analysed by CASA and FC. Semen analysis parameters were collated to build a multi-parametric database of results. From this, normal ranges for parameters were established. The commercial service (SemenRate) has been extended across mainland UK using these normal ranges to classify the quality of semen analysed. The means and medians of different populations of semen used in practice were compared using Student's t-test and two sample t test respectively. Processing along with farm and sire type were evaluated to establish whether they were significant in this variation.

Results For conventional semen normal ranges were established for motility (n=79, $\bar{x}$= 37.443, min=0.000, max=66.900), progressive motility (n=79, $\bar{x}$=26.105, min=0, max=59.000), morphology (n=82, $\bar{x}$=38.563, min=0.260, max=72.5), mitochondrial activity (n=77, $\bar{x}$=81.823, min=51.400, max=93.900), acrosome integrity (n=82, $\bar{x}$=35.298, min=0.0600, max=68.820) and viability (n=79, min=0, max=67.640, median=47.580). For sexed semen (n=10) the ranges established were: motility ($\bar{x}$= 26.120, min=9.400, max=46.300), progressive motility ($\bar{x}$=14.510, min=3.900, max=31.500), morphology ($\bar{x}$=17.915, min=6.94, max=26.74), mitochondrial activity ($\bar{x}$=75.080, min=64.900, max=84.800), acrosome integrity ($\bar{x}$=36.450, min=15.340, max=52.620) and viability (median=47.605, min=17.080, max=66.310). For conventional vs sexed semen significant differences were seen for motility (p=0.0182), progressive motility (p=0.0024), morphology (p=0.0257) and mitochondrial activity (p<0.0001). Significant differences were also noted for dairy vs beef sires for viability (n=40, n=39, p=0.0432) and morphology (n=38, n=39, p<0.0001) as well as for dairy vs beef farms for acrosome integrity (n=59, n=23, p=0.0043), motility and progressive motility (n=22, n=57, p=0.0129 and p=0.0243 respectively) and morphology (n=19, n=58, p=0.0271).

Conclusion The use of FC in multi-parametric analysis is not routinely occurring in SPCs in the UK or in Europe. There are significant differences between the different types of semen in commercial use. The correlation between semen quality parameters and field fertility was not being assessed in the UK. An EU collaboration aims to establish figures relevant to the UK and across the EU (Sellem *et al* 2015). CASA technologies are principally used by semen companies to assess semen ensuring minimum standards prior to commercial release. SemenRate offers an independent service to vets and farmers. The results of semen analysis are clearly only part of an overall fertility solution on participating farms. As well as the technical laboratory-based skills required to run the analysis and scientific knowledge required to interpret results, the veterinary herd health input required to identify and manage risks associated with poor semen quality on farm are needed in partnership with the referring veterinary surgeon. Solutions to the issues causing poor quality semen may be identified and corrected, resulting in use of better semen in the future. Alternatively managing the risk of suboptimal semen usage can help minimise the impact on fertility on that farm. For example, a farmer may be advised to use extra straws of semen per insemination of batches found to have 'compensable' defects (Hudson *et al* 2012). Likewise appropriate advice on semen identified as having non-compensable defects can be given.

Acknowledgements: Innovate UK

References Hudson, C, Kerby M, Statham J M E and Wapenaar, W 2012. Managing Herd Reproduction. In Dairy Herd Health. Eds M. Green, A. Bradley, J. Breen, L. Green, A. Hayton, H. Higgins, C. Hudson, J. Huxley and J. Statham. CABI Publishing. p.73-111.
Sellem, E., Broekhuijse, M.L.W.J., Chevrier, L., Camugli, S., Schmitt, E., Schibler, L. and Koenen, E.P.C. 2015. Use of combinations of in vitro quality assessments to predict fertility of bovine semen. Theriogenology v.84, n.9, p.1-8
Vincent, P., Underwood, S.L., Dolbec, C., Bouchard, N., Kroetsch,T and Blondin, P 2012. Bovine semen quality control in artificial insemination centers. Anim Reprod, v9, n.3, p.153-165

https://doi.org/10.1017/S2040470018000109 Published online by Cambridge University Press

A descriptive analysis of bull sperm methylome using reduced representation bisulphite sequencing (RRBS)

J-P Perrier[1], E Sellem[1,2], A Prézelin[1], M Gasselin[1], L Jouneau[1], F Piumi[1], H Al Adhami[1,3], M Weber[3], S Fritz[2,4], D Boichard[4], C Le Danvic[2,5], L Schibler[2], H Jammes[1], H Kiefer[1]
[1]UMR BDR, INRA, ENVA, Université Paris Saclay, Jouy-en-Josas, France, [2]ALLICE, Paris, France, [3]CNRS, Université de Strasbourg, UMR7242 Biotechnologie et signalisation cellulaire, Illkirch, France, [4]UMR GABI, INRA, AgroParisTech, Université Paris Saclay, Jouy-en-Josas, France, [5]UMR CNRS/USTL 8576, UGSF, Villeneuve D'Ascq, France
helene.kiefer@inra.fr

Application In the context of genomic selection, more information on the epigenetic features transferred to the embryo alongside the paternal genetic heritage is necessary in order to improve semen quality control procedures as well as to guarantee semen fertility and proper embryo development.

Introduction Spermatozoa have a remarkable epigenome in line with their degree of specialization, their unique nature and different requirements for successful fertilization. Accordingly, the incorrect establishment of DNA methylation patterns during male germ cell differentiation has been associated with male infertility in mouse and Human (1). While bull semen is widely used in artificial insemination, the literature describing DNA methylation in bovine spermatozoa is still scarce. Yet domestication, the creation of highly specialized breeds and decades of genetic improvement have shaped the bovine genome. This undoubtedly has had a profound impact on the methylome, since DNA methylation is directly affected by the CpG content of the genome and its alteration by DNA polymorphism. The purpose of this study was therefore to characterize the bull sperm methylome relative to bovine somatic cells.

Material and methods Genomic DNA from Holstein sperm, fibroblasts and monocytes (collected from two individuals for each cell type) was extracted according to standard procedures, in the presence of 50 mM dithiothreitol for sperm. RRBS libraries were prepared from 200 ng of MspI-digested genomic DNA and sequenced on an Illumina HiSeq2500 sequencer to produce 75 bp paired-end reads (Integragen). Sequences were analyzed using an integrated pipeline combining homemade scripts together with external tools (2). High quality reads were aligned on the bovine reference genome (UMD 3.1 assembly) or on an artificial genome containing the consensus sequence of each bovine repeat (Repbase database) using Bismark (3). Only CpGs covered by 5 to 500 uniquely mapped reads for each sample were retained for subsequent analyses. For each pair of cell types, differentially methylated CpGs (DMCs) were identified using methylKit (4). A CpG was considered as a DMC when the associated q-value was weaker than 0.001 and the methylation difference between two conditions was at least 25%. DMCs were then annotated relative to gene features, CpG islands and repetitive elements, and genes containing DMCs subjected to GO enrichment analysis. Validations were carried out by means of bisulphite-pyrosequencing.

Results Bull sperm was compared with bovine fibroblasts and monocytes. The samples were clearly clustered according to the cell type using both hierarchical clustering and principal component analysis. Interestingly, the distance between sperm and other cell types was more important than the distance between monocytes and fibroblasts, highlighting the methylation specificities of germinal cells compared to somatic cells. DMCs were next identified for each pairwise comparison. A subset of 174,103 sperm-specific DMCs was isolated, as being differential in both sperm vs. monocyte and sperm vs. fibroblast comparisons, but not in that involving monocytes vs. fibroblasts. Interestingly, 79% of these sperm-specific DMCs were hypomethylated in sperm. Consistent with previous studies in other species, these hypomethylated sperm-specific DMCs were enriched for genes relevant to the germline differentiation program and sperm functions. The most remarkable observation was a dramatic enrichment for repeats (24.5% vs. 13.2% in background), and particularly for satellites (64.7% vs. 17.8%). We suspected that only a small fraction of the repetitive elements was represented in the uniquely mapped reads, and hypothesized that more information on the repeats hypomethylated in sperm could be extracted from the ambiguous reads. We therefore built an artificial genome containing one copy of each bovine repeat and aligned the totality of the reads on this artificial genome. The hypomethylation of sperm was clear in satellites and also in rDNA repeats encoding ribosomal RNAs. Hypomethylation of the most abundant satellite in the bovine genome, BTSAT4, was confirmed by bisulphite-pyrosequencing.

Conclusion These results highlight the hypomethylation of bull spermatozoa when compared with somatic cells. The genes hypomethylated in bull sperm are conserved across species, which may denote an important role in germline differentiation. In addition, the sperm-specific hypomethylation especially targets bovine repetitive elements. Whether hypomethylation of repetitive elements is of functional significance and contributes to the establishment of normal spermatogenesis needs to be ascertained.

Acknowledgements Grant ANR-13-LAB3-0008-01 'SeQuaMol' and grant ANR-1-INBS-0003

References (1) Boissonnas *et al*., Fertility and sterility 2013, 99(3) 624-631.
(2) https://github.com/FAANG/faang-methylation/tree/master/RRBS-toolkit/
(3) Krueger *et al*., Bioinformatics 2011, 27(11):1571-1572. (4) Akalin *et al*., Genome biology 2012, 13(10) R87.

The relationship between the functional status and miRNA profile of cryopreserved bovine semen

E Malama, S Bauersachs, M Siuda, F Janett, H Bollwein
Clinic for Reproductive Medicine, Vetsuisse Faculty, University of Zurich, Switzerland
emalama@vetclinics.uzh.ch

Application Interest in the implication of sperm microRNA patterns on bull fertility and their use as fertility biomarkers is constantly growing; however, their role in sperm function is not clear yet. Our results highlight the relationship between sperm functional status and miRNA expression levels.

Introduction Bovine sperm are equipped with a wide array of non-coding transcripts, with a length of 20-24 nucleotides, known as microRNAs (miRNAs) (Du *et al*., 2014) as well as other small non-coding RNAs. The involvement of miRNAs in testicular development and post-transcriptional regulation of spermatogenesis has been well documented (Kotaja, 2014). Furthermore, there is strong indication that sperm miRNAs are delivered to the oocyte upon fertilization and play a critical role in early embryonic gene expression and development (Liu *et al.*, 2012). The application of sperm miRNAs as biomarkers of male fertility appears promising, but studies focusing on the functional role of miRNAs in mature sperm are lacking. Our study focused on: a) the investigation of the miRNA population in cryopreserved bovine sperm, and b) the relation between andrological parameters and miRNA profile of sperm obtained from bulls of diverse fertility status.

Material and methods Cryopreserved sperm samples (n=101) of five Brown Swiss, two Swiss Fleckvieh, two Red Holstein and one Simmental bull in station A (n_A=10), and nineteen Holstein-Friesian bulls in station B (n_B=19) were analysed at 0 (0 h) and 3 hours (3 h) of post-thaw incubation. The annual 56-day non-return rate (NRR, %) of the examined bulls ranged from 50.0% to 79.0% (after 1191±1468 first artificial inseminations) and from 51.9% to 72.6% (after 1731±798 first artificial inseminations) in station A and B, respectively. Total, progressive and rapid motility (%) were determined using computer-assisted sperm analysis, while the mean DNA fragmentation index (DFI), the SD of DFI and the percentage of sperm with high DFI (DFI%) were assessed with the Sperm Chromatin Structure Assay (SCSA^{TM}). A multicolour flow cytometric panel including calcein violet AM, propidium iodide, Fluo4 AM and Mitoprobe^{TM} DiIC1(5) was employed for the assessment of sperm esterase activity, plasma membrane integrity, intracellular Ca^{2+} levels and mitochondrial membrane potential, respectively. Sperm acrosomal status and the inducibility of acrosome reaction (at 3 h) were evaluated through a dual staining with propidium iodide and fluorescein isothiocyanate-conjugated agglutinin of *Arachis hypogea.* Sperm total RNA was extracted with a modified TRIzol® protocol at 0h. Small RNA libraries were analysed with an Illumina® HiSeq 2500 sequencer. Unique sequences were mapped to a collection of non-coding RNA sequence databases using BLAST. The Spearman's correlation coefficient (r_s) was calculated to describe the relation between 15 sperm functional parameters and miRNA expression levels.

Results The analysis of sequencing data revealed 435 and 225 unique miRNA isoforms (isomiRs) in samples of stations A and B, that were assigned to 90 and 78 mature miRNA families, respectively; 63 miRNAs were common in samples A and B. MIR34b, MIR100 and MIR191 were the most abundant miRNAs in samples of both stations. All sperm parameters were correlated ($P<0.05$) with the expression levels of at least two miRNAs. MIR21-5p expression levels were related to all sperm traits ($0.71\leq|r_s|\leq0.82$, $P<0.05$ for station A; $0.46\leq|r_s|\leq0.63$, $P<0.05$ for station B). The percentage of sperm simultaneously exhibiting intact plasma membrane, low Ca^{2+} levels, high mitochondrial and esterase activity (0h) showed a correlation with the expression levels of 12 out of 90 miRNAs in samples A ($P<0.05$). The above mentioned sperm sub-population was related to only 2 out of 78 miRNAs (MIR21-5p, MIR30a-5p) in samples B ($P<0.05$); the two miRNAs were detected in samples obtained from both stations. Five miRNAs (MIR146a, MIR16-5p, MIR30a, MIR6089-1, MIR7) were significantly related to the NRR of the bull ($P<0.05$), but showed no correlation to any of the assessed sperm parameters ($P>0.05$). On the other hand, seven miRNAs (MIR146b, MIR106b, MIR29a, MIR29b, MIR151-5p, MIR186, MIR2284x) were not related to NRR values, but significantly related to at least 10 out of the 15 assessed sperm parameters.

Conclusion A wide variety of miRNAs was detected in cryopreserved bovine semen; however, station-dependent differences of sperm miRNA profile were observed. The expression levels of several miRNAs were related to sperm functional parameters and/or the NRR of the bull.

Acknowledgements We acknowledge the support of Dr. Erwin Hasenpusch, Rinderzucht Schleswig-Holstein eG, Neumünster Germany, and Dr. Ulrich Witschi, Swissgenetics, Zollikofen Switzerland

References Du, Y., Wang, X., Wang, B., Chen, W., He, R., Zhang, L., Xing, X., Su, J., Wang, Y., Zhang, Y. 2014. Molecular Reproduction and Development. 81, 1042–1052
Kotaja, N. 2014. Fertility and Sterility. 101, 1552–1562
Liu, W.-M., Pang, R.T.K., Chiu, P.C.N., Wong, B.P.C., Lao, K., Lee, K.-F., Yeung, W.S.B. 2012. Proceedings of the National Academy of Sciences. 109, 490-494.

Natural service bulls in Australian pasture-based dairy herds: management practices, breeding soundness evaluations, and risk factors associated with pre- and post-mating breeding soundness

A Hancock[1,2], P Younis[2], D Beggs[1], P Mansell[1], M Pyman[1]
[1]*The University of Melbourne, Australia,* [2]*The Vet Group, Timboon, Australia*
andrew.hancock@zoetis.com

Application There are currently no peer-reviewed studies which report the management of natural service bulls in dairy herds or which describe the risk factors for reduced fertility both prior to and after the natural mating period in pasture-based dairies. Our data allow for practical recommendations to be made for bull management as well as identifying topics for further research.

Introduction In the pasture-based, seasonally calving dairy herds of southern Australia, the mating period usually consists of an initial artificial insemination period followed by a period of natural service using herd bulls. There are only a few studies which have reported BBSE findings and management practices from natural service dairy herd bulls, and these are not peer-reviewed papers[1-3]. Pre- and post-mating BBSE findings have been reported in beef herds[4], but to our knowledge no studies have reported these in dairy herds. The objectives of this study were, for natural service bulls used in dairy herds, to describe their management; to describe the causes of increased risk of reduced fertility, as measured by a standard bull breeding soundness evaluation (BBSE); to describe the reasons for bull removal by herd managers during mating; and to identify associations between individual bull- and herd-level management factors and bull breeding soundness results.

Material and methods BBSEs were performed on 256 bulls from 32 dairy herds in south-eastern Australia, using guidelines produced by the Australian Cattle Veterinarians, before and immediately after a single natural mating period. The same bulls were evaluated pre- and post-mating. At the same time, herd managers were questioned regarding the management of the bulls. Multivariable mixed effects logistic regression models were used to identify factors associated with bulls being classified as high risk of reduced fertility at the pre-mating and post-mating BBSE.

Results At the premating BBSE, 19.5% of bulls were classified as high risk of reduced fertility, mostly due to physical abnormalities and reduced semen quality. At the post-mating BBSE, 36.5% of bulls were classified as high risk of reduced fertility, mostly due to physical abnormalities, primarily lameness. Of the bulls used, 15.9% were removed from normal mating use by the herd manager, predominantly due to lameness and injuries. Bulls older than 4 years of age at the premating BBSE were more likely to be classified high risk compared with bulls less than 4 years of age (OR 2.16; 95% CI 1.06-4.43; P=0.035). Bulls that were in herds in which concentrates were fed before mating were more likely to be classified as high risk at the post-mating BBSE compared with bulls that were in herds where concentrates were not fed (OR 3.81; 95% CI 1.02-14.6; P=0.03). Univariable analyses also identified areas in need of further research, including breed differences between dairy bulls, leg conformation and joint abnormalities, preventative hoof blocking for bulls, and mating ratios.

Conclusion A premating BBSE is recommended in dairy herd bulls to identify bulls at risk of reduced fertility. Lameness is the most common problem in dairy herd bulls during the natural mating period, and risk factors associated with lameness in these bulls should be identified to better manage herd bulls.

Acknowledgements
The Vet Group (Timboon, Australia), Dairy Australia (Melbourne, Australia), Gardiner Foundation (Melbourne, Australia), the University of Melbourne (Melbourne, Australia), and Chenovet (Wagga Wagga, Australia).

References

Champagne J D, Kirk, Reynolds J P Proceedings 15th Annual Fall Symposium 2002, 15- 21.
Chenoweth, P. J., J. D. Champagne, and J. F. Smith. 2003. Proc. 6th Western Dairy Management Conference, Reno, NV. 107–118
Dwyer, C. 2013b. Proc. Australian Cattle Veterinarians Conference, Darwin, Australia. 123–126
Ellis R W, Rupp G P, Chenoweth P, Cundiff L, and Lunstra D 2005. Theriogenology 64, 657–678.

The genetic background of three fertility disorders in Nordic red cattle breeds

M Andersson[1], H Venhoranta[1], T Iso-Touru[2], K Flisikowski[3], C Wurmser[4], H Pausch[5]
[1]*Department of Production Animal Medicine, Faculty of Veterinary Medicine, Saarentaus, Finland,* [2]*2Natural Resources Institute Finland (Luke), Green Technology, Jokioinen, Finland,* [3]*3Chair of Livestock Biotechnology, Technische Universität München, Freising, Germany,* [4]*4Chair of Animal Breeding, Technische Universität München, Freising, Germany,* [5]*5Institute of Agricultural Sciences, Animal Genomics, ETH, Zurich, Switzerland*
magnus.andersson@helsinki.fi

Application Homozygosity for recessive mutations causing asthenospermia (1) or total asthenoteratospermia (2) compromises the semen quality of affected bulls and makes them unsuitable for use in AI. A mutation in an imprinted gene with paternal expression (3) caused a 44% mortality rate in affected foetuses due to late abortions and stillbirths. The identification of the underlying mutations now enables the implementation of direct gene testing to identify carriers of the respective mutations before they are used for semen collection.

