INTERPRETATIVE SUMMARY:

The ability to predict the fertility of a bull ahead of the widespread use of his semen through artificial insemination (AI) is a crucial factor that can determine the success of an AI program in dairy cattle. The purpose of the current study was to not only identify biomarkers of bull fertility and use these to develop a predictive model, but to enhance our understanding of common aetiologies underlying low fertility in bulls. The application of knowledge gained in this research could lead to the improved detection of bulls of lower fertility, thus avoiding the consequences associated with extensive dissemination of their semen in the field.

RUNNING HEAD: Sperm attributes characterizing dairy bull fertility

TITLE: Comprehensive functional analysis reveals that acrosome integrity and viability are key variables distinguishing AI bulls of varying fertility

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ABSTRACT

In vitro methods of assessing bull semen quality in artificial insemination (AI) centers are unable to consistently detect individuals of lower fertility and attempts to reliably predict bull fertility are still ongoing. This highlights the need to identify robust biomarkers that can be readily measured in a practical setting and used to improve current predictions of bull fertility. In this study, we comprehensively analyzed a range of functional, morphological and intracellular attributes in cryopreserved spermatozoa from a selected cohort of Holstein Friesian AI bulls classified as having either high or low fertility (n = 10 of each fertility phenotype; difference of 11.4% in adjusted pregnancy rate between groups). Here, spermatozoa were assessed for motility and kinematic parameters, morphology, acrosome integrity, plasma membrane lipid packing, viability (or membrane integrity), superoxide production and DNA integrity. In addition, spermatozoa were used for *in vitro* fertilization in order to evaluate their capacity for fertilization and successful embryo development. The information collected from these assessments was then used to phenotypically profile the two groups of bulls of divergent fertility status as well as develop a model to predict bull fertility. According to the results, acrosome integrity and viability were the only sperm attributes that were significantly different between high and low fertility bulls. Interestingly, while spermatozoa from low fertility bulls, on average, had reduced viability and acrosome integrity, this response varied considerably from bull to bull. Principal component analysis (PCA) revealed a sperm phenotypic profile that represented a high proportion of ejaculates from low fertility bulls. This was constructed based on the collective influence of several sperm attributes, including the presence of cytoplasmic droplets and superoxide production. Finally, using the combined results as a basis for modelling, we developed a linear model that was able to explain 47% of the variation in bull field fertility in addition to a logistic predictive model that had a 90% chance of distinguishing between fertility groups. Taken together, we conclude that viability and acrosome integrity could serve as fertility biomarkers in the field and when used alongside other sperm attributes, may be useful in detecting low fertility bulls. However, the variable nature of low fertility bulls suggests that additional, in-depth characterization of spermatozoa at a molecular level is required to further understand the aetiology of low fertility in dairy bulls.

KEYWORDS: Bull fertility, sperm attributes, flow cytometry, principal component analysis, predictive modelling

INTRODUCTION

The success of artificial insemination (AI) programs in cattle is dependent on a wide range of factors, not least the fertility of the bull and semen used (Kumaresan et al., 2017). For this reason, AI centers have implemented quality control measures to ensure bulls destined to be used for insemination have passed minimum post-thaw standards of semen quality, which traditionally consists of microscopic assessments of sperm motility and morphology (Thundathil et al., 2016, Harstine et al., 2018). While the purpose of these *in vitro* assessments is to identify and eliminate bulls (or ejaculates) with poor field fertility, it has been observed that individual bulls that pass the post-thaw quality control checks can still vary in field fertility by up to 25% (Larson and Miller, 2000). This can have major implications at farm level, where the wide-scale use of semen from low fertility bulls could contribute to delays in conception, a prolonged calving season and high levels of involuntary culling, especially in seasonal grass-based systems (Kastelic and Thundathil, 2008). In an attempt to mitigate against this, AI centers often inflate the number of spermatozoa within insemination doses. However, this has been shown to be only effective against compensable sperm defects whereby fertility is typically improved with increasing sperm number until a threshold level is reached (Saacke et al., 1994). This strategy limits the supply of straws that can be processed from an individual ejaculate and disseminated into the field, and is especially problematic for young, genomically selected dairy bulls which are in high demand but produce low quantities of semen.

In an effort to detect these bulls of lower fertility that have normal post-thaw semen parameters, there have been ongoing attempts to identify reliable *in vitro* biomarkers of fertility outside of those routinely assessed in AI centers. The higher throughput and in-depth information provided by technologies such as computer-assisted sperm analysis (**CASA**) and flow cytometry has provided researchers with the tools to study the relationship between bull fertility and different sperm characteristics. Through these studies, bull fertility has been shown to be associated with attributes such as sperm motility and kinematics (Farrell et al., 1998, Kutchy et al., 2019), morphology (Ostermeier et al., 2001, Gillan et al., 2008), viability (Gliozzi et al., 2017), acrosome integrity (Kumaresan et al., 2017), the degree of reactive oxygen species (ROS) production (Simões et al., 2013, Kumaresan et al., 2017), the incidence of DNA fragmentation (Karoui et al., 2012, Dogan et al., 2015) as well as cleavage rate following *in vitro* fertilization (Ortega et al., 2018). One of the most comprehensive functional-based studies published to-date is that by Sellem et al. (2015), who proposed a statistical model that accounted for 40% of the variation in bull fertility based on seven CASA and flow cytometric variables. Despite extensive work already completed in this field, there is still a considerable proportion of variation in bull fertility that is yet to be explained. This highlights the need for further research in order to identify an optimal combination of *in vitro* variables that can be used to reliably and repeatedly predict bull fertility.

To build upon the work that has already been established in this field, predictive models of fertility are required to not only be practical in a research setting but that have the potential to be applied in the field (Harstine et al., 2018). To successfully develop such a model, it is important to first recognize factors that can limit the ability to predict the fertilizing potential of a bull and determine how they can be best managed to minimize their effects (Mocé and Graham, 2008). One of these factors is the

selection of a starting bull population. Studies attempting to predict fertility can be compromised when the observed fertility status of a bull is based on a relatively small number of inseminations. If this issue is then coupled with a minimal divergence in fertility that pre-exists between classified groups, there is a relatively small chance of either detecting or reliably explaining variation in fertility among bulls (Utt, 2016). It is, therefore, imperative that future research in this field takes such factors into consideration in order to more accurately establish cohorts of bulls that are sufficiently divergent in field fertility status. Consequently, this will facilitate a more accurate and robust discrimination of underlying differences in sperm biochemistry and functional capacity.

Aside from the selection of bulls, another limiting factor is the selection of sperm attributes to assess (Mocé and Graham, 2008, Utt, 2016). To focus on the measurement of a small number of sperm attributes, even if these were frequently observed in similar studies, means restricting the capacity to pinpoint where reproductive wastage is occurring in a representative population of bulls. Considering the complexity of events leading up to and during fertilization, it is unlikely that a single sperm attribute will be able to reflect an individual's fertility (Oliveira et al., 2013). For this reason, a multifactorial approach towards selecting sperm attributes for *in vitro* assessment (both compensable and non-compensable) is required (Utt, 2016). By exploring a wide range of sperm attributes, it increases the chances of capturing potential biomarkers associated with lower fertility. The identification of these biomarkers will, not only be vital for predictive modelling, but will help to advance our knowledge of the possible aetiologies underlying lower fertility and provide a scaffold from which to build a phenotypic profile of spermatozoa from these bulls.

Taken together, the objectives of this study were to i) perform a comprehensive analysis of sperm functional, morphological and intracellular attributes spanning from insemination to fertilization in two selected groups of Holstein Friesian AI bulls of divergent field fertility status and ii) use these data to explore the phenotypic profile of spermatozoa from bulls with a specified fertility and develop a predictive model of bull field fertility using sperm attributes of interest.