Introduction The availability of dense molecular markers facilitates the identification of genetic reasons for fertility disorders in cattle. Genetic techniques have improved and diagnostics are getting better because more parameters are used in AI centres and external laboratories. In-depth analyses of these data enabled us to pinpoint three genetic mutations in bulls that were unsuitable for breeding.

Material and methods Initially we studied two bulls with asthenospermia, irrespective of normal sperm plasma membrane integrity and sperm morphology, three bulls with an immotile tail stump sperm defect and one bull with a high proportion of stillborn foetuses and aborted calves (44 % mortality compared to the normal 6 % mortality due to late abortions and stillbirth). We genotyped the affected bulls using the Illumina Bovine 50 Kb DNA SNP chip and included normal bulls and half siblings as a control group. In the case of high mortality due to late abortions and stillbirth, we also genotyped live and dead offspring of the case bull. We performed genome-wide association and homozygosity mapping followed by collecting next generation sequencing (NGS) data in order to identity the underlying mutations.

Results The results are shown in Table 1.

Table 1 Three different phenotypes of bulls unsuitable for breeding in the Nordic red breeds and the genetic mutation findings including diagnosis, number of bulls, mutated gene, nature of mutation and type of inheritance

Diagnosis	Number of bulls	Mutated gene	Nature of the mutation	Type of inheritance
Asthenospermia	4 AI bulls	CCDC189	A splicing variant	Recessive
Tail stump sperm defect	4 AI bulls	ARMC3	A frameshift mutation	Recessive
High rate of abortions and stillbirths	2 (one AI bull)	MIMT1 in PEG3 domain	Deletion	Maternally imprinted gene

Conclusion We were able to identify genetic mutations for three fertility disorders in AI bulls. The use of novel DNA genotyping and sequencing technology combined with an interdisciplinary research approach were the keys for the success. The breeding organisations have implemented direct genetic tests to identify affected animals before they are used for semen collection and breeding.

Acknowledgements All people involved in this study are acknowledged and especially the groups led by the following professors: Ruedi Fries, Marek Switonski, Jeremy Taylor, Heriberto Rodriguez-Martinez, Angelika Schnieke and Hannes Lohi. The funding from Finnish Veterinary Foundation and Academy of Finland is highly appreciated.

References Pausch H, Venhoranta H, Wurmser C, Hakala K, Iso-Touru T, Sironen A, Vingborg RK, Lohi H, Söderquist L, Fries R, Andersson M. 2016. BMC Genet. Feb 29;17:49. doi: 10.1186/s12863-016-0356-7. PMID: 26923438

Infrared thermography and Doppler ultrasonography to evaluate the effects of scrotal insulation on testicular blood flow dynamics in bulls

F Barca Jr[1,2], C Koetz Jr[2], P Favaro[2], G Pereira[2], F Morotti[1], I Dias[2], E M Franco[2], C H Bofinger[2], M Seneda[1]
[1]*Universidade Estadual de Londrina - UEL, Londrina, PR, Brazil,* [2]*Universidade Norte do Paraná - UNOPAR, Arapongas, PR, Brazil*
celsokoetzjr@yahoo.com.br

Application The uses of Infrared Thermography (IRT) and Doppler ultrasound to evaluate thermoregulation assessment of a thermal insult have the potential to become an additional approach that can be included to breeding soundness evaluation in bulls.

Introduction Changes in spermatogenesis can be simulated through scrotal insulation (Brito *et al.*, 2003; Fernandes *et al.*, 2008); however, no information regarding the uses of Doppler ultrasound has been reported to evaluate the effect of scrotal insulation seminal parameters in bulls. Recently, researchers have suggested that the uses of IRT as a noninvasive method to assess scrotal insulation on sperm production in beef bulls is only desirable to evaluate scrotal gradient temperature at the time of insult removal (Menegassi *et al.*, 2018). There is an increasing need to determine emerging technologies tools to evaluate seminal parameters in bulls and the uses of IRT and Doppler Ultrasonography to predict animal reproductive changes that may provide a more reliable and less invasive method of welfare assessment. The aim of this study was to evaluate the dynamics of the scrotal temperature and testicular blood flow through IRT and Doppler ultrasonography in bulls submitted to scrotal insulation.

Material and methods All procedures with animals were approved by the Animal Ethical Committee (CEUA/UEL #18656/2014/58). The study was conducted with 8 Braford bulls (3/8 Nellore - 5/8 Hereford) in Southern Brazil (latitude 23°30'10"S and longitude 51°14'10''W), where the climate in the region is considered Cfa (humid subtropical climate) according to Köppen classification. Braford bulls (n=8), considered approved by breeding soundness evaluation at 18 months of age were randomly assigned as following: insulated for 72 h (INS72; n=2), 96 h (INS96, n=2), 120 h (INS120, n=2), and control animals (CON; n=2) that remained without insulation during all the experimental period. Insulation was performed using a plastic diapers containing two layers of cotton that covered the entire scrotum area, fixed with adhesive tape and carefully placed at the spermatic cord part of the scrotal. IRT and Doppler ultrasonography procedures (mean velocity, pulsatility index and resistivity index) were performed in four different periods: immediately after the scrotal insulation (M0), within 10 min (M10), 30 min (M30) and 60 min (M60) after scrotal insulation. Data were analyzed by ANOVA, *t*-test (paired) and Pearson's correlation with significance level of 5%.

Results None of the observed variables were difference between the insulated groups (INS72, INS96 and INS120, $P>0.05$). The rectal temperature (38.5±0.4) was higher in relation to the scrotal surface (32.7±0.8, $P<0.05$) at the time of scrotal insulation. Insulated animals showed higher scrotal temperature in M0 (33.0±0.7) compared to M10, M30 and M60 periods (30.2±1.3, 31.6±1.5 and 30.6±1.0, respectively; $P<0.05$). There was no difference in pulsatility and resistive indexes after scrotal insulation. However, blood flow velocity was higher in M10 (17.1±4.2) compared to M0, M30 and M60 periods (12.5±5.1, 14.3±4.9 and 14.3±2.9, respectively; $P<0.05$). Positive correlation (93.1%) was found between pulsatility and resistivity indices ($P<0.05$).

Conclusion We conclude that a noninvasive technique IRT is appropriate to evaluate thermal insults, especially short-term thermal changes after scrotal insulation removal. In addition, scrotal insulation resulted in changes in scrotal temperature and testicular blood flow velocity, but either normalized shortly after insulation removal, suggesting a high efficiency of the thermoregulatory mechanisms. Therefore, IRT and Doppler ultrasonography approaches were efficient to evaluate the dynamics of the scrotal temperature and testicular blood flow in bulls submitted to scrotal insulation.

Acknowledgements This study was supported by CNPq/Universal, Brazil (Grant #456724/2014-1) and CAPES, Brazil (Grant CAPES/PNPD). The authors thank Boa Sorte Farm/PR for providing the animals and technical assistance for this study.

References

Brito, L.F.C., Silva, A.E., Barbosa, R.T., Unanian, M.M., Kastelic, J.P. 2003. Animal Reproduction Science. 79, 1-15.
Fernandes, C.E., Dode, M.A., Pereira, D., Silva, A.E. 2008. Theriogenology. 70, 1560-1568.
Menegassi, S.R.O., Pereira, G.R., Dias, E.A., Rocha, M.K., Carvalho, H.R., Koetz Jr, C., Oberst, E.R., Barcellos, J.O.J. 2018. Andrologia. (First View: doi: 10.1111/and.12904).

Hemodynamics evaluation of the supratesticular artery in bulls in different seasons of the year

P Favaro[1], G Pereira[1], F Barca Jr[1], E M Franco[1], M Seneda[2], C Koetz Jr[1]
[1]*Universidade Norte do Paraná - UNOPAR, Arapongas, PR, Brazil,* [2]*Universidade Estadual de Londrina - UEL, Londrina, PR, Brazil*
celsokoetzjr@yahoo.com.br

Application The evaluation of testicular hemodynamics can contribute significantly to understanding the thermoregulatory mechanisms and the supply of oxygen in the testis in domestic animals. Thus the importance to understand bull testicular hemodynamics to achieve success in animal production is essential for reproductive efficiency in this specie.

Introduction Environmental conditions of cattle breeding at tropical regions may be affected by heat stress when the mechanisms of scrotal thermoregulation may result in testicular degeneration in domestic animals (Kastelic *et al.*, 2001). The mechanism of countercurrent action is considered as the main factor for normal thermoregulation, maintaining the testicular parenchyma between 4 and 6^{o}C below the body temperature. Animals under climatic change conditions have mechanisms to promote the adaptation to the environment that may interfere with spermatogenesis and sperm production. Recently, the temperature and humidity index (THI) has been used to measure climate changes in tropical regions in cows (Silva *et al.*, 2007) and bulls (Menegassi *et al.*, 2017). Doppler ultrasonography has been increasingly used to evaluate blood flow in the bovine breeding programmers, generating new information on the physiological and pathological processes of the reproductive tract. Perhaps, the evaluation of testicular hemodynamics can contribute significantly to understanding the thermoregulatory mechanisms. Therefore, the aim of this study was to evaluate the testicular hemodynamics using Doppler ultrasonography in Aberdeen Angus and Polled Hereford bulls during the four climatic seasons of the year and the relation of climatic variations with normal spermatogenesis in bovine breeding animals.

Material and methods All procedures were approved by the Ethics Committee for Care and Use of Experimental Animals (# 18656/2014/58) from UNOPAR Institution, Brazil. We evaluated 28 bulls (Polled Hereford n=12 and Aberdeen Angus n=16) by means of velocimetry (MV), pulsatility index (PI) and resistance index (RI) parameters using Doppler ultrasonography. In addition, we also collected information to calculate the temperature–humidity index (THI) from an automatic meteorological station of the National Meteorological Institute. Ejaculates were collected from each bull using an automatic operated electro ejaculator and sperm classification was performed as previously described by the BBSE of the Western Canadian Association of Bovine Practitioners (Chenoweth *et al.*, 2010). Seminal evaluation was performed twice in each season during one year. Data were analysed using analysis of variance (ANOVA). Tukey's test and Pearson's correlation with significance level of 5% were used.

Results No differences were observed when the interaction between breed and season was evaluated. The THI was lower during autumn (46.8) compared to winter (66.9), spring (71.9) and summer (72.7) ($P<0.05$). The mean velocity (MV) of blood flow (cm/s) was different in autumn (7.53±2.6) compared to winter, spring and summer (13.6±4.2, 13.5±4.3 and 10.9±3.3, respectively; $P<0.05$). In addition, the PI in the autumn (0.45±0.1) showed an increase compared to winter and summer (0.31±0.2 and 0.28±0.1, respectively; $P<0.05$). No difference was observed between the RI and seasons. We observed a positive correlation (0.83) between PI and RI indexes ($P<0.05$). However, a negative correlation between MV x PI (-0.36) and MV x RI (-0.20) was observed. The percentage of sperm total defects observed in spring (31.2±18.8) was higher compared to autumn and winter (20.6±11.4 and 19.7±17.6, respectively; $P<0.05$). Aberdeen Angus animals showed higher scrotal circumference and sperm motility (39.3cm±3.2 and 60.7%±39.4, respectively) compared to Polled Hereford bulls (36.8cm±3.6 and 51.4%±41.3, respectively; $P<0.05$).

Conclusion Low THI affect the values of testicular hemodynamics due to the physiological reflex of the scrotal thermoregulation, causing a peripheral vasoconstriction in response to low temperatures, resulting in a lower average velocity of blood flow of the supratesticular artery. In addition, higher THI in autumn showed higher blood flow in the supratesticular artery that negatively influenced seminal quality. Doppler ultrasound can be used to assess changes in the scrotal blood flow of the pampiniform plexus during the seasons and suggests that differences in VM, PI and RI dynamics are important actors in the physiological requirements for their normal reproductive performance under different environmental conditions.

Acknowledgements This study was supported by CNPq/Universal, Brazil (Grant #456724/2014-1) and CAPES, Brazil (Grant CAPES/PNPD Unopar). The authors thank Três Maria Farm/SC for providing the animals for this study.

References Chenoweth, P.J., Hopkins, F.M., Spitzer, J.C., Larsen, R.E. 2010. Clinical Theriogenology, 2, 43-50.
Kastelic, J.P., Cook, R.B., Coulter, G.H., Wallins, G.L., Entz, T. 1996. Animal Reproduction Science. 41, 153-159.
Menegassi, S.R.O., Pereira, G.R., Dias, E.A., Koetz, C., *et al*. 2017. International Journal of Biometeorology. 60, 151-157.
Silva, R.G., Morais, D.A.V.F., Guilhermino, M.M. 2007. Revista Brasileira de Zootecnia. 36, 1192-1198.

Overview of bull fertility in traditional systems of beef production in Southern Spain

J M Sánchez[1], L M Rosales[2], L Quevedo[3], C C Perez-Marin[2]
[1]*Agriculture and Food Science, University College Dublin, Ireland,* [2]*Animal and Surgery Department, Veterinary Faculty, University of Cordoba, Spain,* [3]*JANDAVET S.L., Medina Sidonia, Cadiz, Spain*
pv2pemac@uco.es

Application The present study shows a snapshot of the beef bull fertility in Spain and describes the importance of the stock bull assessment in order to enhance the profitability in beef farms.

Introduction In Spain, the mean fertility on beef farms (68.0%) is lower than in Europe overall (MAPAMA, 2014). Although nowadays it is known that stock bulls are an essential component of cattle production systems, and that the profitability of farms depends on their fertility, the criteria for the selection of stud bulls is still mainly based on their conformation or morphotype instead of based on their reproductive merit. Moreover, it has been reported that one or two out of 5 stock bulls are subfertile or infertile (Kastelic and Thundathil, 2008). Thus, we aimed to evaluate the fertility of stock bulls by breeding soundness evaluation (BSE) in traditional systems of beef production in Southern Spain.

Material and methods A total of 427 BSEs (general condition and reproductive tract evaluation, mounting ability and libido assessment, and semen analysis) in 227 stock bulls of different breeds (Limousin=162; Charolais=46; Pirenaico= 12; Blonde d'Aquitane=4; Retinto del Guadalquivir=3) ranging from 1 to ≥5 years old on 14 beef farms in Andalucia (Southern Spain) were carried out. This study was performed from 2004 to 2014 and some of the bulls were evaluated several times throughout this period. All BSEs were conducted by two expert technicians. Semen collected by electroejaculation was analysed as follow: macroscopic (volume, colour, density) and microscopic (total and individual motility, concentration, % live spermatozoa, and morphology). Semen concentration was analysed using a photometer and motility was analysed either subjectively and/or objectively by CASA (Computer Assisted Semen Analysis). The effect of the variables (age, farm, sire, scrotal circumference, year) on the ability to become an optimum stock bull (approved or not) was analysed by ANOVA using SPSS 17.0 package (SPSS, Chicago, IL, USA). Significant differences were considered when $P<0.05$.

Results Overall, 86% (361/420) of the assessments were satisfactory (approved), 14% (59/420) failed to pass (non-approved), and 7 animals could not be evaluated due to some missing data. The highest percentage of approved bulls was found in the 3-year-old group and the lowest in the ≥5 year-old group ($P<0.05$). There was also an effect of farm on the percentage of approved bulls suggesting that it is influenced by selection criteria and/or management ($P<0.02$). Charolais bulls showed a lower ability to pass the BSE in comparison with Limousin and Pirenaico bulls ($P\leq0.01$). When scrotal circumference was >40 cm the percentage of non-approved animal increased ($P\leq0.01$). Moreover, Charolais bulls had higher scrotal circumference values than the other sires ($P\leq0.01$).

Conclusion The main challenge in systems of beef production is to achieve high conception rates in a short interval of time. Although, cow fertility undoubtedly plays an important role in reproductive efficiency, bull fertility is essential to successfully achieve conception, early embryo development, implantation and completion of pregnancy. Therefore, BSE in stud bulls is an important management tools and should be implemented routinely in beef farms.

References Kastelic, J.P., Thundathil, J.C. 2008. Reproduction in Domestic Animals 43(Suppl. 2), 368-373.
MAPAMA, 2014. http://www.mapama.gob.es/es/ganaderia/temas/produccion-y-mercados-ganaderos/estudio_nodrizas_2014_tcm7-267234.pdf [accessed 18.01.2018]

https://doi.org/10.1017/S2040470018000109 Published online by Cambridge University Press

Relationship between scrotal circumference and body weight in pasture-raised Jersey and Holstein bulls

R Waite[1,2], C Dwyer[2], D Beggs[1], P Mansell[1], M Stevenson[1], M Pyman[1]
[1]*University of Melbourne, Victoria, Australia,* [2]*Smithton Veterinary Service, Smithton, Tasmania, Australia*
waiter@student.unimelb.edu.au

Application This study contributes information on pasture-raised dairy breed bulls that can help with determining their fertility.

Introduction: Natural sire use in pasture-based dairy herds in south eastern Australia is common. Bulls are typically run with a herd to ensure that cows that did not conceive following a period of artificial breeding can become pregnant. Often considered unimportant, natural service sires account for the parentage of 32% of the known breed herd recorded cows in Australia in 2016 (National Herd Improvement Association and Australian Dairy Herd Improvement Scheme, 2016). The bull breeding soundness examination (BBSE), as outlined for Australian Cattle Veterinarians (Beggs *et al*., 2013), stipulates scrotal circumferential thresholds for Holstein and Jersey bulls but information for pasture-raised animals is not available. Here, we describe body weight (BW), scrotal circumference (SC) relationships, average daily gain (ADG) and threshold measurements for weight classes for pasture-raised dairy breed bulls.

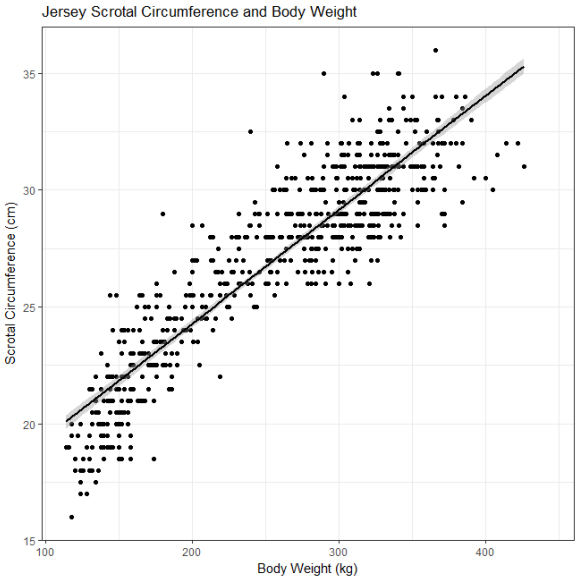


Figure 1 Scatter plot of SC and BW in Jersey bulls

Material and methods Two groups of bulls were enrolled in north western Tasmania, one Holstein (n = 124) and the other Jersey (n = 84). Both groups had BW and SC measurements recorded at approximately 8-week intervals between 6 to 18 months of age. BW and SC data were plotted, a relationship via linear regression was defined and ADG was calculated. Tables of weight classes and mean and standard deviations of SC were constructed. Data was analysed using R version 3.4.3 (R Foundation for Statistical Computing, Vienna, Austria).

Results The relationship between BW and SC for both breeds was demonstrated in a scatter plots (Figure 1 shows data for Jersey bulls, Holsteins not shown). For Holsteins, a one kilogram increase in a bull's BW was associated with a 0.059 (95% CI 0.057 to 0.061) cm change in SC (P= <2.2e-16). For Jerseys, a one kilogram increase in a bull's BW was associated with a 0.048 (95% CI 0.047 to 0.05) cm change in SC (P= <2.2e-16). ADG for Holsteins was 0.55kg/day and for Jerseys 0.52kg/day. Table 1 shows weight classes and mean SC observations.

Table 1 The mean and standard deviation (S.D.) of SC for BW classes in Holstein and Jersey bulls. Included are the 5th and 10th percentile values for SC and the total number of animals in that class (* too few data points to calculate).

	Holstein				Jersey			
BW (kg)	Mean SC (cm) ± S.D.	5th % SC	10th % SC	Total	Mean SC (cm) ± S.D.	5th % SC	10th % SC	Total
<250	20.9 ± 3.3	16	16.5	474	23.2 ± 3.0	18.5	19	269
250-300	27.8 ± 2.7	24	24.5	134	28.6 ± 1.7	26.0	26.5	133
300-400	31.0 ± 1.9	28	28.5	263	30.6 ± 1.8	28.0	28.4	232
>400	34.7 ± 1.9	31	32.5	127	31.3 ± 0.8	*	*	6

Conclusion This study augments the information available to assess pasture raised dairy breed natural service sires.

Acknowledgements The authors gratefully acknowledge support and funding from Dairy Australia, DairyTas, Smithton Veterinary Service and the University of Melbourne.

References Beggs D S, Bertram J D, Chenoweth P J, Entwistle K W, Fordyce G, Johnston H, Johnston P, McGowan M R, Niethe G, Norman S and Perry VEA 2013. Veterinary Bull Breeding Soundness Evaluation. Australian Cattle Veterinarians, Eight Mile Plains, Queensland, Australia.
National Herd Improvement Association and Australian Dairy Herd Improvement Scheme 2016. Australian Dairy Herd Improvement Report.

https://doi.org/10.1017/S2040470018000109 Published online by Cambridge University Press

Predicting semen characteristics in Holstein and Jersey natural service sires using classification and regression tree analysis

R Waite[1,2], C Dwyer[2], D Beggs[1], P Mansell[1], M Stevenson[1], M Pyman[1]
[1]*University of Melbourne, Victoria, Australia,* [2]*Smithton Veterinary Service, Smithton, Tasmania, Australia*
waiter@student.unimelb.edu.au

Application This study describes how measurement of the scrotal circumference (SC) and bodyweight (BW) can be used to predict when young bulls are ready for service.

Introduction Natural sire usage in pasture-based dairy herds in south eastern Australia is common. Bulls are typically run with a herd to ensure that cows that did not conceive following a period of artificial breeding can be mated and become pregnant. Often considered unimportant, natural service sires account for the parentage of 32% of the dairy herd recorded in Australia in 2016 (National Herd Improvement Association and Australian Dairy Herd Improvement Scheme, 2016). Despite their widespread use, little information is available to assist producers and veterinarians to select natural service animals at an appropriate age. The bull breeding soundness examination (BBSE), as outlined for Australian Cattle Veterinarians (Beggs *et al*., 2013), stipulates SC thresholds for Holstein and Jersey bulls but information for pasture raised animals is not available. We describe an approach whereby easily measurable characteristic (SC and BW) can be used to predict if the semen quality of a young Holstein or Jersey bull is sufficient to be submitted for a BBSE.

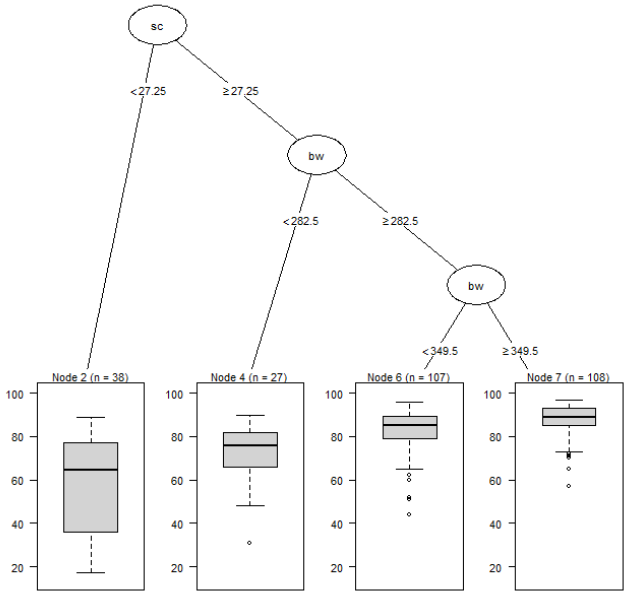


Figure 1. Holstein CART analysis of scrotal circumference, body weight and percent normal sperm (NS) >70%.

Material and methods Two groups of bulls were enrolled in north western Tasmania, one Holstein (n = 124) and the other Jersey (n = 84). Both groups had SC and BW measurements recorded at 8-week intervals between 6 to 18 months of age. Once SC reached 25 cm semen samples were collected via electroejaculation and submitted for morphological analysis (ChenoVet Animal Andrology). Semen samples continued to be collected at each visit until a 70% normal sperm threshold was reached. Classification and regression tree analysis (CART) was used to predict if a bull's semen had exceeded a 70% normal sperm threshold using SC and BW as predictors (Therneau *et al*., 2017).

	Node 2	Node 4	Node 6	Node 7
Correctly classified >70%NS	15	18	95	106
Total in group	38	27	107	108
Classified correct (%)>70%	39.5	66.6	88.8	98.1

Results Our CART analyses show that 98.1% of Holstein bulls that weighed greater than 349.5kg and had a SC of at least 27.25 cm had a percent normal sperm score greater than 70% (see Figure 1). For Jersey bulls 87.5% that weighed greater than 259kg and had SC of at least 29.25cm had a percent normal sperm score greater than 70% (table not shown).

Conclusion CART analysis provides a means for predicting percent normal sperm based on body weight and scrotal circumference in pasture raised Holstein and Jersey natural service sires.

Acknowledgements The authors gratefully acknowledge support and funding from Dairy Australia, DairyTas, ChenoVet Animal Andrology, Smithton Veterinary Service and the University of Melbourne.

References

Beggs D S, Bertram J D, Chenoweth P J, Entwistle K W, Fordyce G *et al*., 2013. Veterinary Bull Breeding Soundness Evaluation. Australian Cattle Veterinarians, Eight Mile Plains, Queensland, Australia.
National Herd Improvement Association and Australian Dairy Herd Improvement Scheme 2016. Australian Dairy Herd Improvement Report.
Therneau T, Atkinson B and Ripley B 2017. rpart: Recursive Partitioning and Regression Trees.

https://doi.org/10.1017/S2040470018000109 Published online by Cambridge University Press

Relationship between the echotexture of testicular parenchyma and the quality of spermatogenesis in bulls

A Echegaray[1], S Aventín[1], I Muñoz[1], S Marcantonio[3], N Escartín[1], G Gnemmi[2]
[1]*Humeco, Huesca, Spain,* [2]*Bovinevet, Lombardia, Italy,* [3]*Buenos Aires University, Buenos Aires, Argentina*
araechegaray@humeco.net

Application These algorithms will be useful in prediction of fertility of young males before entering into a breeding schema and also to evaluate testicle health in valuable sires.

Introduction Incorporation of scrotal ultrasound could be of great interest for the diagnosis and prognosis of the reproductive capacity of bulls. In this context, we have developed several algorithms to try to quantify the echotexture of the testicles.
These algorithms analyze ultrasound images, according to the distribution of black, white and grey pixels and also according to the size and density of hypoechogenic areas. The aim of this work is to investigate the relationship between these ultrasound characteristics and epididymal semen quality in the ipsilateral testicles of 31 slaughtered bulls.

Material and methods Testicles (n = 62), were scanned immediately postmortem. Ultrasound of the testicular area was performed, using an EXAGO (ECM, France) connected to a 5-7.5 MHz linear probe. Five transverse sonographic images of each testicle were taken. The echographic images were analyzed by the algorithms, selecting a region of interest (ROI) and obtaining the average of the 5 images for the following parameters: (EC1) black pixels, (EC2) white pixels, (EC3) mean gray level of pixels, (Density) density of hypoechogenic areas, (Diameter) mean diameter of hypoechogenic areas and (Area) percentage of hypoechogenic areas in the total area of the ROI. Afterwards, a semen sample was taken from the ipsilateral epididymis and analyzed by microscopy at 1000X. The percentage and type of sperm abnormalities (head, middle piece, proximal droplet and distal droplet) of each epididymal sample were determined as a way to evaluate the quality of sperm production in the ipsilateral testis. A total of 400 spermatozoa were counted per testicle, finding the corresponding percentage of each type of anomaly.

In our analysis, we established a cut-off value of 30% major sperm abnormalities (head and proximal cytoplasmic droplets) to differentiate fertile and subfertile samples. When computing total abnormal forms, we did not consider distal cytoplasmic droplet as an anomaly in the epididymal semen. The relationship between the echotexture parameters and the percentages of each type of sperm morpho-anomalies were analyzed by means of a Pearson correlation test, ANOVA, and logistic regression analysis.

Results There were no differences between ipsilateral testicles in either echotextural nor seminal quality parameters ($p>0.05$). The Pearson correlation shows a strong relationship between the percentage of major abnormalities and EC2, EC3 and density of hypogenic areas in the ultrasound image ($p <0.01$). In general, correlation coefficients were higher for major abnormalities than for total abnormal forms. On the other hand, all the echotexture parameters were also significantly related to each other. The ANOVA indicated that testicles producing subfertile samples differed significantly in 5/6 echotexture parameters from testicles producing fertile sperm samples ($p<0,01$). Logistic regression indicated that the density of hypoechogenic areas in the ultrasonogram of a testicle could predict the fertility or subfertility of an epydidimal semen sample. Sensitivity was 100% and specificity was 42.9%. No predictive equation was found to predict total abnormal sperm.

Findings from this study could be compared with results from Arteaga *et al*. (2003). They evaluated the sensitivity and specificity of mean grey level as parameters to predict total abnormal forms in the ejaculate (more or less than 30%). Their echotexture test yielded a lower sensitivity and specificity than ours (60% and 33%, respectively). The greater performance of our test may be partly due to the fact that we related the parenchymal echotexture with the parameters directly related to spermatogenesis. Tail anomalies may have causes that do not reside directly in the testicle, so they can be a source of error in this kind of study. In our experiment sperm tail anomalies were not related to any echotexture parameter.

Conclusion This study demonstrates that testicular ultrasonography may be useful in veterinary practice to investigate testicular function. The new echotexture parameters seems to be related with the spermatogenesis quality in the testicle.

Acknowledgements
This work was supported by Eureka E!11188 and IDI-20170220

References
Theriogenology. 2005, 64(2) 408-15.

Effects of sperm selection by discontinuous Percoll® gradient with reduced volume and centrifugation force on sperm oxidative status of thawed bull semen

L de Cássia Bicudo[1], A F P Siqueira[1], L S Castro[1], V S C Pinto[1], C M Mendes[1], J D Losano[1], D de Souza R. Angrimani[1], I J Fernández[2], M Nichi[1], J A Visintin[1], M E O A Assumpção[1]
[1]*University of Sao Paulo, Brazil,* [2]*Universidad de La Frontera, Temuco, Chile*
meoaa@usp.br

Application Oxidative stress can be detrimental to semen quality and bull fertility. Thus, the aim of this study was to evaluate the effect of discontinuous Percoll® gradient centrifugation on different products involved in sperm oxidative stress.

Introduction The discontinuous Percoll® gradient centrifugation is widely used to select sperm with higher quality and viability, increasing the efficiency of fertilization (Bergstein *et al*., 2016). However, the effects of this procedure on the sperm oxidative status are still unknown. This knowledge is of great importance because the oxidative stress generated by high amounts of reactive oxygen species (ROS) (i.e. mitochondrial ROS) and lipid peroxidation products (i.e. malondialdehyde) can cause injuries to sperm cells (Kawai *et al*., 2017). ROS are important to several physiological events; as an example, superoxide anion was described to be involved in the process of sperm capacitation (O'Flaherty *et al*., 2003).

Material and methods Frozen semen from 15 bulls (n=15) from commercial reproduction Centers was used. Two straws from the same batch from each bull were thawed, homogenized and evaluated at two different times - after thawing (Pre-Percoll®) and after discontinuous Percoll® gradient centrifugation (Post-Percoll®). For this, 250 µL of semen were deposited on a Percoll® density gradient of 45%-90% (v/v) with final volume of 400 µL, centrifuged (6600x*g*/5 minutes), and washed with 1mL of Fert-TALP (1100x*g*/3 minutes). The final pellet of 100 µL was recovered and resuspended to a final concentration of 25×10^6 spermatozoa/mL in Fert-TALP, in three replicates. Three different fluorescent probes (DHE for intracellular superoxide anion, MitoSOX Red for the mitochondria-specific ROS and CellROX green in association with propidium iodide for total nonspecific ROS and sperm viability) were used to detect oxidative status in flow cytometry (Guava EasyCyteTM Mini System), analyzing 20,000 events per sample. Malondialdehyde, a lipid peroxidation product, was measured using the induced TBARS (Thiobarbituric Acid Reactive Substances) assay in a spectrophotometer. The difference between times were analyzed using the Student t or Wilcoxon tests ($p\leq0.05$).

Results No differences between Pre-Percoll® and Post-Percoll® were found in malondialdehyde concentration and mitochondria-specific ROS production. However, the Percoll® centrifugation induced the production of total nonspecific ROS (CellRox) and intracellular superoxide anion (DHE - Figure 1).

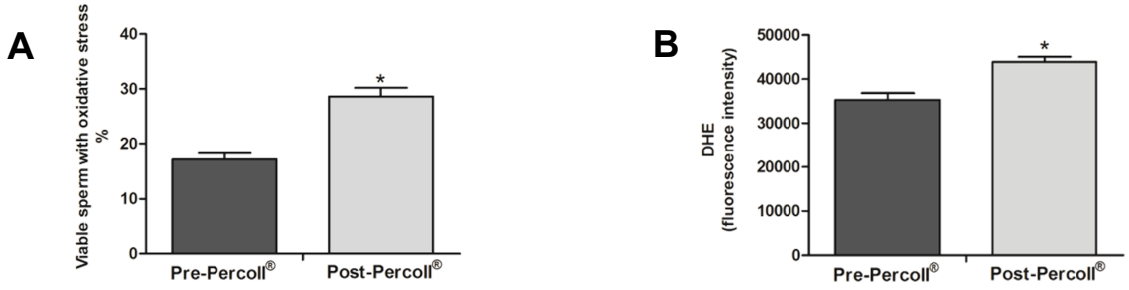


Figure 1- Effect of Percoll® (Pre and Post) on detection of total nonspecific ROS in viable sperm evaluated by CellRox green/PI (A) and intracellular superoxide anion evaluated by DHE (B) in thawed bull semen.
* indicate significant differences between groups ($p<0.05$).

Conclusion Discontinuous Percoll® gradient (45%-90%) centrifugation (6600x*g*/5 minutes) does not promote increase of harmful oxidative products (malondialdehyde and mitochondrial ROS); however, an increase of total nonspecific ROS by superoxide anion production detected by DHE was observed, suggesting that this may be a mechanism by which Percoll® centrifugation increases the number of capacitated sperm.

Acknowledgements The authors acknowledge CAPES for granting the doctoral scholarship.

References

Bergstein, T., Bicudo, L., Rodello, L., Weiss, R., and Bicudo, S. 2016. Arquivo Brasileiro de Medicina Veterinária e Zootecnia. 68(6), 1539-1547.
Kawai, G.K.V., Gurgel, J.R.C., de Agostini Losano, J.D., Dalmazzo, A., Rocha, C.C *et al*., 2017. Journal of Equine Veterinary Science. 55, 76-83.
O'Flaherty, C, Beorlegui, N, Beconi, M. 2003. International Journal of Andrology. 26, 109-114.

https://doi.org/10.1017/S2040470018000109 Published online by Cambridge University Press

Non-infectious progressive proximal post-testicular obstruction (PPPTO) in two half-sibling AI bulls - work in progress

S Björkman[1], T Iso-Touru[2], O Peltoniemi[1], M Andersson[1]
[1]*Helsinki University, Saarentaus, Finland,* [2]*Natural Resources Institute Finland, Helsiki, Finland*
stefan.bjorkman@helsinki.fi

Application Non-infectious PPPTO is not rare in Ayrshire bulls. This disorder is probably caused by different genetic reasons and therefore involves several phenocopies (Williams *et al.*, 2010). Thus, the identification of genetic reasons for this disorder is difficult. We recently identified two halfsiblings (initially intended for use in AI) with non-infectious PPPTO. Both bulls showed progressive oligozoospermia with an elevated proportion of abnormal spermatozoa in the ejaculate. Post-mortem examination revealed several sperm granulomata at the head of the epididymis and extravasation of spermatozoa under the visceral vaginal tunic dorsal to the caput epididymis and spermatic cord. We intend to find some possible genetic reason for this syndrome in these two bulls. The inheritance of this type of disorder in cattle is not well defined (König *et al.*, 1972)

Introduction A fertile bull must ejaculate sperm that contains a high concentration of motile and morphologically normal spermatozoa. Any major and long-lasting reduction in sperm characteristics will eventually lead to culling of AI bulls. One major cause of culling is reduction of sperm concentration (oligozoospermia) and can be caused by, for instance, non-infectious PPPTO. The outcome of PPPTO varies. Usually the post-testicular tract is underdeveloped or absent and bulls may develop testicles increased in size. As a result, sperm granulomata or fluid-filled cavities at the caput epididymis can be found. Some bulls may also have extravasation of spermatozoa under the visceral vaginal tunic. These spermatozoa are trapped and will degenerate.

Material and methods Two Ayrshire AI bulls with poor sperm quality were assessed. Bull A was culled at 15 months of age and always had poor sperm quality. Bull B was culled at 18 months of age and had satisfactory sperm production at the beginning of his reproductive career. Both bulls were examined because of oligozoospermia and morphologically abnormal spermatozoa. After changes in the ejaculate were observed, the bulls underwent palpation and ultrasound examination. After that, the bulls were culled and a post-mortem examination was performed. The pedigrees of the two bulls were studied as well as testicular tissue collected for genotyping with Illumina Bovine 50K SNP BEADSHIP. ROH analysis is currently underway for homozygosity testing of these two bulls and GWAS will be done in future of the two bulls and 100 control bulls.