MATERIALS AND METHODS

Bull selection

A base population of Holstein Friesian bulls was formed (840 bulls; Figure 1) from which the panel of bulls used in this study were selected. The cryopreserved semen from these bulls was used commercially in Ireland and all bulls had a minimum of 500 inseminations. Data on the field fertility for these bulls that formed the base population were obtained from the Irish Cattle Breeding Federation (ICBF) database based on a previously validated sire fertility index (Berry et al., 2011). Sire fertility was defined as pregnancy to a given service identified retrospectively either from a calving event or where a repeat service (or a pregnancy diagnosis) deemed the animal not to be pregnant to the said service. Cows and heifers that were subsequently culled or died on farm were omitted. These raw data were then adjusted for factors including semen type (frozen, fresh), cow parity, month of service, day of the week when serviced, service number, cow genotype, herd and AI technician. The adjusted sire fertility

index given for each bull was then weighted for the number of service records, resulting in an adjusted pregnancy rate (Figure 1; mean = 0%). For this study, bulls were selected from this base population ensuring that the minimum divergence in adjusted pregnancy rate between high and low fertility groups was 10% (n = 20; Table 1). Those selected bulls classified as having high fertility (HF) had an average adjusted pregnancy rate of +3.9±0.19% (n = 10; inseminations (mean) = 22,229; inseminations (median) = 10,048), whereas those classified as low fertility (LF) had an average of -7.5±1.47% (n = 10; inseminations (mean) = 6587; inseminations (median) = 1001). The divergence in adjusted pregnancy rate between the selected fertility cohorts was 11.4%.

Experimental design

Experiment 1a: Assessment of sperm functionality pre-fertilization

This experiment was designed to characterize functional, morphological and intracellular attributes in spermatozoa from bulls of divergent field fertility status with the purpose of identifying potential biomarkers of *in vivo* fertility. For each of the bulls selected (HF = 10 bulls; LF = 10 bulls), a minimum of three ejaculates were assessed for DNA integrity along with a range of sperm attributes that are required up until fertilization (60 ejaculates in total) at 0, 3 and 6 h post-thaw. These included motility and kinematic parameters, morphology (0 h only), viability, acrosome integrity, plasma membrane fluidity as well as superoxide production.

Experiment 1b: In vitro fertilization (IVF) and assessment of embryo development

This experiment was designed to examine differences in oocyte cleavage and embryo development following *in vitro* fertilization (IVF) in bulls of divergent field fertility status. A subset of the panel of bulls from Experiment 1a were selected (HF = 6 bulls; LF = 4 bulls), with a minimum of three ejaculates per bull used for IVF. Oocyte cleavage (48 hours post insemination; 48 hpi) was assessed on Day 2 and blastocyst yield was assessed on Days 6, 7 and 8-post insemination.

Experiment 2: Predictive models for bull field fertility

This experimental analysis was designed to build predictive models (linear and logistic regression) for bull field fertility using the functional, morphological and intracellular sperm attributes assessed in Experiment 1a. For the linear regression, all selected bulls (HF = 10 bulls; LF = 10 bulls) were used for the development of this model, where the response variable was the observed adjusted pregnancy rate (%) determined for each bull. For the logistic regression, all selected bulls (HF = 10 bulls; LF = 10 bulls) and their respective ejaculates (3 ejaculates per bull) were used for the development of this model. For this model, bulls and their ejaculates were assigned with the binomial response, low fertility or not of low fertility, which corresponded to those bulls presenting with high fertility.

Incubation media

All chemicals for incubation media were obtained from Sigma-Aldrich (Arklow, Ireland) and were of the highest reagent grade possible. The basal medium used for Experiment 1a was a modified Tyrode's

media supplemented with lactate and pyruvate (TALP) and devoid of bicarbonate and Bovine Serum Albumin (BSA) (Parrish et al., 1988). TALP consisted of 2 mM $CaCl_2$, 3 mM KCl, 0.4 mM $MgCl_2$, 90 mM NaCl, 0.3mM NaH_2PO_4, 10 mM HEPES, 25.4 mM Sodium lactate, 5 mM D-glucose and 1 mM Sodium pyruvate. To compensate for the omission of bicarbonate and BSA, the concentration of NaCl was increased from 90 to 115 mM and 0.5 mg/mL of polyvinyl alcohol (PVA) and polyvinylpyrrolidone (PVP) was supplemented to media, respectively. Prior to using modified TALP in experiments, the pH of media was adjusted to 7.3 with NaOH.

Semen Preparation

For Experiment 1a, straws of cryopreserved semen were thawed for 30 s in a 37˚C water-bath following which spermatozoa were washed (300 x *g*, 5 min) once in modified TALP before the concentration of spermatozoa was determined using a haemocytometer. For the assessment of motility, kinematic parameters, morphology and DNA fragmentation, samples were diluted to a final concentration of 15 x 10^6 sperm/mL with modified TALP. For all remaining flow cytometric-based assessments, samples were diluted to 2 x 10^6 sperm/mL with modified TALP. All samples were incubated for up to 6 h, with measurements taken at 0, 3 and 6 h, unless otherwise stated.

Computer-assisted sperm analysis

Motility and kinematic parameters were assessed by CASA using the Sperm Class Analyzer system (SCA; Microptic S.L.; Barcelona, Spain). At each time point, 3 µL of sample was placed in a pre-warmed Leja chamber (20 µm depth; IMV Technologies; L'Aigle, France) and analyzed for sperm motion characteristics using factory programmed CASA settings for bull spermatozoa. A minimum of 8 randomly selected fields were recorded and analyzed (at least 200 spermatozoa) using a phase contrast microscope (CX41; Olympus; Centre Valley, USA) with a fitted heated stage set at 37 °C. Manual corrections to the videos captured were performed as required (i.e. adding or deleting sperm and/or debris). The following CASA-derived parameters were assessed for each sample: total motility (TM), progressive motility (PM), straight-line velocity (VSL; µm/s), average path velocity (VAP; µm/s), curvilinear velocity (VCL; µm/s), amplitude of the lateral head displacement (ALH; µm), linearity (LIN; %), straightness (STR, %), beat cross frequency (BCF; Hz) and wobble (WOB).

Morphology assessment

Sperm morphology was assessed using nigrosin-eosin as previously described (Holden et al., 2017). An aliquot of sample was combined with nigrosin-eosin (1:1; sample:stain) before being smeared onto a glass slide and air dried. Morphological abnormalities were categorized according to those present in the head, acrosome, mid-piece and tail as well as the presence of cytoplasmic droplets (proximal and/or distal; categories for morphology assessment adapted from (Boe-Hansen et al., 2018)). All slides were examined under 1000 X with oil immersion (BX60; Olympus) and a minimum of 200 spermatozoa were assessed per sample.

Flow cytometric assessment of sperm function

Sperm functional assessments were performed on a CytoFLEX flow cytometer from Beckman Coulter (Labplan; Dublin, Ireland). CytoFLEX daily quality control fluorospheres (Beckman Coulter) were used prior to each experiment to verify the optical alignment. A sperm specific population was gated following identification with side and forward scatter. The positioning of this gating to detect the sperm population was verified by labelling a semen sample from a single reference bull (reference sample) with 1 µg/mL Hoechst 33342 (excited by 405 nm laser and detected with a 450/45 nm band-pass filter). For all assessments, 10,000 events were recorded (unless otherwise stated) and analysis performed in CytExpert software. For each parameter measured, the area of the signal pulse was used during data collation and a reference sample was included for each functional assessment to monitor day-to-day variation. Merocyanine 540 (**M540**) and all chemicals associated with buffers or staining solutions were sourced from Sigma Aldrich (Arklow, Ireland) whereas Alexa Fluor 647-peanut agglutinin (**AF647-PNA**), 4′,6-diamidino-2-phenylindole (**DAPI**), Sytox Green, MitoSOX Red and Acridine Orange (**AO**) were all sourced from Invitrogen (Biosciences; Dublin, Ireland).