Results The microscopic examination of the ejaculate revealed a high percentage of degenerated sperm such as detached heads and tails. The sperm concentration in the ejaculate was low. On palpation, one of the testicles was enlarged and edematous. Real time ultrasound examination showed increased and hypoechoic areas in the region of the rete testis. Postmortem examination showed several sperm granulomata in the region of the epididymal head in one bull and extravasation of spermatozoa under the visceral vaginal tunic in the caput epididymis and in the spermatic cord in the other bull. The rete testis were dilated. The pedigree of the two bulls showed that they had the same sire. All other genetic work (SNP, ROH, and GWAS) is currently under way.

Conclusion Non-infectious PPPTO can be caused by the following reasons: 1) Firstly, blind ending efferent ducts that do not join with the epididymal duct. This would cause fluid-filled areas of the duct that might compress other efferent ducts joining with the epididymal duct. 2) Secondly, too low reabsorption of fluid in the ductuli efferentes and proximal epididymis. 3) Finally, too effective reabsorption of fluid in rete testis may cause too high density of spermatozoa leading to obstruction in some of the efferent ducts and caput epididymis. The fact that this condition was observed in two half-siblings gives the opportunity to identify a genetic cause for a specific phenotype of PPPTO. The genetic work for these cases is currently under way.

Acknowledgements The authors would like to thank Mari Niemi, Heli Nordgren and Henri Simonen for identifying these bulls at the AI-station.

References

König, H., Weber, W., Kupferschmied, H. 1972. Schweizer Archiv fur Tierheilkunde, 114(1), 73-82.
Williams, H. J., Revell, S. G., Scholes, S. F. E., Courtenay, A. E., Smith, R. F. 2010. Reproduction in domestic animals, 45(5).

The effect of natural heat stress on bull semen quality and subsequent embryo development

N Llamas Luceño[1], D Angrimani[2], L Bicudo[2], K Demeyere[1], B Leemans[1], E Meyer[1], A Van Soom[1]
[1]*Ghent University, Belgium,* [2]*University of São Paulo, Brazil*
nuria.llamasluceno@ugent.be

Application At present, breeding companies are having concerns about the possible impact of natural heat stress on animal fertility. The goal of our project is to address those concerns and to determine the effects of increased temperature on bull fertility.

Introduction In previous work, we induced artificial heat stress for 48 hours in Holstein and Belgian Blue bulls by scrotal insulation and found that 14 to 42 days after the insult, the negative impact on frozen semen quality was most obvious as shown by lower sperm motility and protamination. Moreover, changes in the methylation of paternal pronuclei in resulting zygotes was observed (Rahman *et al*., 2011). Here, we investigated the effects of natural heat stress in Holstein bulls. Frozen bovine semen samples that were obtained from 6 bulls exposed on three consecutive days to natural heat stress (HS) (August 2016, in a range of 13 to 32ºC), and to lower temperature (control) (March 2016, in a range of -4 to 11ºC) were examined. The effect of heat stress on bull semen quality was assessed by sperm motility and viability, quantifying reactive oxygen species (ROS), lipid peroxidation (LPO) and DNA breaks. Moreover, we evaluated the development of embryos generated by heat-stressed semen.

Material and methods In order to assess the effect of heat stress on viable spermatozoa, frozen/thawed sperm were passed through a discontinuous Percoll gradient (45/90% (v/v)) and adjusted to a final concentration of $2.5x10^6$ cells/ml in PBS for semen quality analysis or $1x10^6$ cells/ml in IVF TALP medium for fertilization. Sperm motility was evaluated using computer-assisted sperm analysis (CASA). For ROS evaluation, 100 µM of 2′,7′-dichlorofluorescein diacetate (DCFH-DA) or 5µM CellROX Green Reagent was added to the sperm suspensions and incubated at 37ºC for 30 minutes. Next, 1.5 µM of propidium iodide (PI) was added to assess membrane integrity. All samples were analysed using a Cytoflex flow cytometer. For LPO assessment, 10 µM of BODIPY 581/591 C11 was added to the sperm samples and incubated at 37ºC for 15 minutes before flow cytometry analysis. Overall 10,000 spermatozoa per sample were screened. TUNEL assay was performed using In Situ Cell Death Detection Kit to evaluate breaks in the sperm DNA. Bovine blastocysts were produced by routine *in vitro* methods (Wydooghe *et al*., 2014). Cleavage rates were determined at 48 h post-insemination and blastocyst rates at 7 and 8 days post insemination. Data were analysed using general linear model (GLM) procedure or the Student's t-test ($p \leq 0.05$) and Spearman correlation. The dataset was checked for normal distribution by UNIVARIATE procedure and Shapiro-Wilk test. LSD test was applied to determine significant differences.

Results No significant differences between HS semen and the control group were found for total and progressive motility (CASA), ROS production (DCFH-DA and CellROX), lipid peroxidation (BODIPY), cell death (PI) and DNA breaks (TUNEL). However, a significant decrease was observed in cleavage and total blastocyst rates at day 7 and 8 in the HS group. Moreover, hatching was delayed in the HS group since only the control group showed hatched blastocysts at day 7. We grouped early and normal blastocysts in 'early stage' blastocysts, and expanded, hatching and hatched blastocysts in 'advanced' stage blastocysts. When comparing HS and control periods, we found a significant reduction of advanced blastocysts at day 7 in the HS group. Furthermore, we observed an overall decrease of blastocyst rates produced from HS semen.

Conclusion Quality parameters of Percoll-purified sperm did not differ significantly between HS semen and the control group. The decrease in blastocyst rates and the delayed hatching observed in embryos produced with HS semen indicates that molecular mechanisms for advanced blastocyst development were affected. Next, we will evaluate differentially expressed genes in blastocysts (including imprinted, pluripotency and DNMT genes), which are generated by semen from HS and control period.

Acknowledgements This project is funded by "European Union, Horizon 2020 Marie Sklodowska-Curie Action, REPBIOTECH 675526".

References Rahman, M.B., Vandaele, L., Rijsselaere, T., Maes, D., Hoogewijs, M *et al*., 2011. Theriogenology. 76, 1246–1257.
Wydooghe, E., Heras, S., Dewulf, J., Piepers, S., Van den Abbeel, E *et al*., 2014. Reproduction, Fertility and Development. 26, 717-24.

316

Droplet digital PCR provides robust, reproducible quantitation of sex-skewed bovine semen

N Cray, M Wagner, J Hauer, E Roti Roti
GenusPLC, Windsor, WI, USA
elon.rotiroti@genusplc.com

Application A validated ddPCR assay quantifies sex skew in bovine semen with 0.5% standard deviation for % X chromosome sperm cells.

Introduction Quantifying the relative population of sperm cells bearing the X or Y chromosome in a sexed semen sample has historically been limited to methods that are either low throughput and subjective (like fluorescent *in situ* hybridization), or relatively insensitive (like qPCR with a change detection threshold of 2X). Customers pay a premium for sexed semen, and should have access to reliable quality control data, which include an accurate, precise test for sex skew that is orthogonal to the method used to generate sexed semen. Droplet digital PCR (ddPCR) has the capacity to provide an accurate and precise sex skew measurement by subdividing a pool of template DNA into nanoliter scale droplets containing either one or zero copies of template DNA. PCR amplification occurs in these droplets, and the number of copies of the amplicon of interest can be counted as the number of fluorescence-positive droplets[1]. We have optimized and validated a multiplexed ddPCR assay that uses this copy counting method to quantify the sex skew (ratio of X or Y chromosomes) in bovine semen.

Material and methods Primer and probe combinations for X, Y, and autosome-associated loci were screened using PCR and qPCR for specificity and reproducibility across *bos taurus* breeds. Specific, robust primers/probes were then optimized to develop a multiplex ddPCR reaction to reproducibly maximize separation between populations in reactions probing X, Y, and the autosomal control in each droplet using a commercial ddPCR system. Droplet fluorescent intensity data were analysed using a custom analysis pipeline that stringently assessed sample size and variance for each reaction to ensure high confidence measurements and produces unbiased sex skew results.

Results Standard PCR identified primer combinations that reproducibly amplified product of the predicted molecular weight for genomic DNA template isolated from Holstein, Jersey, and Angus, n = 16. Candidate primer/probe combinations were then required to exhibit variance in the qPCR cycle threshold (CT) less than 0.5 CTs across DNA templates isolated from 8 different sires prior to testing in the ddPCR platform.

The multiplexed ddPCR Sex Skew Assay was optimized for maximal separation between droplet populations positive for X and Y chromosomes and the autosome-linked housekeeping gene, respectively, as demonstrated by repeat measure of semen samples resulting in sex-skew percentages (%X) with standard deviations of ± 0.5%. To ensure assay stringency, we confirmed the Sex Skew Assay interrogated a minimum of 2000 cells/assay, and the measured copies of the autosome-linked control equalled the total copies of X+Y chromosomes within assay variance. Dose-response curves varying the X and Y chromosome input demonstrated linear assay sensitivity from 8-93% X chromosome (slope = 0.99, R^2 = 0.998).

A custom algorithm eliminated potential user bias in data interpretation by analysing the scatter plot of droplet fluorescence data, ultimately quantifying % X chromosome cells in the sample population, and identifying a sample as pass or fail based upon quality control requirements. The algorithm flags individual assays for repeat if the measurements fall outside acceptable ranges, including minimum total copy number per reaction, maximum variance for % X in replicate reactions, and maximum deviation between total housekeeping gene copies and total X+Y copies.

Conclusion The robust, precise ddPCR Sex Skew Assay provided a broad linear dynamic range suitable for quantifying the relative sex skew in bovine semen enriched for either the X or Y chromosome. Stringent, automated quantification provides unbiased quantification, ensuring accurate quantification of sexed semen.

Acknowledgements Thank you to Matt Campbell and his group for technical input and lab resources.

References

Hindson C M, Chevillet J R, Briggs H A, Gallichotte E N, Ruf I K, Hindson B J, Vessella R L, and Tewari M 2013. Nature Methods. 10, 1003-1005.

https://doi.org/10.1017/S2040470018000109 Published online by Cambridge University Press

Polyunsaturated fatty acids influence offspring sex ratio in cows

W F A Marei[2,1], W A Khalil[4], A P G Pushpakumara[1,2], M A El-Harairy[4], A M A Abo El-Atta[4], D C Wathes[1], A A Fouladi-Nashta[1]

[1]*Royal Veterinary College, Hatfield, UK,* [2]*Cairo University, Faculty of Veterinary Medicine, Giza, Egypt,* [3]*University of Peradeniya, Sri Lanka,* [4]*Mansoura University, Department of Animal Production, Faculty of Agriculture, Mansoura, Egypt*
afouladi@rvc.ac.uk

Application Sex ratio is a key factor in cattle breeding. Semen sexing technologies can achieve high success rates in producing the desired fetal sex in dairy and beef cow industries. However, the technology is costly, and the pregnancy rate from artificial insemination using sexed sperm is significantly lower than conventional semen. Therefore, other potential methods of altering the sex ratio are of significant interest. Despite the widespread use of various feeds which alter dietary PUFA concentrations and ratios, the effects of PUFAs on the sex of the offspring in cattle have not been investigated to date.

Introduction Dietary polyunsaturated fatty acids can influence fertility in farm animals. Some evidence in mice (Fountain *et al* 2008) and sheep (Green *et al* 2008) have suggested that PUFAs may influence offspring sex ratio, which may have significant value for cattle production.

Material and methods ***Experiment 1*** Sixty Holstein cows were stratified and randomly divided into three groups according to BW and parity, and individually fed according to the nutrient requirements recommended by NRC. The diets were supplemented with either 0%, 3% or 5% protected fat (PF) in the form of calcium salt of fatty acids (rich in omega-6) from 14-21 days pre-partum until conception. Proven-fertile frozen semen from the same ejaculate was used for insemination. The sex of the offspring was recorded at birth.

Experiment 2 To test if the effect of diet on fetus sex ratio was caused by a direct influence on the oocyte, bovine cumulus oocyte complexes collected from abattoir-derived ovaries, were supplemented during *in vitro* maturation with either omega-3 alpha-linolenic acid (ALA), omega-6 linoleic acid (LA) or trans-10, cis-12 conjugated linoleic acid (CLA). Sex ratio of the produced transferable embryos was determined using PCR of SRY gene.

Results Calf sex recorded at birth was 8/19 (42.1%) male offspring in the control group, increasing to 14/20 (70%, $P>0.05$) and 17/20 (85%, $P<0.05$) in 3% and 5% PF, respectively.
Similar to the *in vivo* results, sex ratio was skewed to the male side ($P<0.05$) in the embryos derived from LA- and CLA-treated oocytes (79% and 71%) compared to control and ALA-treated oocytes (44% and 54%, respectively).

Conclusion These results indicate that both dietary and in vitro supplementation of omega-6 PUFAs can skew the sex ratio towards the male side in cattle. Further experiments are required to confirm this effect on a larger scale and to study the mechanisms of action that might be involved.

Acknowledgements The authors gratefully acknowledge funding from RVC, The Commonwealth Commission and Cairo University.

References

Green, M.P., Spate, L.D., Parks, T.E., Kimura, K., Murphy, C.N., Williams, J.E., Kerley, M.S., Green, J.A., Keisler, D.H., and Roberts, R.M. 2008. Reproductive Biology Endocrinology. 6, 21.
Fountain, E.D., Mao, J., Whyte, J.J., Mueller, K.E., Ellersieck, M.R., Will, M.J., Roberts, R.M., Macdonald, R., and Rosenfeld, C.S. 2008. Biology of Reproduction. 78, 211-217.

Novel portable quantitative assessment of semen motility

V Martinez[1], A Jepson[1], J Statham[2], M Spilman[2], K Burton[2], J Arlt[1], T Wood[1], W Poon[1]
[1]*School of Physics & Astronomy, The University of Edinburgh, UK,* [2]*RAFT Solutions Ltd., Ripon, UK*
vincent.martinez@ed.ac.uk

Application While bull semen motility is crucial to high fertilisation rates and thus to profitable cattle livestock production, subjective visual assessment of motility is widely used on-farm. We have developed a portable device for objective, quantitative characterisation of semen motility at bull-side on-farm.

Introduction Characterisation of semen motility is important for both semen research and animal reproduction industries. There are two commonly used methods of measuring bull semen motility: visual assessment by a trained technician or computer-assisted sperm analysis (CASA). On-farm, visual assessments are used, found to have a variation of 20-40% [1]. In-lab, CASA systems are often used if available. Based on high-resolution optical microscopy movies combined with specialized particle tracking software, CASA returns detailed information on kinematic parameters. However, CASA systems are composed of expensive bulky hardware with very limited portability. Additionally, they are restricted to low cell concentration and thus dilution of raw sample is typically required, which affects its motility. We have developed a novel portable device that returns key motility parameters of bull semen. Here, we demonstrate its use on both fresh and thawed samples, performed on-farm and in-lab, respectively.

Material and methods Fresh semen samples were measured during a field study conducted in the south east of Scotland on bulls undergoing routine bull breeding soundness examinations, approved by the Royal (Dick) School of Veterinary Studies Veterinary Ethical Review Committee. Frozen bovine semen, produced for standard artificial insemination, from Belgian Blue bulls (purchased from Cogent Breeding Ltd.), pooled Holstein bulls and Charolais bulls, diluted with extenders to $(80 \pm 10) \times 10^6$ cell/ml) were supplied by RAFT Solutions Ltd. Straws were removed from liquid nitrogen storage using forceps and thawed in a 37 °C water bath for 30 seconds. Samples were loaded into 20 μm deep glass capillaries and then sealed with Vaseline and placed in our device (a purpose-built microscope). Low-magnification movies were recorded, using a camera at high framerate, at the centre of the capillary. The time-lapsed images were then analysed using bespoke image-processing algorithm to quantify the statistics of spatial-temporal fluctuations of the cell density, from which key motility parameters were extracted.

Results We demonstrate that our device gives a fast, high-throughput method to characterise bull semen motility. We show our device can measure mean values of the swimming speed, head oscillation amplitude, head oscillation frequency, and motile fraction, averaged over approximately 10^3 cells. We validate the extracted motility parameters using particle tracking on a dilute thawed sample (see Fig 1a&b for an example). High-statistics, high time-resolution monitoring of motility using our device shows rapid changes in swimming speed in a sealed capillary at 37 °C due to oxygen depletion (fig 1c&d). The results could be useful in metabolism characterisation as well as for thermal incubation tests. Finally, we confirm that diluting semen to low densities gives rise to higher swimming speeds, caused by a reduction in viscosity (see poster).

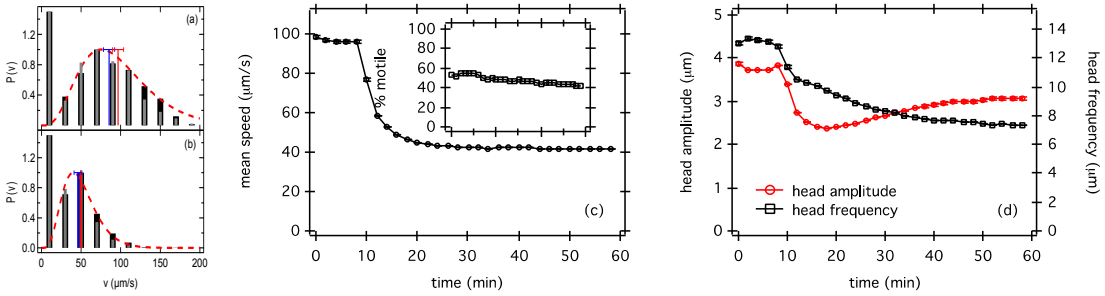


Figure 1 (a&b) A comparison of tracking (histograms) and DDM (dotted red lines with red bar the mean speed) methods for measuring the swimming speed for a sample heated for (a) 15 min and (b) 50 min. (c&d) Time-dependency of motility parameters measured with DDM.

Conclusion Our device allows measurements of key motility parameters of bull semen on-farm, providing potentially more accurate semen characterisation prior to natural service or artificial insemination. For research purposes, our method provides high-time-resolution, unrestricted by sample concentration, which can be used to assess the motility of microorganisms in a variety of environmental conditions and at every stage of sample manipulation.

Acknowledgements We acknowledge funding from EPSRC (PII017) and ERC Proof of Concept (PorCSperm).

References

Davis, R.O., Katz, D.F. 1993. Operational standards for CASA instruments. Journal of Andrology, 14:385–94.

https://doi.org/10.1017/S2040470018000109 Published online by Cambridge University Press

Interest in the study of the echotexture of the testicular parenchyma to evaluate the maturity of young bulls

G Gnemmi[2], C Maraboli[2], N Escartín[1], I Muñoz[1], S Marcantonio[3], A Echegaray[2]
[1]*Humeco, Humeco, Spain,* [2]*Bovinevet, Lombardia, Italy,* [3]*Buenos Aires University, Argentina*
araechegaray@humeco.net

Application These algorithms will be useful in prediction of fertility of young males before entering into a breeding schema and also to evaluate testicle health in valuable sires.

Introduction Several authors reported changes in echogenicity of the testis ultrasonograms during the development of the gonads in young bulls. The echogenicity of the testes increases between 8 and 46 wk of age, with an inflection at 30 wk of age (around puberty) until 70 weeks of age (around maturity)[1,2,3]. Mean grey level of pixels in the ultrasonogram is a good predictor of puberty and maturity in young bulls, but it is less efficient than the measure of the scrotal circumference[3]. In this context, we have developed several algorithms to try to quantify the echotexture of the testicles, allowing for a better predictive value of ultrasonography. These algorithms analyze ultrasound images, according to the distribution of black, white and grey pixels and also according to the size and density of hypoechogenic areas. The aim of this work is to investigate the relationship between these ultrasound characteristics and maturity in young bulls.

Material and methods
Ultrasound scans of the testicular area (n =45) were performed in a total of 33 Holstein bulls aged between 11 and 32 months and housed in artificial insemination centres. Ultrasonograms were carried out using an EXAGO scanner (ECM, France) connected to a 5-7.5 MHz linear probe. Three transverse sonographic images of each testicle were taken. The echographic images were analysed by the algorithms, selecting a region of interest (ROI) and obtaining the average of the 3 images for the following parameters: (EC1) black pixels, (EC2) white pixels, (EC3) mean gray level of pixels, (Density) density of hypoechogenic areas, (Diameter) mean diameter of hypoechogenic areas and (Area) percentage of hypoechogenic areas in the total area of the ROI. Afterwards, 2 ejaculates were collected per bull and ejaculate volume, sperm concentration and sperm motility were recorded. A sample of spermatozoa was stained by eosin-nigrosin and analysed by microscopy at 1000X to investigate sperm morphoanomalies. The percentage and type of sperm abnormalities (head, middle piece, proximal droplet and distal droplet) of each sample were determined. A total of 200 spermatozoa were counted per sample. We established a cut-off value of maximum 15% major sperm abnormalities in the ejaculate (sperm head anomalies, intermediate piece formation anomalies, and proximal cytoplasmic droplets) as a picture of a mature semen sample.The relationship between the echotexture parameters and semen quality was analysed, by means of a Pearson correlation test, ANOVA, and logistic regression analysis.

Results There were no differences between ipsilateral testicles in echotextural parameters ($p>0.05$). The Pearson correlation showed a significant relationship between the age of the bull and the density of hypoechogenic areas in the ultrasonogram ($R=0,42$ $p<0.01$). Also, this parameter was highly positively correlated with the total number of motile ($R=0,65$) and total number of normal sperm ($R:0,63$) in the ejaculates ($p<0.01$) but negatively correlated with the percentage of major abnormalities in the ejaculates ($R:0,34$ $p<0.05$). However, the age of the bull was not related to the percentage of sperm morphoanomalies. It is possible that factors other than the age of the bull have been implicated in the percentage of sperm morphoanomalies of some ejaculates.
Bulls producing immature ejaculates had significant differences in the mean density of hypoechogenic areas of the testicular ultrasonograms ($p<0,05$). Logistic regression, including the density of hypoechogenic areas in the ultrasonogram of paired testicles, could predict the maturity of a young bull. Sensitivity was 97.1% and specificity was 63.6%. Brito *et al*. (2012)[3] reported lower values regarding the predictive capacity for the maturity of the echogenicity of the testis ultrasonogram. However, direct comparison with their data is not possible, because their criteria for ejaculate maturity of bulls were different to ours.

Conclusion This study suggests that a more profound analysis of testicular ultrasonograms could provide useful parameters to investigate testicular maturity in young bulls, but it would be necessary to collect and analyze more data to confirm these results.

Acknowledgements
This work was supported by Eureka E!11188 and IDI-20170220

References
Evans A C ., Pierson R A, Garcia A, McDougall L M, Hrudka F, Rawlings N C 1996. Theriogenology 46, 345-357
Chandolia RK, Honaramooz A, Omeke B C, Pierson R, Beard AP, Rawlings N C 1998. Theriogenology 48 119-32
Brito L F, Barth A D, Wilde R E, Kastelic J P 2012. Theriogenology, 78(1) 69-76.

Advances in the proteome of electroejaculated seminal plasma from tropical-adapted bulls

N Satake[1], H Skovsgaard Pedersen[2], M McGowan[1], G Brandt Boe-Hansen[1]
[1]*The University of Queensland, School of Veterinary Science, Gatton, Queensland, Australia,* [2]*Aarhus University, Department of Animal Science, Tjele, Denmark*
g.boehansen@uq.edu.au

Application A deeper understanding of the seminal plasma proteome of bulls is needed in order to optimize protocols for extension and storage of bull semen, to enable full use of assisted reproductive technologies to accelerate rate of genetic gain in beef herds.

Introduction The potential benefits of widespread dissemination of superior genetics into beef herds is well recognised in intensive management systems. However, in extensive systems such as those operating in northern Australia the cost of frozen semen is significant and pregnancy rates after AI are variable. Furthermore, specific semen preparation protocols for electroejaculated (EEJ) semen have not been developed. This is despite documented differences in composition of seminal plasma between semen collected by EEJ compared to that collected using an artificial vagina (Rego *et al.*, 2015). This study assessed the seminal plasma proteome of semen collected by EEJ using a powerful modern mass spectrometric approach and next-generation proteomics.

Material and Methods Droughtmaster bulls (23-26 mo; n=6) underwent semen collection by EEJ. The bulls were selected based on a previous normal bull breeding soundness including normal spermiogram. Raw semen samples were centrifuged at >20,000*g* twice to remove spermatozoa and solid materials. Ice-cold acetone was added to seminal plasma samples at 4:1 ratio in order to precipitate proteins. A filter-aided sample preparation method using trypsin was used (Wisniewski *et al.*, 2009). Samples were then desalted, dried and re-suspended in 0.1% formic acid/2% acetonitrile solution and profiles were assessed using an advanced shot-gun proteomics approach using 95 min data dependent acquisition method on a discovery platform with nano-LC/MS chromatographic sample input with peptides analysed by ABSciex™ 5600 Triple TOF (AB Sciex Pte. Ltd, MA, USA) MS. Protein identification were mined using up-to-date transcriptome available and Protein Pilot™ software (ver4.5; AB Sciex Pte. Ltd, MA, USA).

Results Across the six samples, a total of 417 proteins were identified, with verification of 43 of the 46 proteins previously identified in a similar population (Rego *et al.*, 2014). Using online software and associated public databases, 186 proteins were annotated and associated with processes, components and function (Table 1). An *in-silico* protein interaction analysis was conducted, with six distinctive groups identified presenting interactions between in redox reactions; proteasomes for ATP-dependent proteolytic activity; binding/enzymatic/regulatory; molecular chaperones and metabolic.

Table 1. Proportions of proteins according to biological processes, cellular component and metabolic function.

Biological processes		Cellular component		Metabolic function	
Cellular	25%	Extracellular	37%	Binding	49%
Regulation	24%	Intracellular organelles	27%	Catalytic	35%
Metabolic	10%	Cytoplasm	7%	Molecular transduction	2%
Response to stimulus	9%	Plasma membrane	6%	Antioxidant	2%
Interactions	9%	Macromolecular complex	5%	Other	12%
Localisation	9%	Cell surface	4%		
Immune system	6%	Other	14%		
Developmental	4%				
Reproduction	1%				
Other	3%				

Conclusion This new methodology has enabled greater definition of the seminal plasma proteome of bulls, identifying more proteins than previously. The proteins identified were predominately of accessory sex glands origin and the interaction analysis showed expected regulatory clusters, and metabolic function similar to previously reported (Rego *et al* 2014). Currently, samples are undergoing data independent sequential window acquisition of all theoretical mass spectra (*SWATH*-MS), to quantitate relative abundances of all identified proteins. This methodology will allow for further investigation into how the proteome is altered in disease and health, and for optimization of semen extenders for dilution and preservation.

Acknowledgements We acknowledge funding from Endeavour Fellowship for HSP.

References

Rego, J.P.A, Crisp, J.M., Moura, A.A., Nouwens, A.S., Li, Y., Venus, B., Corbet, N.J., Corbet, D.H., Burns, B.M., Boe-Hansen, G.B., McGowan, M.R. 2014. Animal Reproduction Science. 148, 1-17.
Rego, J.P.A., Moura, A.A., Nouwens, A.S., McGowan, M.R., Boe-Hansen, G.B. 2015. Animal Reproduction Science. 160, 126-137.
Wisniewski, J.R., Zougman, A., Nagaraj, N., Mann, M. 2009. Nature Methods. 6, 359-362.

https://doi.org/10.1017/S2040470018000109 Published online by Cambridge University Press

Effect of season of collection in cryopreserved semen from the Spanish native cattle breed "Asturiana de la Montaña"

C Hidalgo[1], J Néstor Caamaño[1], C Fueyo[1], C Tamargo[1], A Salman[2], Á Fernández[1], M J Merino[1], M Carbajo[3], F Martínez-Pastor[2]
[1]*Department of Animal Selection and Reproduction, Regional Agri-Food Research and Development Service of Asturias (SERIDA), Gijón, Asturias, Spain,* [2]*INDEGSAL and Molecular Biology, University of León, Castilla y León, Spain,* [3]*Facultad de Veterinaria, University of León, Castilla y León, Spain*
jncaamano@serida.org

Application Semen cryopreservation is a key technique for the conservation of valuable or endangered breeds. This study is part of an overall evaluation of the semen bank from native cattle breed Asturiana de la Montaña (Asturias, Spain), in order to assess its viability, estimate the future usefulness of the stored doses and identify factors of variation on sperm quality.

Introduction Germplasm banks are a safeguard for preserving valuable genetics or for preserving endangered breeds. These indigenous breeds convey cultural and ethnic values, and their genetic traits could become valuable in the future, because of adaptation to their specific environments and to their rusticity and endurance. Standard cryopreservation protocols may not be entirely suitable for these breeds. Moreover, because of their rusticity and sometimes lower selection, they are more sensitive to seasonality. Seasonality could affect the quality and freezability of their semen and this should be taken into account in order to properly manage germplasm banking. The aim of this study was to assess the overall quality of the Asturiana de la Montaña sperm bank by computer-assisted sperm analysis (CASA) and flow cytometry and to estimate effects of the season of collection in the post-thawed sperm quality.

Material and methods We used semen doses from 34 Asturiana de la Montaña bulls stored in the SERIDA cryobank and collected in different seasons (spring-summer-autumn-winter, 8 bulls were sampled in the 4 seasons, 16 in 3 and 10 in 2). Three semen doses per bull were thawed in a 37 °C water bath for 30 s, pooled and assessed after 10 min and after incubating them at 37 °C for 5 h. Motility was recorded and analysed in a CASA system (ISAS 1.2, Proiser, Valencia, Spain) and sperm viability, acrosomal status and mitochondrial activity were assessed by flow cytometry (CyAn, Beckman Coulter, Brea, CA) after staining with propidium iodide, PNA-FITC (peanut agglutinin) and Mitotracker deep red, respectively, with Hoechst 33342 to discard debris (Martínez-Pastor *et al*., 2010). Data were analysed with the R statistical environment v.3. The effects of season and incubation on sperm post-thawing quality were determined by using linear mixed-effects models.

Results Season affected sperm viability, acrosomal status or mitochondrial activity, both total and ratio in viable spermatozoa, while effects on CASA parameters were not significant (Table 1). Incubation of the samples at 37 °C for 5 h caused a general decrease in sperm quality, but there was no interaction with season. A Tukey test on season as main effect showed significant differences between autumn and spring for viability, acrosomal status, mitochondrial activity and its ratio, and also between summer and spring for the mitochondrial activity ratio.

Table 1 Effect of season and incubation time on sperm quality (all values in % of positive cells, mean±SEM).

Season	Spring		Summer		Autumn		Winter		Significance[1]	
Incubation	0	5	0	5	0	5	0	5	Season	Incubation
MOT[2]	40.7±1.5	20.8±1.7	40.2±2.1	23.1±2.2	43.7±2.3	19.9±2.0	40.7±1.8	20.2±2.0	NS	***
PROG[3]	29.6±1.1	17.2±1.5	30.7±1.9	18.3±1.8	33.2±2.0	15.9±1.7	29.7±1.3	16.4±1.7	NS	***
Viability	50.7±1.2	39.8±1.2	52.1±1.7	40.9±1.6	55.5±1.4	44.1±1.3	53.4±1.3	43.1±1.5	*	***
ACR[4]	21.1±0.9	51.8±1.3	21.3±1.2	51.4±1.7	20.7±1.4	47.1±1.1	20.1±0.8	49.3±1.5	*	***
MITO[5]	43.0±1.5	34.2±1.2	45.0±1.7	37.6±1.6	47.7±1.4	40.1±1.4	44.9±1.5	39.3±1.4	**	***
MITOr[6]	83.6±1.9	84.3±1.9	86.4±1.8	90.3±1.2	85.7±1.8	89.6±1.2	83.1±1.9	89.3±1.2	*	***

[1] * P<0.05, ** P<0.01, *** P<0.001; [2]MOT: Total motility; [3]PROG: Progressive motility; [4]ACR: Damaged acrosomes; [5]MITO: Active mitochondria; [6]MITOr: Proportion of MITO within viable spermatozoa.

Conclusion Season of collection affected the post-thawing quality of Asturiana de la Montaña bulls when assessed by flow cytometry. Semen collected during spring could present a lower freezability, especially compared with autumn, which coincide with the peak of the non-breeding and breeding seasons in ruminants. The bull effect was high for most variables, showing the strong individual effect in this autochthonous breed.

Acknowledgements This study was supported by INIA (Project RZP2013-00006-00-00). We acknowledge the support of Asociación Española de Criadores de Ganado Vacuno Selecto de la Raza Asturiana de la Montaña (ASEAMO).
References Martínez-Pastor F., Mata-Campuzano M., M Álvarez-Rodríguez M.A., Álvarez M., Anel L. and de Paz P. 2010. Reproduction in Domestic Animals. 45 (suppl. 2), 67-78.

https://doi.org/10.1017/S2040470018000109 Published online by Cambridge University Press

Artificial insemination using two different AI-sheaths for training of AI in the cow in veterinary medicine

J Parlevliet
Utrecht University, Faculty of Veterinary Medicine, Department of Farm Animal Health, Utrecht, The Netherlands
j.m.parlevliet@uu.nl

Application No differences were found in the use of two different AI-sheaths to train veterinary students in artificial insemination of cows.

Introduction In the Netherlands artificial insemination (AI) in cattle is performed by licenced lay people (trained technicians) which differs from other countries such as Belgium where only veterinarians inseminate the cows. Since herd size increased, farmers have been trained to inseminate their own cows. This training is carried out on cows at the slaughterhouse or on slaughterhouse material. Nowadays the percentage of farmers who inseminate their own cows on farm has increased tremendously. Therefore, the supervising veterinarian of the farm needs to know more about appropriate heat detection to estimate the right moment of AI, frozen semen storage (liquid nitrogen), semen handling and thawing and artificial insemination. For this reason, second year master veterinary students are trained in AI-procedures on the teaching herd of the bovine clinic of the faculty of Veterinary Medicine of Utrecht University (this training was approved by the animal welfare committee of Utrecht university). We compared to different protective AI-sheaths for training of non-surgical AI to see if there was a difference in usability of the devices used by untrained students.

Material and methods Non-pregnant cows (n=45) of the teaching herd were used for the insemination training. As we are not allowed to synchronise these cows for training purposes, cows may or may or not be in heat during this training of unexperienced students. Cows were sham inseminated at the maximum of two times per session with three months in between each session. 240 cows were sham inseminated using a conventional AI-sheath (Bovine medium plastic AI sheath, frontal delivery; NIFA, Leeuwarden, the Netherlands) and 256 cows were sham inseminated using the ALPHA pipet AI (crystalline plastic sheath, dual delivery (sideways) with polished rounded tip; NIFA, Leeuwarden, the Netherlands). Both protective plastic sheets (minimal contamination) were used in combination with a spiral AI-gun loaded with AI-straws containing 0.25 ml 0.9% NaCl. After sham AI the sheaths were checked on abnormal content or blood. Results of both sheaths were compared by a T-test (Microsoft Excel 2007; Microsoft, Redmond, USA).

Results Comparison of both AI-sheaths showed that there was no significant difference in usability of both AI-sheaths ($P>0.05$). In 0.03 of the cases the conventional AI-sheaths did contain blood on the tip of the sheath after AI, which was in 0.02 of the cases when the ALPHA AI-sheaths were used which was not significant ($P>0.05$).

Conclusion In conclusion, there is no difference in usability of conventional or ALPHA AI-sheaths for training of veterinary students in artificial insemination of the cow.

Acknowledgements We would like to thank NIFA, Leeuwarden, the Netherlands for providing the AI-sheaths.

Interaction of Bovine Sperm with Cervical Mucin is Sialic Acid Dependent

S Gedair[1], H Al Mhanna[1,2], M Gallagher[1], S Carrington[1], C Reid[1]
[1]*University College Dublin, Ireland,* [2]*Kufa University, Iraq*
colm.reid@ucd.ie

Application Understanding the role of cervical mucus in cervical-sperm transit and function will allow for the development of interventions that can positively and negatively influence fertility.

Introduction The bovine cervical mucosa is lined by a mucus-secreting epithelium which contributes to a protective barrier between the external environment and the uterus. Cervical mucus is thin and watery during oestrus to allow sperm access, but thick and viscoelastic secretions at other times (Pluta *et al*. 2011). Mucins are the structural glycoproteins of mucus and Sialic Acid (SA) is a common type of sugar in mucins which may influence trans-cervical sperm transit (Tollner 2008). In addition, the type of SA on cervical mucins is hormonally regulated (Pluta *et al*. 2011).
We hypothesise that 'sperm interact directly with cervical mucins in a SA-dependent manner' and 'the interaction between sperm and SA is dependent on the type and relative amount of SA on cervical mucin'. To test this, we compared the binding of sperm to purified cervical mucin before and after the removal of peripheral SA and the ability of different SAs to compete with the binding of sperm to cervical mucin was determined.

Material and Methods Mucins were purified from the cervix of synchronised heifers (Pluta *et al*. 2011) at discrete stages of the oestrous cycle and treated with sialidase treated. Untreated and treated mucin were coated on slides and probed with labelled sperm to compare sperm-mucin interactions. The effect of SA on sperm SA/mucin interactions was determined by incubating sperm with Neu5AC or Neu5GC (the most common forms) and assessing their binding to mucin coated slides.
Results Sperm associated strongly with both follicular and luteal mucin and this was reduced after sialidase treatment. The addition of increasing amounts of SA resulted in a decrease in the binding of sperm to both follicular and luteal purified mucin.