Membrane fluidity, acrosome and viability

AF647-PNA, M540 and DAPI were used in combination for the simultaneous analysis of acrosome integrity, membrane fluidity and viability (as detected by membrane integrity), respectively. At each time point during incubation, samples were labelled with a final concentration of 0.5 µg/mL AF647-PNA, 0.8 µM M540 and 3 µM DAPI for 15 min at 37˚C prior to assessment. DAPI, M540 and AF647-PNA were excited using a 405, 488 and 635 nm laser and detected with a 450/45, 585/42 and 660/10 nm band-pass filter, respectively (Supplementary Figure 1). Compensation was performed through the CytExpert software to correct for spectral overlap.

Superoxide production

The combination of MitoSOX Red and Sytox Green was used to assess superoxide (SO) production in viable (as detected by membrane integrity) spermatozoa. At each time point during incubation, samples were labelled with a final concentration of 2.5 µM MitoSOX Red and 30 nM Sytox Green for 20 min at 37˚C prior to assessment. As a positive control for SO production, a reference sample was incubated with 25 µM arachidonic acid for 10 min (Aitken et al., 2013) prior to labelling. MitoSOX Red and Sytox Green were excited using a 488 nm laser and detected with a 585/42 and 525/40 nm band-pass filter, respectively (Supplementary Figure 2). Compensation was performed through the CytExpert software to correct for spectral overlap.

DNA integrity

The susceptibility of sperm chromatin to DNA fragmentation was assessed using AO. As a positive control for DNA fragmentation, a reference sample was incubated with 0.8 M HCl for 5 min at 37˚C prior to assessment. Samples were prepared and stained with AO according to the protocol described by Evenson and Jost (2000). AO was excited using a 488 nm laser and green and red fluorescence was detected with a 525/40 nm or 690/50 nm band-pass filter, respectively. During data acquisition, the flow

rate was adjusted to approximately 200 events/s and 5000 events (in the sperm specific gate) were recorded for analysis. The population with high red and low green fluorescence was identified as spermatozoa with high DNA fragmentation (Supplementary Figure 3). In addition, a population of spermatozoa with low red and very high green fluorescence was identified as having high DNA stainability (HDS). It is hypothesized that this population represents a decrease in chromatin condensation owing to inadequate sperm maturation (Evenson and Jost, 2000).

Oocyte recovery and *in vitro* maturation, fertilization and embryo culture

In vitro maturation was carried out as previously described by Ward et al. (2001). Briefly, cumulus-oocyte complexes (COCs) were recovered by aspirating follicles (2-8 mm) from the ovaries of slaughtered heifers and cows. Good quality COCs were washed in PBS and matured for 24 h in groups of 50 in 500 µL of TCM-199 supplemented with 10% fetal calf serum (v/v) and 10 ng/mL epidermal growth factor at 39˚C under an atmosphere of 5% CO_2 in air with maximum humidity.

The concentration of frozen-thawed spermatozoa from high and low fertility bulls was assessed using a haemocytometer and diluted to 2 x 10^6 sperm/mL with fertilization medium. Before insemination, matured COCs were washed twice in fertilization medium and transferred in groups of 50 into four-well dishes containing 250 µL of fertilization medium. To each well, 250 µL of fertilization medium containing 2 x 10^6 sperm/mL was added to give a final concentration of 1 x 10^6 sperm/mL with mature COCs (Day 0). Each bull was allocated approximately 100 COCs (in two groups of 50) for IVF. Gametes were co-incubated for 20 h at 39˚C in an atmosphere of 5% CO_2 in air with maximum humidity. Presumptive zygotes were denuded by gentle vortexing and cultured in synthetic oviduct fluid droplets (25 µL droplets under mineral oil; 25 embryos per droplet) at 39˚C in a humidified atmosphere with 5% CO_2 and 5% O_2 up until Day 8.

Statistical analysis

All statistical analyses were performed using R (Team, 2019). Data collected from Experiment 1a and 1b were first analyzed using a linear mixed model in order to identify sperm attributes that differed between fertility groups. Fertility and incubation time (where assessed) were set as fixed effects in the model, whereas bull and ejaculate were included as nested random effects. Other confounding factors such as cryopreservation media or AI center were also included in the random model. Normality and homoscedasticity of the residuals were assessed for all models by use of Shapiro-Wilk test and Bartlett's test, respectively. Identification of statistical outliers was assessed by Cooks Distance and observations were removed when necessary. Pairwise comparisons between levels of significant fixed effect/s were determined using a Tukey adjustment. If a log transformation was performed to improve normality or homoscedasticity of the residuals, the results were back-transformed and presented as the geometric mean ± back-transformed s.e.m. Otherwise, data are presented as the mean ± s.e.m.

A principal component analysis (PCA) was performed in addition to linear mixed models as a way to further explore patterns in the data associated with fertility groups and the relationship between the sperm attributes measured in Experiment 1a and 1b. For Experiment 1b, fertilization and embryo development data were combined with functional data collected in Experiment 1a in order to observe

the contribution of both on fertility group-associated patterns. Prior to analysis, variables were log transformed if necessary, then standardized by centering and scaling (mean = 0; SD = 1) (Boligon et al., 2016). Linear combinations of the original measured variables were generated using the method, singular value deposition (SVD), to construct principal components (PCs). The PCs successively model maximum variation in the data, where each measured variable will contribute differently to the PCs (Ringnér, 2008). This contribution is best observed by the loadings of a variable, which represent the strength and direction of that contribution on a PC. PCs with an eigenvalue > 1.0 were retained for further analysis (Kaiser, 1958), which was followed by an assessment of the percent variation in the data explained by each PC. If the first two PCs were able to explain close to 50% of the variation in the data, these were used to create a biplot, a visual representation of the PCA output. This biplot presents the loadings of each variable (arrows) and the distribution of bulls according to their position on the PC1 and PC2 axes. The distribution of bulls within each fertility group was highlighted by means of 95% confidence interval ellipses. In addition to the biplot, the top 10 variables with the highest loadings for PC1 and PC2 axis were determined.

Finally, the extensive functional data measured in Experiment 1a were used to build two predictive models for bull fertility, a linear and logistic regression model (*Experiment 2* describes modelling outcomes in Results). These two modelling approaches were taken in order to examine their differential predictive ability. While a linear regression uses fertility estimates as the response variable and is more commonly used in similar studies, the logistic regression uses a classified group of bulls belonging to a fertility cohort. As such, this allows for the identification of sperm attributes that are best able to predict this classified group as opposed to a specific fertility estimate. Prior to the development of both the linear and logistic regression, variables were log transformed, if necessary and the full model was systematically reduced to only include the most significant predictors. The following diagnostics were performed for the final models: identification and removal of outliers using Cooks Distance, checking for model normality (linear regression only) or overdispersion (logistic regression only) and multicollinearity. For the final linear model, the percent variation in bull fertility explained (adjusted r^2) was determined and for the final logistic model, the ability to reliably discriminate between the fertility groups was assessed by a Receiver Operating Characteristic (ROC) curve and associated area under this curve (AUC).

RESULTS

Experiment 1a – Viability and acrosome integrity represent biomarkers of fertility

In this analysis of sperm attributes in bulls with high and low fertility, there was no interaction between fertility and time for any of the variables assessed ($P>0.05$). There was also no difference between fertility groups in motility and kinematics, morphological abnormalities, plasma membrane fluidity, superoxide production, DNA fragmentation or high DNA staining (Table 2). However, the percentage of viable spermatozoa and those with intact acrosomes were found to differ between fertility groups across the incubation period (Table 2 and Figure 2; $P<0.05$). Bulls classified with high fertility were found to have a 13.5% increase, on average, in the percent of viable spermatozoa when compared to low fertility

bulls (HF: 47.6±3.0% vs LF: 34.1±5.8%). Comparably, the high fertility bulls also presented with a 9.1% higher, on average, in the percent of spermatozoa with intact acrosomes when compared to low fertility bulls (HF: 75.5±2.0% vs LF: 66.4±4.2%). Interestingly, the bull to bull variation for these sperm attributes was much higher in the low compared to the high fertility group (Table 2 and Figure 2). Here, the percentage of viable spermatozoa or those with intact acrosomes ranged from 0.2-68.9% and 32.1-84.6%, respectively, for bulls in the low fertility group. In addition to the differences observed between fertility groups, all variables were found to vary across time, with the exception of the kinematic parameter, straightness, viable spermatozoa with high membrane fluidity, membrane fluidity as assessed by M540 median fluorescence, DNA fragmentation and high DNA staining (Supplementary Table 1). Those variables that differed over time primarily decreased over the 6 h period, a response that would be expected following an extended incubation of processed spermatozoa.