Conclusion Sperm interact with follicular and luteal purified bovine mucins in a SA dependent manner and this interaction is influenced by the amount and type of Neu5AC. This points to a central role for SA on mucins in mediating sperm interactions in cervical mucus function.

Acknowledgements This work was supported by Science Foundation Ireland and a Government of Saudi Arabia Scholarship.

References
Pluta, K., *et al*., 2011. Journal of Animal Science, 89, 4032–4042.
Tollner, T.L *et al*., 2008. Reproduction. 23 2523–2534.

https://doi.org/10.1017/S2040470018000109 Published online by Cambridge University Press

Identification of seminal parameters predictive of conception rates in Angus and Nelore bulls used in TAI

É Nogueira[1,2], W B Rodrigues[2], C Sanches Silva[2], E V Costa e Silva[2], J R Potiens[3], J C Borges Silva[1], P Sutovsky[4]
[1]Embrapa Pantanal, Corumbá- MS, Brazil, [2]Universidade Federal de Mato Grosso do Sul, PostGraduation Program of Veterinary Medicine, Campo Grande-MS, Brazil, [3]SELEON, SP, Brazil, [4]Division of Animal Sciences and Departments of Obstetrics, Gynecology & Women's Health, University of Missouri, Columbia MO 65211, USA, Columbia-MO, USA
eriklis.nogueira@embrapa.br

Application Our data demonstrate that differences in pregnancy rates per artificial insemination (P/AI) are breed-dependent. Seminal parameter analysis has a significantly predictive value for pregnancy in TAI.

Introduction The ability to predict male fertility is highly desirable for bulls used in AI. Timed artificial insemination (TAI) represents a breakthrough in the use of AI in Brazil and other countries. Numerous causes contribute to the wide range of results and/or unsatisfactory pregnancy rates in TAI programs, highlighting the factors inherent in the bovine female in addition to several factors inherent to quality of semen used. Regarding the quality of semen used in AI programs, differences reported in fertility could be attributed to variation in sperm qualitative characteristics. Consequently, the success of bovine AI programs largely depends on the use of good quality semen. When only high fertility bulls are used, better conception rates are achieved, reducing costs of reproductive programs. Thus, some authors have shown that semen used in TAI has great impact on pregnancy rates, and various biomarkers of sperm quality are required to predict the fertility of bull spermatozoa (Oliveira *et al*., 2013, Holden *et al*., 2017). Our goal is to correlate different methods of post-thaw semen evaluation with the P/AI of Nelore (zebu) cows subjected to TAI to identify the candidate predictors of conception rate.

Materials and methods P/AI data from 7258 Nelore cows, inseminated in fixed time with protocols using Estradiol, P4, PGF and ECG, with frozen-thawed semen from 35 Angus bulls and 17 Nelore bulls, were used. Pregnancies were evaluated by transrectal ultrasonography 30 days after TAI. Four samples of each semen batch were analysed for physical, functional and morphological aspects, including subjective means [gross motility, thermal resistance test (TRT), morphology, sperm concentration per ml (total and viable)], Computer Assisted Semen Analysis [CASA- total motility, progressive motility, average path velocity (VAP), straight line velocity (VSL), linearity, straight-line path (STR), amplitude of lateral head displacement (ALH) and curvilinear velocity (VCL)], hyposmotic swelling test (HOST), thiobarbituric acid-reactive substances assay (TBARS), assessment of plasma membrane integrity (PI) and mitochondrial membrane potential (JC-1) measured by flow cytometry. Data was analysed using ANOVA (GLIMMIX), Partial Least Squares (PLS) regression with use of Wolds criterion to explore the importance of sperm variables related to fertility (P/AI). Simple regression analysis has been used to correlate variables of interest and pregnancy ($P<0.05$ being considered significant).

Results The differences between bulls were found in P/AI ($P<0.001$) and in the pattern of semen quality according to breeds ($P<0.05$, except for plasma membrane integrity and viable sperm concentration). P/AI in the Nelore group was 55.62%, and in the Angus group 48.06% ($P<0.001$). The following in vitro sperm variables were determined to be important predictors of P/AI: plasma membrane integrity, gross motility, vigor, CASA variables (total motility, progressive motility, VAP, VSL, linearity, ALH, and VCL, viable sperm concentration, and TBARS (Fig 1). The pregnancy of Nelore cows subjected to TAI was significantly correlated with the following parameters: CASA (Linearity (R^2= -0.28, $P<0.001$), VAP (R^2= 0.207, $P<0.001$), VCL (R^2= 0.222, $P<0.001$), total motility (R^2= 0.235, $P<0.001$)), gross motility (R^2 = 0.210, $P<0.001$), vigor (R^2= 0.240, $P<0.001$), TBARS (R^2= 0.204, $P<0.001$). The other parameters evaluated were not correlated with pregnancy rate.

Conclusion Angus and Nelore bulls differ in P/AI when mated to Zebu cows. While the individual laboratory sperm tests are predictive of pregnancy in TAI, a combination of multiple tests will most likely be needed to increase the accuracy of this prediction. Multiplex test studies correlating seminal parameters and differences in fertility rates observed in TAI and programs are under way.

Acknowledgements – This research was supported by Embrapa (Mais Cria Project) and Fundect-CNPQ

References Holden, S.A., Fernandez-Fuertes, B., Murphy, C., Whelan, H., O'Gorman, A., Brennan, L., Butler, S.T., Lonergan, P., Fair, S. 2017. Relationship between in vitro sperm functional assessments, seminal plasma composition, and field fertility after AI with either non-sorted or sex-sorted bull semen Theriogenology. 87, 221–228
Oliveira, L.Z., de Arruda, R.P., de Andrade, A.F., Celeghini, E.C., Reeb, P.D., Martins, J.P., *et al*. 2013. Assessment of in vitro sperm characteristics and their importance in the prediction of conception rate in a bovine timed-AI program. Animal Reproduction Science. 137, 145–55.

https://doi.org/10.1017/S2040470018000109 Published online by Cambridge University Press

Relationships between the content of seminal plasma proteins and bull fertility

J M Morrell[1], S Resjö[2], J Willfors[3], T Hallap[4], F Levander[3], E Andreasson[2], D-J de Koning[1], P Humblot[1]
[1]*Swedish University of Agricultural Sciences, Uppsala, Sweden,* [2]*Swedish University of Agricultural Sciences, Alnarp, Sweden,* [3]*Lund University, Sweden,* [4]*Estonian University of Life Sciences, Tartu, Estonia*
jane.morrell@slu.se

Application Identification of markers of bull fertility in seminal plasma (SP) from protein patterns

Introduction Differences in fertility between males are most often attributed to variation in sperm quality whereas the contribution of SP is rarely considered. Differences in fertility among bulls with apparently similar sperm quality have been related to variations in SP content and its effects on spermatozoa (Maxwell *et al*., 2007). Sets of proteins from seminal plasma have been associated with sperm motility, resilience to freezing and survival in the genital tract in the sheep (Rickard *et al*., 2014, 2015; Soleilhavoup *et al*., 2014). In addition, SP from bulls of low fertility negatively affected the viability of bovine endometrial epithelial cells in culture (Nongbua *et al*., 2018). The present work was performed to investigate the possibility of identifying predictive markers of bull fertility from SP protein patterns.

Material and methods Following collection and centrifugation of full ejaculates, SP samples were prepared from the semen of 20 bulls (2 ejaculates per bull at 2 weeks´ intervals, in winter). The fertility performance (as estimated by non-return rates at 56 days post-AI) ranged from 34 to 68% based on 500 to >1000 inseminated females, respectively. Aliquots of SP (5 µl) were then frozen (- 196°C) and transferred on dry ice to the laboratory for proteomic analyses. Proteins were extracted, digested with trypsin and protein patterns were then quantified by liquid chromatography - mass spectrometry analysis of the protein digests. The data processing was performed using a label free workflow in the Proteios Software Environment (www.proteios.org). Peptide quantities were normalised using the Normalyzer software and differential expression analysis was conducted using empirical Bayes statistics in the LIMMA R package with Benjamini-Hochberg False Discovery Rate (FDR) correction of the p-values.

Results A total of approximately 1000 proteins was identified in seminal plasma samples. Overall, from clustering analysis, the proteomic profiles were found to be repeatable between ejaculates of the same individuals. Out of these, 38 proteins had significantly different abundance between low and high fertility bulls (at an FDR of 0.05). In addition, for a set of 18 proteins, significant correlations between their content in seminal plasma and fertility indexes were found. Among those, 14 correlated negatively (Pearson correlation coefficients from -0.85 to -0.43; $p<0.05$) and 4 correlated positively (0.52 to 0.44) with fertility. From this set of 18 proteins, 4 had previously been found to be related to sperm quality, motility and/or freezing ability in sheep (Rickards *et al*., 2014, 2015, Soleilhavoup *et al*., 2014). An interesting example is Ubiquitin carboxyl-terminal hydrolase isozyme L3, which increases in abundance with fertility. It has also been shown previously to be involved in the differentiation of spermatocytes into spermatids and to correlate with sperm count, motility and fertilization in humans (Wang *et al*., 2016). To our knowledge, the remaining 14 proteins have not been associated to sperm quality or fertility in published literature thus representing new candidates to screen for bull fertility.

Conclusion Several proteins had different abundance in SP from bulls of different fertility. This study reports the existence of putative new markers that could be of interest to identify bulls with different fertility profiles. However, the existence of such differences should be demonstrated from repeatable results obtained from several ejaculates of the same bulls collected at different time-points and confirmed from a complementary set of individuals.

Acknowledgements Funding for this contribution was provided by Mistra Biotech, a research program financed by Mistra – the Swedish foundation for strategic environmental research, and SLU.

References

Maxwell *et al*., 2007, Society of Reproduction and Fertility Supplement, 64, 13-38
Nongbua *et al*., 2018, Reproduction in Domestic Animals, 53:85-92
Rickards *et al*., 2015, Journal of Proteomics, 126,303-311
Rickards *et al*., 2014, Reproduction, 148, 469-478
Soleilhavoup *et al*., 2014, Journal of Proteomics,109, 245-260
Wang *et al*., 2016, PLOSone, 11, doi.org/10.1371/journal.pone.0165198

Seminal plasma of AI-bulls stimulates cytokine production by bovine endometrial epithelial cells in culture in a fertility-dependent manner

T Nongbua[1,2], Yi Guo[1], P Humblot[1], M Rubér[3], H Rodriguz-Martinez[3], T Ntallaris[1], J M Morrell[1]
[1]*Swedish University of Agricultural Science, Uppsala, Sweden,* [2]*Mahasarakham University, Thailand,* [3]*Linköpings University, Sweden*
jane.morrell@slu.se

Application Some seminal plasma (SP) is included in bull semen doses for insemination, which may have a detrimental effect on the uterus. Since SP from bulls of lower fertility had an adverse effect on cultured bovine endometrial epithelial cells (bEEC) compared to SP from bulls of higher fertility, it may be possible to modulate the cytokine response by removing SP from the semen of low fertility bulls or by replacing it with SP from high fertility bulls. Such manipulation may improve fertility in dairy cattle bred by artificial insemination.

Introduction Seminal plasma contains cytokines involved in immune-regulation of the female reproductive tract [1, 2]. Variations in fertility among bulls could be due to differences in SP composition and their effect on cytokine production by bEEC [3]. The objective of this study was to investigate the response of cultured bEEC in terms of cytokine production after treatment with SP.

Material and methods Bulls were categorized as above or below average fertility (H and L, respectively) according to a fertility index calculated from the 56-day non-return rate based on at least 1,000 artificial inseminations; a bull of average fertility scores 100. The H bulls (n=3) had a score of >104 whereas L bulls (n=2) scored ≤ 92. The bEEC (passage 5; approximately 5 – 13 x 10^5 per flask) were challenged with 1% or 4% SP from H- or L-fertility bulls or 1% or 4% PBS as control with cells from the uterus of 8 cows. After 72h and staining with trypan blue, cells were counted in a haemocytometer. The supernatant was analysed for IL-6 by ELISA (Bovine IL-6, MABTECH, Sweden) and IL-10 by Luminex (MILLIPLEX™MAP, Merck Millipore, USA), and cytokine content was expressed as pg/million cells. Data analysis was performed using the mixed model in SAS® (Proc Mixed, SAS® 9.3, USA). Concentration of SP, fertility of bull, and their interaction were fixed parts of the model, with cytokine response as variable parameter. Post-hoc comparisons were adjusted for multiplicity using Tukey's. All values are presented as LSMEAN ± SEM.

Results Challenge with SP had a significant effect on IL-6 production due to concentration of SP ($p<0.01$) and fertility of bull ($p<0.001$), but there was no significant interaction between concentration and bull fertility. Thus, even if the initial amount of IL-6 present in SP differed between low and high fertility bulls (1407±837 and 487±708 pg/mL, respectively), the magnitude of the increase after challenge was independent of this amount. For IL-10, the levels were below the limit of detection.

Conclusion Higher concentrations of SP stimulated more IL-6 production, which could be associated with impaired cell adhesion or cell damage. The SP from bulls of lower fertility had more of an effect than SP from higher fertility bulls.

Acknowledgements We are very grateful to VikingGenetics, Sweden, for supplying the semen. TN was supported by Mahasarakham University, Thailand, and the Faculty of Veterinary Medicine, SLU; the co-authors were funded by FORMAS, Stockholm, JMM by grant 221-2010-1241 and MR and HR-M by grant 2017-00946.

References

Robertson, S.A., 2005. Cell and Tissue Research 322, 43-52.
Rodriguez-Martinez, H., Kvist, U., Ernerudh, J., Sanz, L., Calvete, J.J., 2011. American Journal of Reproductive Immunology 66 Suppl 1, 11-22.
Nongbua, T., Guo, Y., Edman, A., Humblot, P., Morrell, J.M., 2018. Reproduction in Domestic Animals 53, 85-92.

Is there value in doing Bull Breeding Soundness Evaluation in a first-opinion veterinary practice?

MTomlinson[1], P Wood[2], A Macrae[2], M Mihm-Carmichael[1]
[1]*University of Glasgow, UK,* [2]*University of Edinburgh, UK*
martin.tomlinson@glasgow.ac.uk

Introduction Bull Breeding Soundness Evaluation (BBSE) is commonly undertaken to identify bulls that are potentially unfit for use as breeding sires. Improving reproductive efficiency is vital for cow-calf (suckler) operation profitability, and a potential area for a veterinary business to generate cash flow through value added work. However**,** the bio-economic cost benefit of improved reproduction and calf output resulting from BBSE is still debatable, as overall BBSE results may not be consistent with pregnancy results. This is in contrast to individual measurements such as scrotal circumference [1]. Yet, one study did show an increase in calf crop by 31% when sub-fertile animals were excluded from breeding[2].Thus the collection of more economic data following BBSE should not only improve farming practice, but may also provide guidance for veterinary businesses allowing these to become more sustainable[3]. The aim of our BBSE outcome and practice income/expenditure analysis, therefore, was to determine primarily the investment cost of diagnosing a sub-fertile bull to farmers, and the potential economic benefit to their veterinarians.

Material and methods BBSE data from a 3 vet, farm animal only, first opinion practice in the South East of Scotland between March 2014 and May 2015 was reviewed. Bulls from 35 farms were assessed using British Cattle Veterinary Association (BCVA) guidelines [4]. A cost benefit analysis of the BBSE's was performed to assess income to a veterinary practice. Vet professional time was included as an easily appreciated figure accounting for such variables as staff salaries, cars, related practice costs etc. BBSE could then be determined as an opportunistic revenue stream to a practice compared to other uses of professional time. The cost in generating a management decision after detecting a sub-fertile bull to a farmer in this study was calculated via: (Total overall BBSE costs/No. Bulls)(Average Investment required to detect subfertility).

Results Of 162 bulls tested in this study, 61 animals (37%) failed BBSE1, with 41 (25%) failing due to poor semen quality. 33 animals underwent a repeat BBSE 6 to 8 weeks later with 22 (64%) failing BBSE2. Reasons for failure of BBSE and are described in Table 1. Results from a cost benefit practice cash flow analysis can be seen in Table 2. The cost to a farmer to generate a management decision after detecting a sub-fertile bull can be seen in table 3.

Table 1 Reasons for failure of BBSE

No. of animals failing BBSE1	n=61	No. of animals failing BBSE2	n=22
<60% motility& <70% morph.	25	<60% motility & <70% morph.	14
<60% motility only	11	<60% motility only	4
<70% morphology only	5	<70% morphology only	4
Lameness	11		0
Scrotal circumference <34cm	4		0
Other relevant BBSE lesions	5		0

Table2 Results from a cost benefit practice cash flow analysis of BBSE

BBSE visit type	Charge£	No.	Income£	Expenditure	Charge£	No.	Income£
Single BBSE	125	8	1000	training	500	2	-1000
Multiple BBSE's	100	134	13400	equipment	2000	1	-2000
Partial(excl.semen)	40	20	800				
Repeat exam	60	33	1980				
Total (£)			17180	Total (£)			-3000
Total Cash flow into vet practice (£)							14180
Vet time cost/hour (£) (Consensus charge in practice area)							120
Potential hours generated by income							118
BBSE/hour for opportunity breakeven							1.4

Table 3 Farmer BBSE expenditure to a sub-fertile bull

Total BBSE charges (£)	17180
Total No. BBSE's	162
Average BBSE charge (£)	106
Failure rate (%)	37
Average No. of BBSE to detect a subfertility	3
Average Cost to detect a sub -fertile bull (£)	**318**

Conclusions BBSE failure rate was normal in this practice [5].

In this study, BBSE generated **farmer economic gain** if management of sub-fertile bulls produce **> £318 output.**

Using the charges in this study BBSE generated **veterinary practice economic gain** at **> 1.4 BBSE/hour** or 45 mins. per BBSE. This does not take into account other revenue that may be generated as a result of BBSE.

References:
1)Menegassi 2011. Revista Brasileira de Zootecnia. 40, 441. 10. 1590.
2)Waldner 2010. Theriogenology. 74, 5. 871-883.
3)Henry 2018. Exploring the future sustainability of farm animal veterinary practice.
4)Penny 2010.Veterinary Record 2010; 167,551-554.
5)Kastelic (2008). Reproduction of Domestic Animals. 43. 368-373

https://doi.org/10.1017/S2040470018000109 Published online by Cambridge University Press

Characterization of bull fertility through multicolour flow cytometric analysis of cryopreserved sperm

E Malama, K Bucher, M Siuda, F Janett, H Bollwein
Clinic for Reproductive Medicine, Vetsuisse Faculty, University of Zurich, Switzerland
emalama@vetclinics.uzh.ch

Application The establishment of a simple methodology for the prognosis of male fertilizing ability remains an issue of great interest for animal biotechnologists. In the present study, we evaluated the use of a five-colour flow cytometric assay for the assessment of bull fertility.