To further explore potential differences between fertility groups, the dataset was subjected to a PCA. Using PC1 and PC2 to produce a visual representation of the analysis (biplot), it was notable that low fertility bulls tended to cluster to the upper left quadrant of the biplot, while high fertility bulls were more dispersed across the remaining quadrants (Figure 3). The clustering of low fertility bulls in this quadrant was owing to the contribution of several sperm attributes, most notably, superoxide production in viable spermatozoa, the presence of cytoplasmic droplets, DNA fragmentation, high DNA staining as well as kinematic parameters like beat-cross frequency, linearity and wobble. In contrast, the distribution of high fertility bulls was influenced by sperm viability, total and progressive motility, the presence of an intact acrosome, plasma membrane fluidity and kinematic parameters such as curvilinear velocity and amplitude of lateral head displacement (Figure 3). For high and low fertility bulls, these sperm attributes were also confirmed as having the greatest contribution on PC1 and PC2 as shown by the length of the arrows in the biplot (Figure 3) and calculated loadings (Table 3). While a high proportion of bulls (and ejaculates) presented with sperm attributes that represented their respective fertility group, there were cases where bulls (particularly those of low fertility) presented with a profile that contrasted their true fertility group (Figure 3).

Experiment 1b – Fertilization and early development attributes are not reliable indicators of fertility in this subset of bulls

In this subset of Holstein Friesian AI bulls, there were no differences in the rate of cleavage (48 hpi) or blastocyst development between high and low fertility bulls following IVF (Supplementary Table 2; $P>0.05$). Although, it was interesting to note that again, there was more variability in each of these measured variables for low fertility bulls compared to those of high fertility (Supplementary Table 2). Using cleavage rate as an example, this variable ranged from 50.0-96.4% in high fertility bulls whereas for low fertility bulls, cleavage rate ranged from 15.6-93.1%.

On examining the PCA for this subset of bulls with fertilization, embryo development and functional data, there was again clustering of fertility groups, although this time, the low fertility bulls were clustered in the lower half of the plot while the high fertility bulls were primarily in the upper half (Figure 4). This profile suggests that variables that contribute predominantly to PC2 are the ones of most interest in clustering the fertility groups. This did not include fertilization or embryo development

attributes, as evident by the length of the arrows in the biplot and the calculated loadings. Although, cleavage rate (48 hpi) and blastocyst rate (Day 7 and 8) were identified as strong contributors to PC1 (Figure 4 and Table 4). Instead, the kinematic parameters, straightness, linearity and wobble as well as the presence of cytoplasmic droplets appeared to contribute most to clustering this subset of low fertility bulls (Figure 4 and Table 4). Contrastingly, variables such as total and progressive motility, the percentage of spermatozoa with intact acrosomes or viable spermatozoa with high membrane fluidity appeared to predominantly contribute to the clustering of high fertility bulls (Figure 4 and Table 4).

Experiment 2 – Linear and logistic predictive models

The final linear regression model was composed of the following predictors, all of which were necessary in the model (P<0.05); the presence of tail abnormalities (TaAbLN) and cytoplasmic droplets (CytAbLN) as well as the percentage of spermatozoa with an intact acrosome (ACI).

$$Adjusted\ pregnancy\ rate\ (\%) = -14.12 - (3.74 \times TaAbLN) - (7.15 \times CytAbLN) + (0.27 \times ACI)$$

Together, these three predictors were able to explain 47% of the variation in bull fertility for this selected population (adjusted r^2 = 0.474; Figure 5).

The final logistic regression model following systematic reduction was composed of the following predictors, all of which were necessary in the model (P<0.05); the presence of cytoplasmic droplets (CytAbLN), DNA fragmentation (DFILN), high DNA stainability (HDSLN) and the percentage of viable spermatozoa (VIA).

$$\log(odds\ of\ Low\ Fertility) = -1.21 + (2.98 \times CytAbLN) + (4.44 \times DFILN) - (2.71 \times HDSLN) - (0.11 \times VIA)$$

The AUC of the ROC curve for this logistic regression was a value of 0.90, indicating that there is a 90% chance that this model is able to distinguish between low fertility or not of low fertility bulls, otherwise classified as low or high fertility bulls. The plot of the model's predicted probability of a bull (and ejaculate) having low fertility also appears to reflect the ability of discriminating between groups, with a concentration of ejaculates from low fertility bulls present where the probability of predicting this group is high (~0.7-1.00; Figure 6).

DISCUSSION

The purpose of quality control measures in AI centers is to ensure all bulls pass post-thaw standards of semen quality before dissemination in the national herd (Harstine et al., 2018). However, it has become increasingly apparent that these assessments are not always sufficient to accurately predict a bull's field fertility. Consequently, the reliable identification of low fertility bulls within a population prior to release into the field remains a hindered key objective of the industry. Through our comprehensive analysis of sperm attributes in a selected panel of Holstein Friesian AI bulls of divergent field fertility,

414 we identified viability and acrosome integrity as potential key biomarkers in discriminating between bulls
415 of varying fertility and developed sperm phenotypic profiles. Using this information as a basis for
416 modelling, we produced a linear and logistic predictive model, the former explaining 47% variation in
417 bull fertility and the latter having a 90% chance of distinguishing between bulls of low and high fertility.
418 The findings of the multifactorial approach taken to examine sperm attributes between fertility
419 groups are consistent with those of other studies (Sellem et al., 2015, Gliozzi et al., 2017, Kumaresan
420 et al., 2017; Christensen et al., 2011). This verifies the importance of viability and acrosome integrity
421 when it comes to bull fertility. Sperm viability is actually a measure of the integrity of the plasma
422 membrane (Garner and Johnson, 1995), an extremely dynamic structure that has a diverse range of
423 functions aside from acting as a cellular border (Flesch and Gadella, 2000). Following insemination,
424 spermatozoa must still transit up the female reproductive tract, be able to undergo capacitation and
425 fertilize the oocyte, all of which could be compromised if the plasma membrane is damaged and the cell
426 becomes non-viable. Acrosome integrity is vital for successful fertilization, whereby the outer acrosomal
427 and overlying plasma membrane fuse to cause a release of lytic enzymes either just prior to or upon
428 contact with the zona pellucida surrounding the oocyte (Ickowicz et al., 2012). Therefore, if the
429 acrosome prematurely reacts or is damaged during cryopreservation or soon after insemination, the
430 potential for spermatozoa to successfully fertilize will be reduced (Thundathil et al., 1999). Although it
431 is uncertain what factors have predisposed the integrity of the acrosome and plasma membrane based
432 on this study alone, it is plausible that issues in the composition and/or assembly of these cellular
433 structures may inherently exist in low fertility bulls. Interestingly, several studies have already identified
434 a relationship between the lipid profile and quality of spermatozoa (Andersen et al., 2016, Craig et al.,
435 2019). In humans, examining the sperm lipidome in patients who did or did not obtain a successful
436 pregnancy following intracytoplasmic sperm injection revealed key differences in their profiles, including
437 an increase in lipids such as ceramide and sphingomyelin in those with unsuccessful pregnancies
438 (Rivera-Egea et al., 2018). Furthermore, Evans et al. (2020) demonstrated that the fatty acids, arachidic
439 and oleic acid, were increased in the polar lipid fraction of bull spermatozoa with high post-thaw viability
440 (good freezeability) compared to those with low post-thaw viability (poor freezeability). Given the
441 apparent association between sperm lipids and quality, it would be of interest to further explore the bull
442 sperm lipidome in this panel of bulls to determine whether this may explain the decline in plasma
443 membrane or acrosome integrity.
444 Aside from differences between fertility groups with respect to the sperm attributes measured in this
445 study, an interesting finding was the apparent variability between individual bulls for many of these
446 attributes, particularly those with low fertility. Clear examples of this variability were observed for viability
447 and acrosome integrity, in addition to head abnormalities, presence of cytoplasmic droplets as well as
448 fertilization and early embryo development. This inherent variability suggests that bulls within the low
449 fertility population are unlikely to have lower fertility for the same reason. Since it is generally accepted
450 that the fertilizing capacity of an individual is far too complex to be dictated by a single or even a couple
451 of sperm attributes (Amann and Hammerstedt, 1993, Mocé and Graham, 2008, Oliveira et al., 2013), it
452 would be unreasonable to assume that viability and acrosome integrity can completely explain
453 differences in bull fertility. For this reason, exploring the data using a PCA offered the opportunity to

examine the collective influence of all measured sperm attributes on patterns that may exist owing to differences in fertility. By doing so, an apparent sperm phenotypic profile for high and low fertility bulls that was described by several sperm attributes was observed. This phenotypic profile is not only useful to select additional biomarkers that could be employed to predict bull fertility, it also provides information on the potential aetiologies underlying lower fertility in these bulls.