Introduction Given the complex nature of male gametes, a multi-parametric approach of sperm functional status would contribute to the characterization of male fertility. Advancements in flow cytometry have enabled the simultaneous assessment of multiple cellular characteristics in large cell populations. In the present study, we aimed: a) to develop a five-colour panel configured by a three-laser flow cytometer for the assessment of viability, acrosomal status, mitochondrial membrane potential and intracellular Ca^{2+} levels in commercially produced cryopreserved bovine semen, and b) to evaluate the use of multi-colour flow cytometry for the characterization of bull fertilizing potential.

Material and methods Twenty Holstein-Friesian bulls serving as mature sperm donors in an artificial insemination (AI) centre were selected based on existing data on their annual 56-day non-return rate (NRR, %) after at least 1000 field AI. The selected sires showed extreme NRR values compared to the mean NRR of total bull population in the AI centre (NRR_{total}) and were accordingly assigned to a High (HF; n_{HF}=10 bulls with NRR>NRR_{total}+SD) or Low Fertility group (LF; n_{LF}=10 bulls with NRR<NRR_{total}-SD). Three to four cryopreserved ejaculates of each bull (n=91) were examined at 0 (0 h) and 3 hours (3 h) of post-thaw incubation. A multicolour panel including calcein violet AM, propidium iodide, the R-pycoerythrin-conjugated lectin of *Arachis hypogea*, Fluo-4 AM and Mitoprobe^{TM} DiIC1(5) was configured by means of a three-laser flow cytometer, in order to assess sperm esterase activity, plasma membrane integrity, acrosomal status, intracellular Ca^{2+} levels and mitochondrial membrane potential, respectively. Sperm sub-populations showing one or a combination of the following favourable features were defined: high esterase activity (C_{pos}); intact plasma membrane (PI_{neg}); intact acrosome (PNA_{neg}); low intracellular Ca^{2+} levels (F_{neg}); high mitochondrial membrane potential (M_{pos}). The % relative size of 20 sperm sub-populations assessed with multicolour flow cytometry (MC parameters) was used as input for the statistical analysis. Between-group differences of MC parameters (HF vs LF bulls) were examined using mixed-effects linear models. The Random Forest^{TM} algorithm (RF) was used to evaluate the 20 MC parameters as predictors of the fertility group computing the % out-of-bag (OOB) error rate and the importance of each MC parameter as fertility classifier.

Results Sperm sub-populations combining two favourable features accounted for more than 50% of the total sperm population at 0 h, except for sperm combining low Ca^{2+} levels with another feature (i.e. $PI_{neg}F_{neg}$, $PNA_{neg}F_{neg}$, $M_{pos}F_{neg}$ as well as $C_{pos}F_{neg}$ in LF group). Furthermore, M_{pos} cells comprised more than 90% of $PI_{neg}PNA_{neg}$ sperm at 0 h (94.8%±3.82% and 93.2%±3.74% in the HF and LF group, respectively); these values were limited to 84.5%±6.05% and 85.1%±5.31% at 3h, respectively. Sperm simultaneously exhibiting three, four or five features at 0h comprised less than half of total sperm population, with exception of $PI_{neg}PNA_{neg}M_{pos}$ sperm (0h) that exceeded 50% in both fertility groups (51.8%±8.81% and 51.4%±8.87% for the HF and LF group, respectively). The percentage of F_{neg} cells within the $PI_{neg}PNA_{neg}$ and $PI_{neg}M_{pos}$ sperm populations at 0 h was higher in the HF (87.7%±5.14% and 81.3%±5.76%, respectively) compared to the LF bulls (80.9% ±8.18% and 73.5%±8.54%, respectively; P<0.01 in both cases). Thus, HF bulls showed higher $PI_{neg}PNA_{neg}F_{neg}$ (35.8%±7.40%) and $PI_{neg}M_{pos}F_{neg}$ sperm (34.9±7.40%) at 3h than LF bulls (39.4%±7.33% and 29.8%±7.18%, respectively; P<0.05 in both cases). Applying RF to the 0 h and the 3 h dataset, the OBB error rate was estimated to 34.1% and 30.8%, respectively, suggesting that approximately two thirds of ejaculates could be correctly assigned to their fertility group. The percentage of F_{neg} cells within the $PI_{neg}PNA_{neg}$ and $PI_{neg}M_{pos}$ sperm populations as well as $C_{pos}F_{neg}$ and $C_{pos}PI_{neg}PNA_{neg}$ sperm at 0h turned out to be the MC parameters of highest importance for the prediction of fertility group.

Conclusion The vast majority of viable sperm exhibited an active mitochondrial membrane potential. However, the ability of viable sperm to retain low Ca^{2+} levels differed between bulls of diverse fertility status. A classifier based on selected sperm parameters assessed through multicolour flow cytometry could accurately predict the fertility class of approximately two out of three cryopreserved ejaculates.

Acknowledgements We acknowledge the support of Dr. Erwin Hasenpusch, Rinderzucht Schleswig-Holstein eG, Neumünster Germany, and Dr. Ulrich Witschi, Swissgenetics, Zollikofen Switzerland

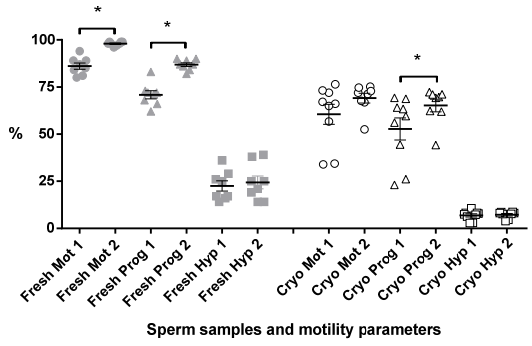
Sperm motility, semen production efficiency and field fertility in different aged Norwegian Red bulls

A Khezri, B Narud, J Mbuma, F D Myromslien, E Kommisrud
Inland Norway University of Applied Sciences, Hamar, Norway
elisabeth.kommisrud@inn.no

Application The introduction of genomic selection has created challenges for semen production in young bulls. Increased genetic improvement and shortening of the generation interval conflicts with onset of puberty and efficient semen production.

Introduction In agriculture and livestock production, high reproductive efficiency is crucial for efficient and thereby economically sustainable production. Norway enjoys advantages due to long term and on-going breeding goals focusing on low heritable traits including fertility. Male fertility, as a prerequisite to success with artificial insemination (AI), is critical since a selected and restricted number of males is used extensively as semen donors in livestock breeding programs. Genomic selection has challenged semen production efficiency in young bulls. It has been shown that age can significantly affect semen quality (Lambert *et al*., 2018; Vince *et al*., 2017), and thereby, result in reduced field fertility. Therefore, the aim of this study was to evaluate the effect of age on sperm quality, semen production and fertility in young genomically-selected Norwegian Red bulls.

Material and methods Ejaculates were collected from eight Norwegian Red bulls during a period of nine months starting at approximately 14 months of age. Semen was processed using a two-step dilution procedure before cryopreservation, quality control and distribution to all parts of Norway. Samples for sperm quality analyses were collected twice, when the bulls were approximately 14 and 17 months old. Both pre-diluted (non-glycerol containing extender) and cryopreserved samples (straws) were evaluated for motility parameters including total motile sperm cells, progressive motility and hyperactive sperm cells using computer-assisted sperm analyses (CASA). Moreover, field fertility data for first inseminations from the nine-month semen production period were obtained from the national AI recording database (Geno) and calculated as 56 days non-return rate (NRR). Returns within 0-3 days were excluded from the analyses. Data are presented as average for the bulls, for the whole period and for periods at different ages. Data have not yet been analysed for the effect of e.g. month of AI and parity of the females.



Results Results indicate that fresh diluted sperm cells from 17-month-old bulls, exhibit significantly higher motility and progressivity compared to samples from the same bulls at 14 months of age ($p < 0.05$). A similar trend was observed for cryopreserved sperm cells. However, the only parameter with a significantly higher value at 17 months was progressive motility (Figure 1). Field fertility data revealed an overall 56 NRR of 72 % for 27,879 first AIs. Splitting data into NRR for collection number 1-4, 5-8, 9-12, 13-16 and 20-30 did not reveal significant differences, NRR being 73.1, 72.4, 74.1, 71.4 and 70.2, respectively. Semen production increased with age, due to increased number of doses produced per week and due to reduced number of semen doses discarded.

Figure 1 Sperm motility parameters at different aged Norwegian Red bulls. Fresh: first dilution samples Cryo: cryopreserved sample, Mot: % motile sperm cells, Prog: % progressive motility, Hyp: % hyperactive sperm cells. 1: 14 months old bulls, 2: 17 months old bulls.

Conclusion The results of this study indicate that age affects sperm motility in both fresh and cryopreserved semen samples from Norwegian Red bulls. However, field fertility is not influenced by age while semen production efficiency is affected.

Acknowledgements The authors acknowledge funding from Inland regional research fund (project 257606) and the Research Council of Norway (project 268048).

References

Lambert, S., P. Blondin, C. Vigneault, R. Labrecque, I. Dufort, and M.-A. Sirard. 2018. Spermatozoa DNA methylation patterns differ due to peripubertal age in bulls. Theriogenology. 106:21-29.
Vince, S., I. Žura Žaja, M. Samardžija, I. Majić Balić, M. Vilić, D. Đuričić, H. Valpotić, F. Marković, and S. Milinković-Tur. 2017. Age-related differences of semen quality, seminal plasma, and spermatozoa antioxidative and oxidative stress variables in bulls during cold and warm periods of the year. animal, 1-10.

https://doi.org/10.1017/S2040470018000109 Published online by Cambridge University Press

Is genomic selection challenging the production?

K Kupisiewicz, S Borchersen
Viking Genetics, Randers, Denmark
kakup@vikinggenetics.com

Application Flow cytometry, optimization of semen collection and processing

Introduction Genomic selection has revolutionized modern cattle breeding with continuous efforts to increase the reliability of genomic proofs and reduce generation interval in pedigree breeding. For bull studs, genomic selection leads to: 1) reduction in the number of available animals; 2) reduction in the age of collected animals and 3) challenges in obtaining good quality semen. Thus, the aim of our study was to describe changes in semen quality depending on the number of ejaculate and bull's age at collection. Obtained results can be used as an aid for production management tools and prompt for new production strategies and intense research in the biology of young bulls.

Material and methods Presented work is based on production and field results obtained at Viking Genetics in 2016 and 2017. In total 151 Holstein bulls aged between 8 and 16 mths. Average age at first approved collection was 11.2 mths. Average number of collections per bull was 29. Semen qualified for processing was evaluated for concentration and viability using flow cytometry, and processed as described in Christensen *et al*. (2011). Semen quality variables were processed using Microsoft Excel (version 2013 O64) and concentration, viability and volume of neat ejaculate were plotted against ejaculate number or the age of the bull. Mean values of quality variables for first and 20th ejaculate were compared using F-test for variances. Field data from 104 bulls were extracted from Finish cattle database as the percentage of animals without detectable signs of oestrus within 56 days (NRR 56) and the number of inseminations.

Results Mean values of quality variables for first and 20th ejaculate and P values are presented in Table 1. Our results show that only concentration of neat semen differs significantly between 1st and 20th ejaculate. Volume, viability and NRR 56 are not significantly different between 1st and 20th ejaculates. Even though NRR 56 values are higher for 20th ejaculate than for 1st, more data are needed to statistically prove the difference between them. Discard rates are very high when collecting first ejaculate (80,9%) from the subsequent ejaculates (11,9 for 20th ejaculate). 122 ejaculates out of 151 were discarded at first collection and only 16 at collection number 20.

Table 1. Mean values for 1st and 20th ejaculate ±SD and confidence intervals in parentheses. Table shows also P values for calculated difference and their level of significance.

	1st ejaculate (n=151)	**20th ejaculate (n=112)**	**P value**	Significance level
Concentration (milliard/ml)	0,76±0,25 (0,51 – 1,01)	1,18±0,47 (0,71-1,65)	P<0.001	***
Volume (ml)	3,5±1,35 (2,15 – 4,85)	4,2±1,37 (2,83 – 5,57)	P=0,43	NS
Viablility (%)	81,3±5,65 (75,6 – 86,95)	86,3±5,85 (80,45 – 92,15)	P=0,35	NS
NRR 56	57,3±29,67 (27,63 – 86,97)	62,8±19,1 (43,7 – 81,9)	P=0.01	NS

Conclusion Our study describes production and semen quality variables from subsequent ejaculates and from bulls of different age. Collected data can give us a tool for more efficient bull management and production.

Acknowledgements Authors would like to acknowledge Viking Genetics personnel that contributed to the study with data collection and evaluation of semen. We thank Danish Cattle and Faba for providing field data.

References

Christensen P, Labouriau R, Brick A, Boe-Hansen GB, Pedersen J and Borchersen S 2011. Journal of Dairy Science. 94, 1744-1754

https://doi.org/10.1017/S2040470018000109 Published online by Cambridge University Press

Sperm DNA fragmentation and kinetics of development of bovine embryos produced *in vitro* using bulls of different field fertility

C Maicas[1,2], C Passaro[1], C J Byrne[1,3], S T Butler[2], P Lonergan[1]
[1]*School of Agriculture and Food Science, University College Dublin, Ireland,* [2]*Teagasc Animal and Grassland Research and Innovation Centre, Moorepark, Fermoy, Co. Cork, Ireland,* [3]*Animal and Bioscience Department, Teagasc, Dunsany, Co. Meath, Ireland*
cliomaicas@gmail.com

Application Currently, the assessment of semen in breeding centres is limited to measures of motility and viability of sperm. A bull effect exists in the early dynamics of embryo development and bulls presenting problems during this period could be identified in vitro using a combination of assays including IVF.

Introduction Currently, the assessment of semen in breeding centres is mainly based on measures of sperm motility and viability. While this allows the identification of bulls with very poor sperm quality, for the majority of bulls, these variables are poorly correlated with pregnancy rate after artificial insemination. The timing of DNA replication (S-Phase) during the first cell cycle after fertilisation (Eid *et al*., 1994) and the kinetics of the early embryonic cleavage divisions (Ward *et al*., 2001) are influenced by the sire and a relationship with field fertility based on non-return rate (NRR). High levels of DNA fragmentation have been associated with poor fertility outcomes and may represent a useful predictor of fertility in combination with other variables. The aim of this study was to assess the incidence of DNA fragmentation in sperm from bulls of different field fertility and to characterise the kinetics of pronuclear formation, pronuclear S-phase, and embryo development following insemination *in vitro*.

Material and methods Holstein-Friesian bulls with more than 1000 inseminations were selected from the Irish Cattle Breeding Federation (ICBF) database, based on an adjusted fertility index (Berry *et al*., 2011). Three bulls from the top 10% and three bulls from the bottom 10% were selected and classified as high fertility (HF), or low fertility (LF). In Experiment 1, the percentage of sperm with fragmented DNA (%DF) was determined by flow cytometry using the Sperm Chromatin Structure Assay (SCSA; Evenson and Jost, 2000). In Experiment 2, frozen-thawed sperm was used to fertilise *in vitro* matured cumulus oocyte complexes at a final concentration of 10^6 sperm/mL. Twelve h post insemination (hpi), a subset of presumptive zygotes (n=50 per bull) were incubated with 1 mM 5-ethynyl-2'-deoxyuridine (EdU), a nucleoside analogue of thymidine that is incorporated into DNA during active DNA synthesis, for 1 h, fixed and labelled with Click-T reaction "cocktail" (that reveals the presence of EdU) and Hoechst 33342. The % of oocytes with 2 pronuclei (zygotes) and the % of zygotes in S-Phase (where one or both pronuclei were labelled with Alexa Fluor 647) were determined under fluorescence microscopy. At 24 hpi, denuded presumptive zygotes were transferred into synthetic oviductal fluid (SOF) supplemented with 5% FCS under mineral oil. Cleavage rate was assessed at 24, 27, 30, 33, 36, 42 and 48 hpi, and blastocyst development was assessed on Days 7- and 8-post insemination. IVF data was analysed using the GENMOD procedure; %DF data was analysed after log transformation using MIXED procedure (SAS, version 9.4).

Results %DF did not differ between HF and LF bulls (1.6 ± 0.4% vs. 2.2 ± 0.4%, respectively, $P > 0.05$). IVF with sperm from HF bulls resulted in more zygotes at 12 hpi than the LF bulls (63.5 ± 3.8% vs. 50.8 ± 4.0%, respectively, $P < 0.05$). However, no difference was found in the percent of zygotes in S-Phase between the groups (HF: 45.7 ± 4.6% and LF: 41.0 ± 4.6%, $P > 0.05$). Cleavage rate was higher in HF than in LF at 36 hpi and 42 hpi, but no difference was found at other time-points assessed (Table 1), nor in Day 7- or Day 8-blastocyst rates (26.9 ± 1.7% vs. 24.5 ± 1.6%, and 33.6 ± 2.0% vs. 29.7 ± 1.9%, both $P > 0.05$).

Table 1 Kinetics of cleavage following IVF with semen from HF and LF bulls (mean ± SEM).

Fertility group	Cleavage (%)						
	24 h	27 h	30 h	33 h	36 h	42 h	48 h
HF	4.9 ± 0.9	23.1 ± 2.6	47.0 ± 2.6	57.6 ± 2.8	64.9 ± 2.3	72.2 ± 2.1	73.8 ± 1.9
LF	4.7 ± 0.9	23.2 ± 2.6	41.3 ± 2.5	51.2 ± 2.7	56.6 ± 2.4*	64.3 ± 2.3*	69.3 ± 2.0

*Difference between bull fertility groups ($P < 0.05$)

Conclusion Differences in pronuclei formation and cleavage kinetics after IVF existed for bulls of extreme fertility (-8 vs. +8) and may provide potential for discriminating between bulls of differing fertility.

Acknowledgements We gratefully acknowledge the contribution of Dr Alfonso Blanco (UCD - Conway Institute) and the National Cattle Breeding Centre (NCBC). Supported by Department of Agriculture Food and The Marine.

References

Berry, D.P., Evans, R.D., Mc Parland, S 2011. Theriogenology, 75, 172-181
Eid, L.N., Lorton, S.P., Parrish, J.J. 1944 Biology of Reproduction 51,1232-7
Evenson, D. and Jost, L. Methods Cell Sci 2000. 22(2-3):169-89
Ward, F., Rizos, D., Corridan, D., Quinn, K., Boland, M., Lonergan, P. 2011 Molecular Reproduction and Development 60(1):47-55

https://doi.org/10.1017/S2040470018000109 Published online by Cambridge University Press

Nuclear protein contents – An epigenetic marking of sperm cells

C Le Danvic[1], F Bray[2], E Sellem[1], H Kiefer[3], H James[3], C Rolando[2], L Schibler[1]
[1]*ALLICE, Département R&D, Paris, France,* [2]*MSAP, USR3290 Lille 1, CNRS, Paris, France,* [3]*UMR BDR, INRA, ENVA, Université Paris Saclay, Jouy en Josas, France*
chrystelle.ledanvic@allice.fr

Application During the past twenty years, numerous studies have highlighted the link between sperm cell function and fertility. Nevertheless, the underlined phenotypes do not allow a strong prediction of male fertility (Sellem *at al.,* 2015). The project SeQuaMol aims to identify new fertility biomarkers based on semen epigenetic marks. The present study introduces an aspect of epigenetic marking that has been under-explored in the bull: the characterization of semen histone/protamine content.