Following the extensive exploratory analysis of the sperm attributes in this study, two different models were built, both distinct in their ability to predict bull fertility. While the vast majority of models developed to predict bull fertility use fertility estimates as the response variable (Sellem et al., 2015, Gliozzi et al., 2017, Kumaresan et al., 2017), including in the current study, we decided to take an additional approach to modelling and develop a logistic model using the defined fertility groups as a categorical response variable. Modelling based on a group of bulls belonging to a fertility classification allows for the identification of sperm attributes that are best able to predict this group as opposed to a fertility estimate. Without being constrained by specific fertility estimates, a model such as this could be implemented into any AI center as a tool to detect bulls of lower fertility.

Upon examining the predictors in the final logistic model, the percentage of viable spermatozoa, the presence of cytoplasmic droplets, DNA fragmentation and high DNA staining were identified as sperm attributes that could best predict the probability of a bull (and ejaculate) being of lower fertility. It is no surprise that these predictors were either significantly different between fertility groups or were in the list of attributes that constructed the low fertility phenotype as observed through PCA. Aside from the importance of viability for fertility, which has been previously explained, the presence of cytoplasmic droplets and concerns with DNA integrity are also well-known sperm attributes associated with lower fertility in bulls. An increased incidence of cytoplasmic droplets is frequently observed in young bulls but in those that are mature, it is considered to be a sign of abnormal spermiogenesis or epididymal function (Thundathil et al., 2001). In fact, a study examining the relationship between the presence of proximal droplets in bulls and fertility indicated that the fertilizing potential of a bull would decline if semen contained >30% of spermatozoa with this attribute (Amann et al., 2000). This value is substantially higher than that observed in the current study, with only a maximum of 6% of spermatozoa showing this attribute. With this in mind, it may suggest that the incidence of cytoplasmic droplets at such a low level is unlikely to be problematic alone, but it could simply serve as an additional discriminating biomarker to identify bulls of lower fertility.

With respect to sperm DNA integrity, bull fertility has also been shown to correlate with both DNA fragmentation and high DNA staining (Dogan et al., 2015, Kumaresan et al., 2017, Narud et al., 2020). The incidence of high DNA fragmentation is associated with reduced embryo quality and implantation in humans (Virro et al., 2004, Simon et al., 2014) and can be caused by a number of factors, including oxidative stress (Aitken and Krausz, 2001, Wright et al., 2014). Conversely, spermatozoa with high DNA staining are suggested to represent an immature population that lack the full exchange of histones for protamines. These protamines are nuclear proteins that effectively replace histones during spermiogenesis and play a crucial role in chromatin compaction, which works to protect and stabilize the DNA during transit through the epididymis and female reproductive tract (Ward, 2010). Though the presence of high DNA staining has been associated with early miscarriage in humans (Jerre et al.,

2019), there is contradictory evidence regarding its usefulness as a robust test for nuclear immaturity (Mohammadi et al., 2020). With this knowledge, it is important to verify current results with alternative methods that assess for indicators of nuclear maturity, such as chromomycin A3 (Lolis et al., 1996). In any case, with so few predictors in this logistic model, it may be possible to assess these sperm attributes in the routine quality control checks if it meant that there was a high probability of detecting bulls of lower fertility. However, further validation with another panel of bulls is required to ensure its predictive capacity can be extrapolated and still provide the same discriminatory ability.

Despite the high proportion of bulls that exhibited a sperm phenotypic profile consistent with a fertility group and/or were correctly identified using the predictive model, there were low fertility bulls (or their respective ejaculates) which were identified as having high fertility. Interestingly, in the PCA that was performed using functional data alone, a number of ejaculates from high and low fertility bulls were found to occupy a similar distribution when examined visually. Given that viability and acrosome integrity were identified as the only sperm attributes that significantly differed between the fertility groups, this finding is not entirely unexpected. As such, this would suggest that these particular bulls are of low fertility owing to sperm attributes outside of the functional analysis performed in this study and that may exist at the molecular level. It is also worth noting that it is possible for high fertility bulls (and their ejaculates) to present with a phenotypic profile that appears to reflect a low fertility bull, which was evident in the PCA performed with functional and IVF data. While this can depend on factors related to the analysis itself (i.e. sperm attributes and bulls selected for the PCA), it could also be attributed to biological factors such as intra-bull variability (Sellem et al., 2015). In any case, the characterization of sperm attributes from bulls of varying fertility is still necessary for any study attempting to predict bull fertility, in fact combining functional and molecular biomarkers could improve upon the predictive ability of a developed model. Narud et al. (2020) were able to explain an increased proportion of the variation in bull fertility when using DNA fragmentation as a predictor alongside a number of intracellular metabolites. As such, future studies should focus on molecular-based characterization with the aim of building upon the sperm phenotypic profiles already created in the current study and improving the ability to reliably and repeatedly predict bull fertility.

In conclusion, through this comprehensive analysis of various pre and post-fertilization sperm attributes, only viability and acrosome integrity were identified as significant biomarkers of fertility. Further exploratory analysis and the development of predictive models illustrated that the presence of cytoplasmic droplets, DNA fragmentation and high DNA staining are likely having a collective influence on the discrimination of bull fertility. While the sperm attributes identified appear to be important in explaining phenotypic differences in fertilizing capacity, additional, in-depth characterization of spermatozoa at a molecular level would help to better understand the aetiology underlying the variation in bull fertility.

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720 **FIGURES AND TABLES**

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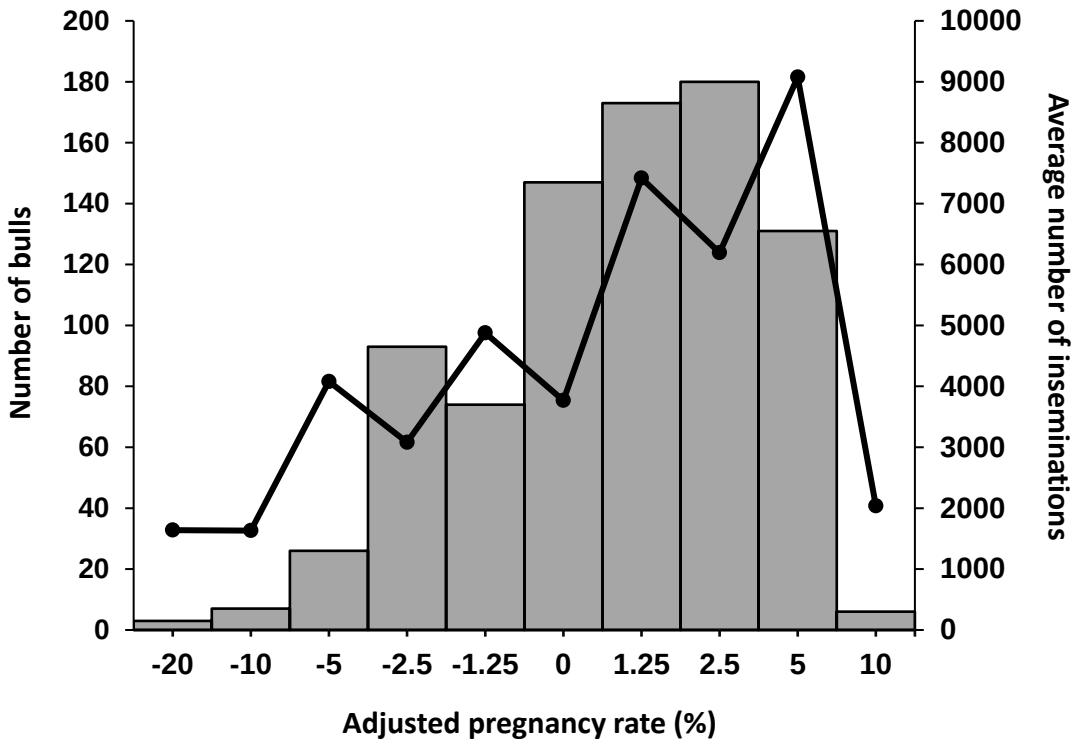
722 

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734 **Figure 1.** Base population of Holstein Friesian AI bulls (n = 840), from which the panel of bulls in this
735 study were selected. Data presented as the frequency of bulls with an adjusted pregnancy rate within
736 a specified range (bars; i.e. < -20 but ≥ -10) and the average number of inseminations for each range
737 (lines).

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Table 1. Panel of bulls selected for experiments in this study including the adjusted pregnancy rate, the number of inseminations used for this estimate and assigned fertility phenotype (High or Low)

Bull number	Adjusted pregnancy rate (mean = 0%)	Number of inseminations	Assigned fertility phenotype
1	-20.23	1723	Low
2	-8.93	1034	Low
3	-7.23	23811	Low
4	-6.63	967	Low
5	-5.73	568	Low
6	-5.63	31148	Low
7	-5.53	908	Low
8	-5.43	4619	Low
9	-4.73	579	Low
10	-4.73	506	Low
11	3.07	11459	High
12	3.07	8637	High
13	3.37	4470	High
14	3.87	17441	High
15	3.87	37849	High
16	3.97	100288	High
17	4.07	2267	High
18	4.07	1771	High
19	4.47	3132	High
20	4.97	34973	High

769 **Table 2.** Range of post-thaw sperm functional, morphological and intracellular variables assessed in
770 Holstein Friesian bulls of high and low fertility (n = 10 bulls per phenotype; Experiment 1a)

Variable[1]	High Fertility[2]	Low fertility[2]	Difference in fertility (P value)[3]
CASA			
Total motility (%)	45.9±5.4 (*24.9-67.0*)	40.5±4.9 (*24.9-64.0*)	NS
Progressive motility (%)	31.9±4.7 (*12.6-50.5*)	29.9±3.9 (*20.8-46.1*)	NS
Curvilinear velocity (VCL; µm/s)	64.7±5.0 (*43.9-84.3*)	70.4±5.2 (*51.2-93.5*)	NS
Straight-line velocity (VSL; µm/s)	36.9±5.0 (*19.5-58.8*)	43.2±4.8 (*28.5-63.3*)	NS
Average path velocity (VAP; µm/s)	43.4±4.8 (*26.3-64.2*)	49.7±4.8 (*37.6-70.5*)	NS
Linearity (LIN; %)	49.1±3.5 (*32.8-62.2*)	54.9±3.4 (*40.6-65.4*)	NS
Straightness (STR; %)	72.1±3.1 (*52.7-82.9*)	77.0±2.8 (*61.9-88.0*)	NS
Amplitude of lateral head movement (ALH; µm)	2.6±0.1 (*2.1-3.3*)	2.6±0.2 (*1.9-3.1*)	NS
Beat cross frequency (BCF; Hz)	8.0±0.6 (*4.8-10.8*)	8.8±0.6 (*6.1-11.2*)	NS
Wobble (WOB; %)	63.9±2.3 (*56.9-72.0*)	67.3±2.3 (*60.7-74.0*)	NS
Morphology (0 h only)			
Normal (%)	68.4±2.3 (*57.5-79.2*)	63.7±3.1 (*51.5-74.8*)	NS
Head abnormalities (%)	5.7±0.7 (*3.3-9.7*)	8.2±1.5 (*3.0-19.2*)	NS
Acrosome abnormalities (%)	18.7±2.1 (*11.3-28.3*)	19.2±2.3 (*9.5-33.5*)	NS
Mid-piece abnormalities (%)	3.3±0.8 (*1.2-8.7*)	3.2±0.9 (*0.8-13.2*)	NS
Tail abnormalities (%)	2.5±0.6 (*1.2-7.2*)	3.3±0.7 (*1.3-6.8*)	NS
Cytoplasmic droplets (Proximal and Distal; %)	0.3±0.1 (*0.0-0.7*)	0.7±0.3 (*0.0-3.0*)	NS
Flow cytometry			
Viable (%)	47.6±3.0 (20.1-71.1)	34.1±5.8 (0.2-68.9)	<0.05
Acrosome intact (%)	75.5±2.0 (59.4-92.8)	66.4±4.2 (32.1-84.6)	<0.05
Viable, low membrane fluidity (%)	39.9±3.0 (*26.9-49.7*)	30.0±5.4 (*5.4-56.0*)	NS
Viable, high membrane fluidity (%)	5.4±1.7 (*1.1-18.5*)	2.9±1.0 (*0.5-7.4*)	NS
M540 fluorescence intensity in viable cells (MFU, 000s)	64.0±11.7 (*23.5-130.4*)	57.5±9.8 (*18.6-98.1*)	NS
MitoSOX fluorescence intensity in viable cells (MFU, 000s)	47.2±2.0 (*28.1-59.3*)	50.1±2.6 (*31.7-69.4*)	NS
DNA fragmentation (%)	1.9±0.5 (0.6-9.6)	2.8±0.4 (1.1-6.2)	NS
High DNA staining (%)	0.6±0.2 (0.1-5.8)	0.7±0.2 (0.1-4.5)	NS

771 [1]Data for all variables were first averaged across ejaculates within bull (3 ejaculates per bull), then
772 across time (0, 3 and 6 h) for presentation (with the exception of morphology and DNA integrity).
773 [2]Results are presented as the mean ± s.e.m. and the range of each measured variable within a fertility
774 group given in parentheses. If the results were log transformed, the geometric mean ± back-transformed
775 s.e.m. is provided.
776 [3]NS = non-significant.
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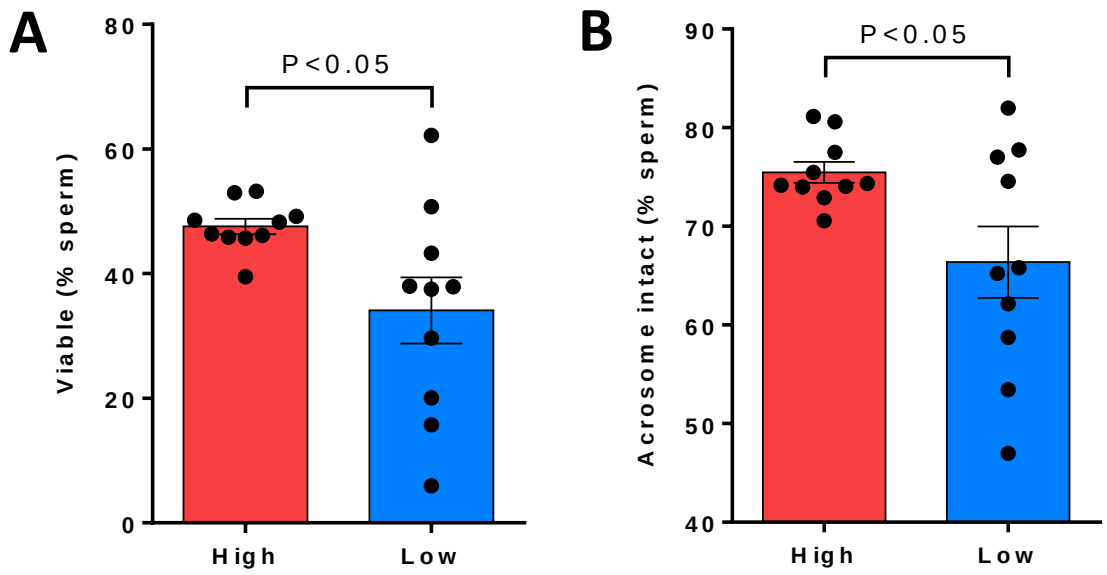

Figure 2. The percentage of frozen-thawed spermatozoa from high (n = 10) or low (n = 10) fertility Holstein Friesian bulls in Experiment 1a that were viable (**A**) or acrosome intact (**B**) following an extended incubation period (6 h). Acrosome integrity and viability (membrane integrity) were assessed with PNA-Alexa Fluor 647 and DAPI, respectively. Each data point represents an individual bull assessed across three separate ejaculates, highlighting the degree of variability observed in these attributes for bulls with low fertility.