Introduction Sperm cells are highly specialized cells, produced by spermatogenesis, a process that involves extensive cellular, epigenetic and chromatin changes. One of the main epigenetic changes involves the replacement of histones by protamines (highly basic proteins). This transition results in a 10-fold compaction of the spermatozoa genome, leading to the protection of the paternal genome from physical and chemical damage (Rathke *at al.*, 2013). An excessive ratio of histone retention during spermatogenesis indicates sperm chromatin immaturity with weaker compaction. This ultimately causes sperm dysfunction. The objective of our study was to determine histone-protamine content in bull frozen sperm by proteomics. The final aim will be to obtain a better understanding of the key role of histone/protamine in fertility of bull frozen sperm.

Material and methods Nuclear proteins were extracted from 30 Montbéliard ejaculates with contrasting fertility (n=10 per group). A combination of peptide-based bottom-up MS/MS strategy with a top-down approach based on the analysis of intact nuclear sperm proteins was used to characterize those proteins. Intact proteins and peptides were identified using a high mass accuracy Orbitrap Q-Exactive$^+$ mass spectrometer (Thermo Scientific). The acquired raw files were analysed with PEAKS 7 studio (Bioinformatics Solutions Inc.) and X!TandemPipeline softwares using a custom-made histones and protamines database. Three posts-translational modifications were included in the searches: acetylation, methylation, phosphorylation. Quantification of identified proteins was performed using the exponentially modified protein abundance index (emPAI) offering a relative quantitation of proteins in an extract.

Results All five histones types (H1, H2A, H2B, H3 and H4) were identified with a minimum of two unique peptides. Interestingly, no H3 and H4 types could be detected in the more fertile group. Overall, a total of 18 PTMs was detected including lysine and arginine methylation, N-terminal and lysine acetylation and tyrosine phosphorylation. However, no link between histones PTMs patterns and fertility seems to stand out. In addition to histones, two isoforms of protamine PRM1, but also of PRM2, were also identified. PRM2 protein is identified in bull sperm for the first time. Although the bull PRM2 gene is translated and transcribed at low levels, no PRM2 protein has been detected until now. Both PRM1 and PRM2 isoforms are modified by PTMs, mainly by serine and threonine phosphorylation. Interestingly, the PRM1a isoform was identified only in the fertile group (n=2/10 bulls) and with a phosphorylation on the serine 30. No significant difference was observed in histone/protamine ratio between fertility groups. Nevertheless, there was a tendency to have a higher ratio in the sub-fertile bull group compare to the fertile group.

Conclusion The results obtained highlight the heterogeneity of the nuclear protein content among bulls with differences appearing between fertile and sub-fertile bulls. These results have to be confirmed by different protein quantitation methods and detailed statistical analysis. Nevertheless, our work clearly shows a diversity of nuclear protein isoforms based on histones, but also protamines, primary sequences and on beard PTMs. Work is in progress to precisely characterize the PTMs pattern (nature and localization) and to relate them with bull fertility potential.

Acknowledgements
This work is supported by an ANR and APIS-GENE (LabCom SeQuaMol).

References
Sellem E, Broekhuijse ML, Chevrier L, Camugli S, Schmitt E, Schibler L, Koenen EP. 2015. Use of combinations of in vitro quality assessments to predict fertility of bovine semen. Theriogenology 84, 1447-1454
Rathke C, Baarends WM, Axe S, Renkawitz-Pohl R. 2013. Chromatin dynamics during spermatogenesis. Biochim Biophys Acta doi:10.1016/j.bbagrm

Effect of altering plane of nutrition during the first and second six months of life on in-vitro fertilizing ability and DNA fragmentation in mature Holstein-Friesian bulls

C J Byrne[1,2], B Fernandez-Fuertes[1], S Fair[3], C Maicas[1], P Lonergan[1], D A Kenny[1,2]
[1]*School of Agriculture and Food Science, University College Dublin, Belfield, Dublin 4, Ireland,* [2]*Animal and Bioscience Research Department, Teagasc Grange, Dunsany, Meath, Ireland,* [3]*Laboratory of Animal Reproduction,Department of Biological Sciences, Faculty of Science and Engineering, University of Limerick, Ireland*
colin.byrne.3@ucdconnect.ie

Application Earlier onset of puberty in young genomically-selected high genetic merit dairy bulls will advance the availability of semen, shorten the generation interval and accelerate genetic improvement. Our data demonstrate that increasing the plane of nutrition during calf-hood does not impact sperm DNA fragmentation or capacity for *in vitro* fertilisation and/or subsequent blastocyst yield of mature bulls.

Introduction With the advent of genomic selection, dairy bulls can now be identified as sires for use in artificial insemination shortly after birth, thus creating demand for their semen as soon as puberty is reached. Recent data from our group (Byrne *et al*., 2018) show that offering bull calves a high plane of nutrition in early life hastens the age at puberty onset. However, the impact of this strategy, if any, on post pubertal sperm fertilising ability or DNA integrity is unclear. The aim of this study was to examine the effect of plane of nutrition during the (i) first 6 months of life and (ii) from 6 months to puberty, on sperm DNA fragmentation, *in-vitro* fertilising (IVF) ability and subsequent blastocyst development.

Material and methods Autumn-born Holstein-Friesian bull calves (n=83) with a mean (±S.D.) age and bodyweight of 17 (±4.4) days and 52 (±6.2) kg, respectively, were blocked on age, bodyweight and sire and assigned to a high [H] or low [L] plane of nutrition for the first 6 months of life. Calves were individually offered milk replacer and concentrate using an electronic feeder. After five days acclimatisation H (n=37) and L (n=46) calves received either 1200 g or 450 g of milk replacer, respectively. H calves were offered concentrate *ad libitum* while those on L received a maximum of 1 kg concentrates daily. Calves were weaned when consuming a minimum of 1 kg concentrates for 3 consecutive days, at a mean age (±S.D.) of 79 (5.1) days. Following weaning, H calves were offered *ad libitum* concentrates while L calves received 1 kg of concentrate daily. All calves had *ad libitum* access to hay. At 24 weeks of age, calves were re-assigned, within treatment, to either remain on their existing plane of nutrition or to change to the opposite diet, until puberty. This resulted in four groups: HH; HL; LL and LH (n=19, 18, 22 and 24, respectively). Animals were turned out to pasture at 26 weeks of age where HH and LH calves received grass and concentrate *ad libitum* while LL and HL calves received grass to appetite plus 0.5 kg concentrate daily. Animals were weighed weekly pre-weaning and fortnightly post-weaning. A sub-group of bulls (n=4, 5, 5 and 4 from HH; HL; LL and LH, respectively) was selected for IVF and DNA fragmentation analyses; two straws from one ejaculate, collected at 16 months of age were analysed with each straw analysed twice for the amount of DNA fragmentation. DNA fragmentation was analysed using the method of Evenson and Lost (2000) and quantified using a flow cytometer (Accuri C6, BD Biosciences, San Jose, CA, US). Data were analysed using GENMOD procedure within SAS (version 9.4). Plane of nutrition pre and post six months of age, with interactions, where appropriate were included in the model.

Results There was no effect of plane of nutrition during either the pre or post six months periods of life, on fertilising ability, blastocyst rates or percentage DNA fragmentation ($P > 0.05$; Table 1).

Table 1 Mean (± SEM) percentage *in vitro* cleavage and blastocyst rates following IVF with sperm from Holstein-Friesian bulls at 64 weeks of age (n = 18) offered either a high or low plane of nutrition from 2 to 24 wk of age followed by either a high or low plane of nutrition until puberty

Treatment	High/High	High/Low	Low/High	Low/Low
AOP[1] (days)	266	255	339	356
Oocytes (n)	924	1355	1345	1060
% Cleaved	75.0 ± 5.22	69.2 ± 3.51	75.5 ± 3.13	70.5 ± 4.53
% Blastocysts[2]	28.6 ± 3.37	28.5 ± 2.76	31.8 ± 3.34	27.7 ± 1.59
% DNA fragmentation	9.7 ± 3.12	16.8 ± 3.62	13.3 ± 4.19	10.9 ± 3.04

[1]AOP = age at puberty
[2]%blastocyst = % of blastocysts from oocytes matured

Conclusion We have previously clearly shown that offering Holstein Friesian bull calves a high compared to moderate plane of nutrition during the first 6 months of life hastens puberty onset. We have now shown that these differences do not impact either sperm DNA fragmentation, IVF capacity or indeed subsequent blastocyst development when ejaculates were collected at 16 months of age. In conclusion, an enhanced plane of nutrition can be used to hasten onset of puberty in Holstein-Friesian bulls without negatively impacting subsequent fertility.

Acknowledgements We acknowledge funding from the Department of Agriculture, Food and Marine. (Project 11/S/116)

References Byrne, C.J., Fair, S., English, A.M., Cirot, M., Staub, C., Lonergan, P., Kenny, D.A. 2018. Journal of Dairy Science. 101, 3447-3459.
Evenson, D. Jost, L. 2000. Methods in Cell Science. 22, 169-189.

https://doi.org/10.1017/S2040470018000109 Published online by Cambridge University Press

Progesterone induces the release of bovine sperm from oviductal epithelial cells

J Romero-Aguirregomezcorta, S Cronin, S Fair
Laboratory of Animal Reproduction, Department of Biological Sciences, Faculty of Science and Engineering, University of Limerick, Ireland
jon.romero@ul.ie

Application To increase our understanding of the interactions of sperm with the female reproductive tract so as to optimise the use of semen during artificial insemination.

Introduction The mechanism which causes the detachment of sperm from oviduct epithelial cells around the time of ovulation remains to be elucidated. As the cumulus cells of the bovine oocyte are known to secrete progesterone (P4), and P4 has been shown to act upon CatSper channels in human sperm (Strunker *et al*., 2011), it was hypothesised that the incubation of bound sperm with P4 would induce hyperactivation, due to an influx of extracellular calcium, and this would stimulate the release of sperm from the bovine oviduct epithelial cells (BOEC) explants. The mechanism of action of P4 on the detachment of sperm from BOECs was also investigated.

Material and methods Initial dose response assessments were carried out to determine the optimum concentration of P4 (10 nM), Mibefradil (non-specific calcium channel antagonist, 5 µM), NNC (CatSper channel antagonist, 2 µM), Mifepristone (intracellular P4 receptor antagonist, 400 nM), and AG205 (membrane P4 receptor antagonist, 10 µM) on sperm hyperactivation. BOEC explants, isolated from the isthmic portion of the oviduct, were incubated in the presence of frozen-thawed sperm (pool of 3 bulls stained with 1% Hoechst 33342) for 30 min at 37.5°C and 5% CO_2 following which loosely bound sperm were removed from the samples through gently pipetting. Two experiments were then completed. Experiment 1: BOECs were treated for 30 mins with: (i) No Treatment (control), (ii) P4, (iii) NNC, (iv) Mibefradil, (v) P4+Mibefradil, (vi) P4+NNC, (vii) P4+Mibefradil+NNC and (viii) P4+EGTA. The calcium chelator EGTA (2.5 mM) was used to chelate the calcium ions in the media. Experiment 2: BOECs were treated for 30 mins with: (i) No treatment (control), (ii) P4, (iii) Mifepristone, (iv) AG205, (v) Mifepristone+AG205, (vi) P4+Mifepristone, (vii) P4+AG205 and (viii) P4+Mifepristone+AG205. For the assessment of unbinding in both experiments a 10µL droplet was placed on a pre-warmed slide and covered with a coverslip. Ten fields of view were assessed per slide on a heated stage (37°C) at 400x, under a half light, half fluorescence. Sperm were classified as bound if the head was in contact with the BOEC explant. A micrometre, placed in the eyepiece of the microscope, was used to measure the surface area of each explant and the number bound per mm^2 was determined. The assessor was blinded to the treatments and three replicates of each experiment were completed. Data were analysed using a univariate ANOVA (IBM SPSS, version 22). Post hoc tests were conducted using the Bonferroni test and results were reported as the mean ± standard error of the mean (s.e.m.). $P<0.05$ was considered statistically significant.

Results P4 stimulated the release of bound sperm from BOEC explants ($P<0.001$; Figure 1). The action of NNC, Mibefradil and the calcium chelator, EGTA, inhibited the release of sperm from BOEC explants induced by P4 ($P<0.001$). In the absence of P4, Mifepristone and AG205 alone or in combination did not affect the release of sperm from BOECs ($P>0.05$). The release of sperm from BOEC explants by P4 was inhibited in the presence of Mifepristone ($P<0.0001$) and AG205 ($P<0.01$; Figure 1). AG205 and Mifepristone in combination had the greatest inhibiting action of P4 to release sperm.

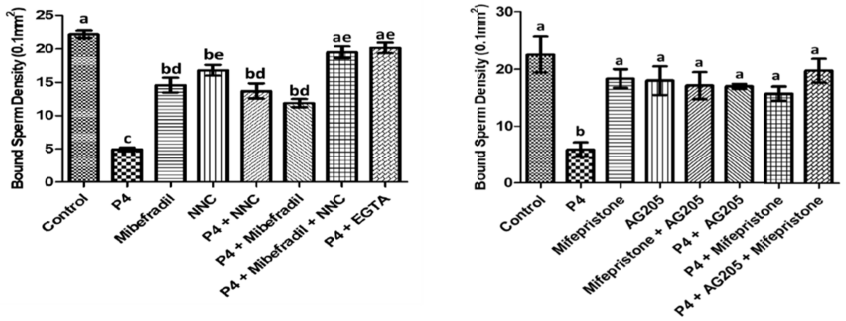


Figure 1 Release of sperm from BOEC explants of frozen-thawed bull sperm treated with A) Progesterone (P4), calcium channel antagonists (NNC and Mibefradil) and calcium chelator (EGTA), or B) Progesterone (P4) and Progesterone receptor antagonists (AG205 and Mifepristone).

Conclusion These findings suggest the presence of a P4 membrane receptor on bull sperm and that P4 is capable of inducing the release of sperm from the bovine oviductal epithelium. This P4 induced release is mediated by extracellular Ca^{2+} . This study has increased our understanding of sperm interaction with the female reproductive tract and may aid in the development of fertility biomarkers in bovine reproduction.

References

Strunker T, Goodwin N, Brenker C, Kashikar ND, Weyand I, Seifert R and Kaupp UB 2011. Nature. 471**,** 382-386.

https://doi.org/10.1017/S2040470018000109 Published online by Cambridge University Press

Variation in the field fertility of dairy bulls used in AI

E M Donnellan[1], M M Kelleher[2], S Fair[1]
[1]*University of Limerick, Ireland,* [2]*Irish Cattle Breeding Federation, Co.Cork, Ireland*
eimear.donnellan@ul.ie

Application This study characterises the variation in field fertility of dairy bulls, used in AI with apparently normal semen quality, which is the first step in addressing its amelioration.

Introduction Using AI semen from an individual bull can be used to inseminate tens of thousands of cows and, therefore, the impact of reduced fertility of individual bulls can have significant economic losses for farmers. To protect against this, animal breeding centres worldwide use a range of microscopy based pre and post-freeze quality control checks of sperm motility and morphology prior to releasing semen into the field. Despite these stringent checks, the fertility of bulls with apparently normal sperm characteristics vary significantly in the field. The objective of this study was to quantify this level of variation.

Material and methods Field fertility data were collated from bulls of dairy breeds (Holstein, Friesian, Jersey, Montbeliarde, Norwegian Red) used on Irish farms over an 8-year period from 2010-2017. A minimum cut off of 500 inseminations per bull was then used to select bulls with a reasonably reliable phenotype (Amann and DeJarnette 2012), leaving 980 bulls in the dataset. Sire fertility was expressed as an overall adjusted mixed regression model which adjusted the conception rate for factors such as herd, cow genotype/parity, price of straw, days in milk, semen type (frozen, fresh), month of service, day of the week when serviced, service number, cow genotype, herd, AI technician and bull breed resulting in an adjusted pregnancy rate centred around 0% (Holden *et al.* 2017). All bulls were plotted relative to the mean of the population. All results are presented as mean ± SD.

Results The mean number of inseminations per bull was 5,397 (range: 500 – 99,953) and their mean adjusted fertility was 0% ± 3.32%. In all, 79% of bulls fell within one SD from the mean (between -3.32% and 3.32 %) while 95.9% of bulls fell within two SD from the mean (between -6.64% and 6.64 %). There was 4% of bulls lower than two SD and <1% of bulls above two SD (Figure 1).

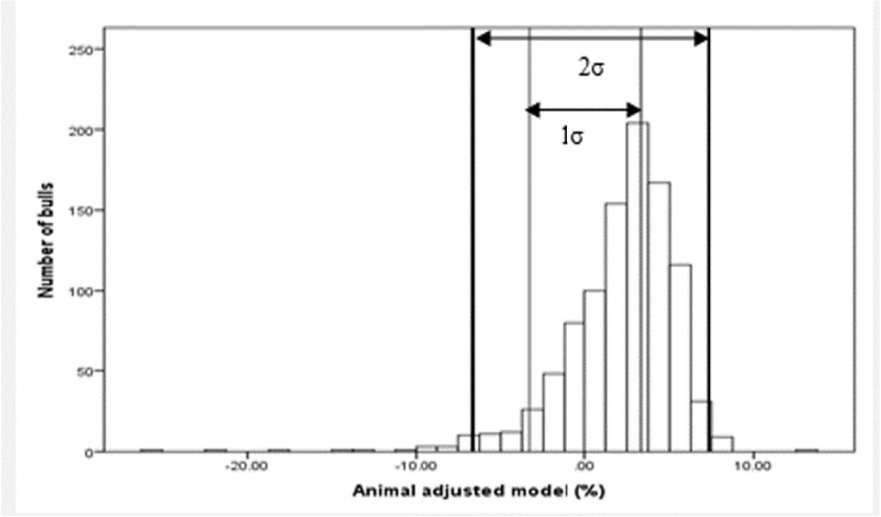


Figure 1 Adjusted animal model of bull fertility phenotypes calculated for all 980 sires with >500 inseminations relative to population mean (0%) and SD (3.32).

Conclusion This database provides a clear, quantifiable overview, based on at least 500 repeated measures per sire, of the variation in bull fertility used for AI in the Irish dairy herd. It shows that the quality control measures used in Irish AI centres are effective in minimising the number of bulls with significantly lower fertility relative to the population mean as only a very small minority of bulls fall below 2 standard deviations of the mean.

Acknowledgements The authors acknowledge the contribution of the Irish AI centres and funding from the Irish Research Council (Grant ID:GOIPG/2017/1884).

References

Amann, R.P. and DeJarnette, J.M. 2012. Theriogenology, 77, 795-817.
Holden, S.A., Fernandez-Fuertes, B., Murphy, E.M., Lonergan, P. and Fair, S. 2017, Reproduction Fertility and Development. 29, 2457-2465.

https://doi.org/10.1017/S2040470018000109 Published online by Cambridge University Press