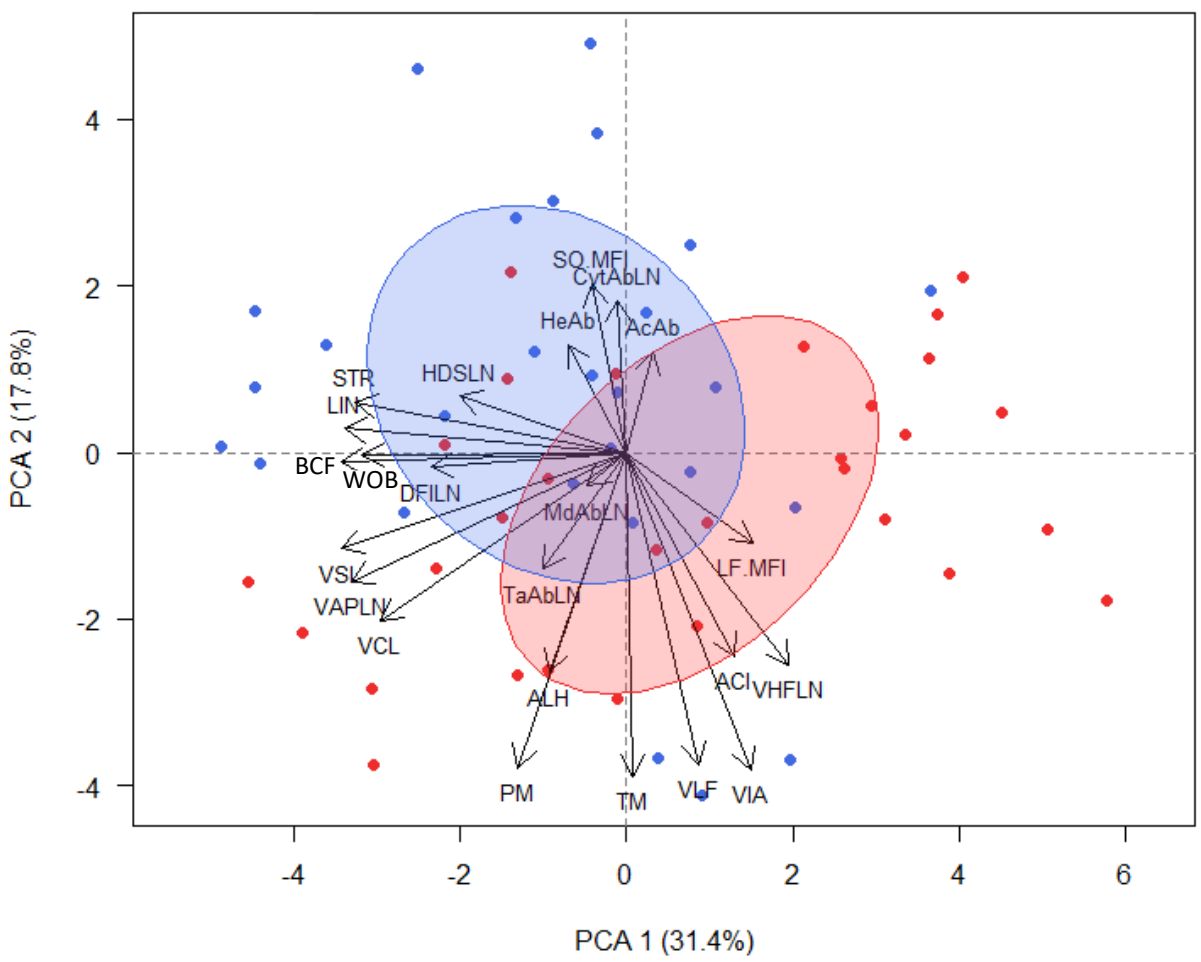

Figure 3. Principal component analysis (PCA) biplot presenting the distribution of bulls (n = 20; Experiment 1a) based on the measurement of various sperm attributes and the contribution of these on Principal Component 1 (PC1) and Principal Component 2 (PC2). The PCs represent linear combinations of the measured variables, for which PC1 and PC2 explain 31.4% or 17.8% of the variation in the data set, respectively. Each sperm attribute is indicated by an arrow, where the length and direction of the arrow reflects the strength and direction of the contribution on a PC and by default, on the distribution of bulls. An individual dot represents the functional profile of an ejaculate from a bull, where all measured variables were first averaged over time (except for morphology) prior to performing this analysis. High and low fertility bulls have been indicated by red or blue dots, respectively, and 95% confidence ellipses were calculated to highlight fertility group clusters.

TM: Total motility; **PM**: Progressive motility; **VCL**: Curvilinear velocity; **VSL**: Straight-line velocity; **VAPLN**: Average path velocity; **LIN**: Linearity, **STR**: Straightness; **ALH**: Amplitude of lateral head movement; **BCF**: Beat cross frequency; **WOB**: Wobble; **HeAb**: Head abnormalities; **AcAb**: Acrosome abnormalities; **MdAbLN**: Mid-piece abnormalities; **TaAbLN**: Tail abnormalities; **CytAbLN**: Presence of proximal or distal cytoplasmic droplets; **VIA**: Viable spermatozoa; **ACI**: Acrosome intact spermatozoa; **VLF**: Viable spermatozoa with low fluidity; **VHFLN**: Viable spermatozoa with high fluidity; **LF MFI**: Median fluorescence intensity of M540 in viable spermatozoa; **SO MFI**: Median fluorescence intensity of MitoSOX Red in viable spermatozoa; **DFILN**: DNA fragmentation index; **HDSLN**: High DNA staining. **LN**=Log transformed prior to analysis.

Table 3. The top 10 variables in Experiment 1a that contribute most strongly to Principal Component (PC) 1 or PC2 (as also observed in Figure 3), which is determined by the loading value

PC1		PC2	
Variable[1]	Loading value[2]	Variable[1]	Loading value[2]
BCF	-0.34	TM	-0.38
VSL	-0.34	VIA	-0.38
LIN	-0.33	PM	-0.37
VAPLN	-0.32	VLF	-0.37
STR	-0.32	ALH	-0.26
WOB	-0.31	VHFLN	-0.25
VCL	-0.29	ACI	-0.24
DFILN	-0.23	SO MFI	0.20
HDSLN	-0.20	VCL	-0.20
VHFLN	0.19	CytAbLN	0.18

[1]**TM**: Total motility; **PM**: Progressive motility; **VCL**: Curvilinear velocity; **VSL**: Straight-line velocity; **VAPLN**: Average path velocity; **LIN**: Linearity, **STR**: Straightness; **ALH**: Amplitude of lateral head movement; **BCF**: Beat cross frequency; **WOB**: Wobble; **CytAbLN**: Presence of proximal or distal cytoplasmic droplets; **VIA**: Viable spermatozoa; **ACI**: Acrosome intact spermatozoa; **VLF**: Viable spermatozoa with low fluidity; **VHFLN**: Viable spermatozoa with high fluidity; **SO MFI**: Median fluorescence intensity of MitoSOX Red in viable spermatozoa; **DFILN**: DNA fragmentation index; **HDSLN**: High DNA staining. **LN**=Log transformed prior to analysis.

[2]The sign of the loading value reflects the direction of the contribution (positive or negative) and the greater this value, the stronger the contribution to a PC.

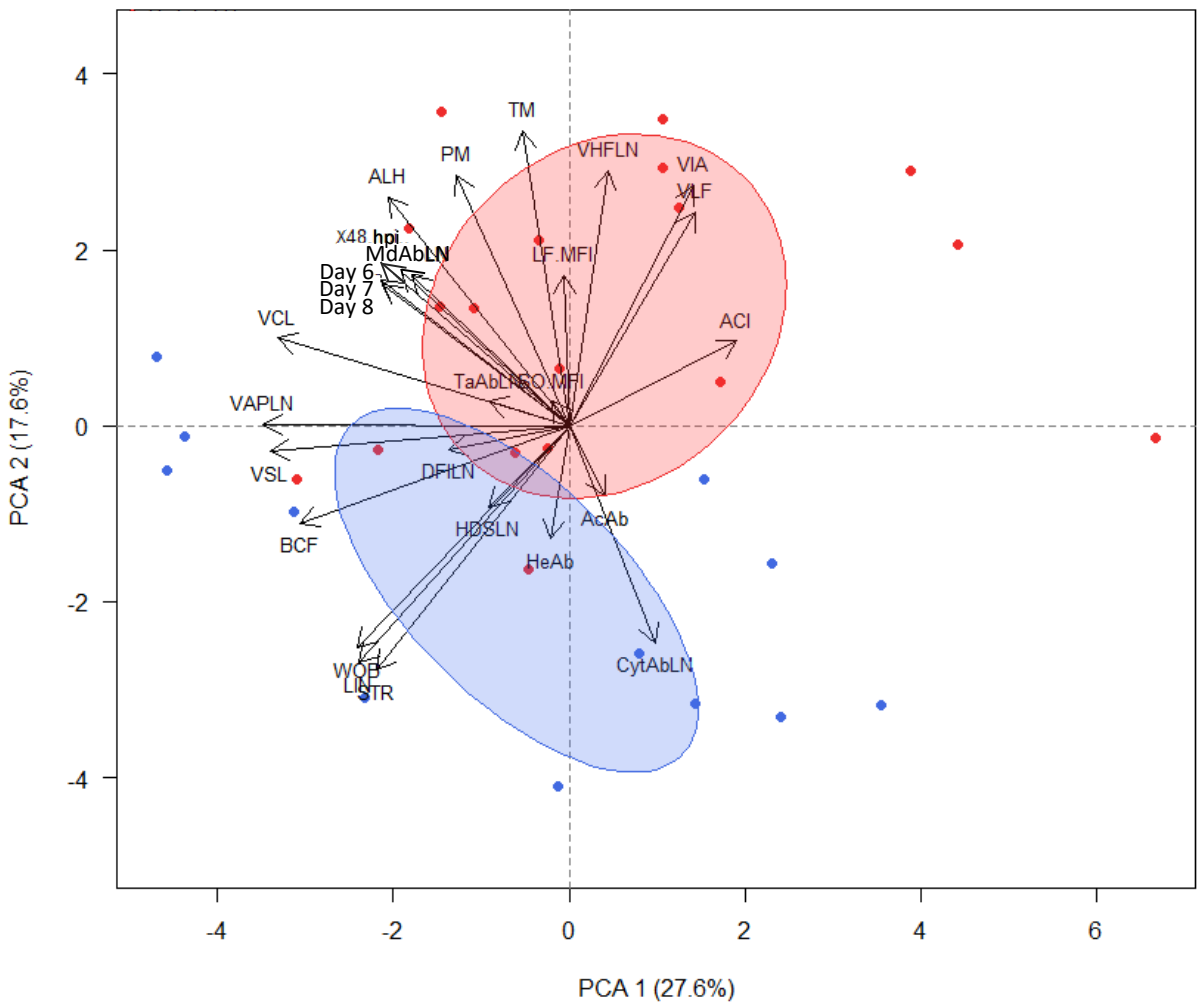

Figure 4. Principal component analysis (PCA) biplot presenting the distribution of a subset of bulls (n=10; in Experiment 1b) based on the contribution of various sperm attributes as well as fertilization and early embryo development parameters on Principal Component (PC) 1 and PC2. The PCs represent linear combinations of the measured variables, for which PC1 and PC2 explain 27.6% or 17.6% of the variation in the data set, respectively. Each sperm attribute is indicated by an arrow, where the length and direction of the arrow reflects the strength and direction of the contribution on a PC and by default, on the distribution of bulls. An individual dot represents the profile of an ejaculate from a bull, where all measured variables were first averaged over time (except for morphology, fertilization and embryo development) before performing this analysis. High and low fertility bulls have been indicated by red or blue dots, respectively, and 95% confidence ellipses were calculated to highlight fertility group clusters.

48 hpi: 48 hours post-insemination (cleavage rate); **Day 6:** 6 days post-insemination (blastocyst rate); **Day 7:** 7 days post-insemination (blastocyst rate); **Day 8:** 8 days post-insemination (blastocyst rate); **TM**: Total motility; **PM**: Progressive motility; **VCL**: Curvilinear velocity; **VSL**: Straight-line velocity; **VAPLN**: Average path velocity; **LIN**: Linearity, **STR**: Straightness; **ALH**: Amplitude of lateral head movement; **BCF**: Beat cross frequency; **WOB**: Wobble; **HeAb**: Head abnormalities; **AcAb**: Acrosome abnormalities; **MdAbLN**: Mid-piece abnormalities; **TaAbLN**: Tail abnormalities; **CytAbLN**: Presence of proximal or distal cytoplasmic droplets; **VIA**: Viable spermatozoa; **ACI**: Acrosome intact spermatozoa; **VLF**: Viable spermatozoa with low fluidity; **VHFLN**: Viable spermatozoa with high fluidity; **LF MFI**: Median fluorescence intensity of M540 in viable spermatozoa; **SO MFI**: Median fluorescence intensity of MitoSOX Red in viable spermatozoa; **DFILN**: DNA fragmentation index; **HDSLN**: High DNA staining. *Log transformed prior to analysis. **LN**=Log transformed prior to analysis.

Table 4. The top 10 variables in Experiment 1b that contribute most strongly to Principal Component (PC) 1 or PC2 (as also observed in Figure 4), which is given by the loading value

PC1		PC2	
Variable[1]	Loading score[2]	Variable[1]	Loading score[2]
VAPLN	-0.34	TM	0.33
VSL	-0.33	VHFLN	0.29
VCL	-0.33	PM	0.28
BCF	-0.30	STR	-0.27
WOB	-0.24	VIA	0.27
LIN	-0.23	LIN	-0.26
48 hpi	-0.22	ALH	0.26
STR	-0.21	WOB	-0.25
Day 7	-0.21	CytAbLN	-0.24
Day 8	-0.21	VLF	0.24

[1]**48 hpi:** 48 hours post-insemination (cleavage rate); **Day 7:** 7 days post-insemination (blastocyst rate); **Day 8:** 8 days post-insemination (blastocyst rate); **TM**: Total motility; **PM**: Progressive motility; **VCL**: Curvilinear velocity; **VSL**: Straight-line velocity; **VAPLN**: Average path velocity; **LIN**: Linearity, **STR**: Straightness; **ALH**: Amplitude of lateral head movement; **BCF**: Beat cross frequency; **WOB**: Wobble; **CytAbLN**: Presence of proximal or distal cytoplasmic droplets; **VIA**: Viable spermatozoa; **VLF**: Viable spermatozoa with low membrane fluidity; **VHFLN**: Viable spermatozoa with high membrane fluidity. **LN**=Log transformed prior to analysis.

[2]The sign of this value reflects the direction of the contribution (positive or negative) and the greater this value, the stronger the contribution to a PC.

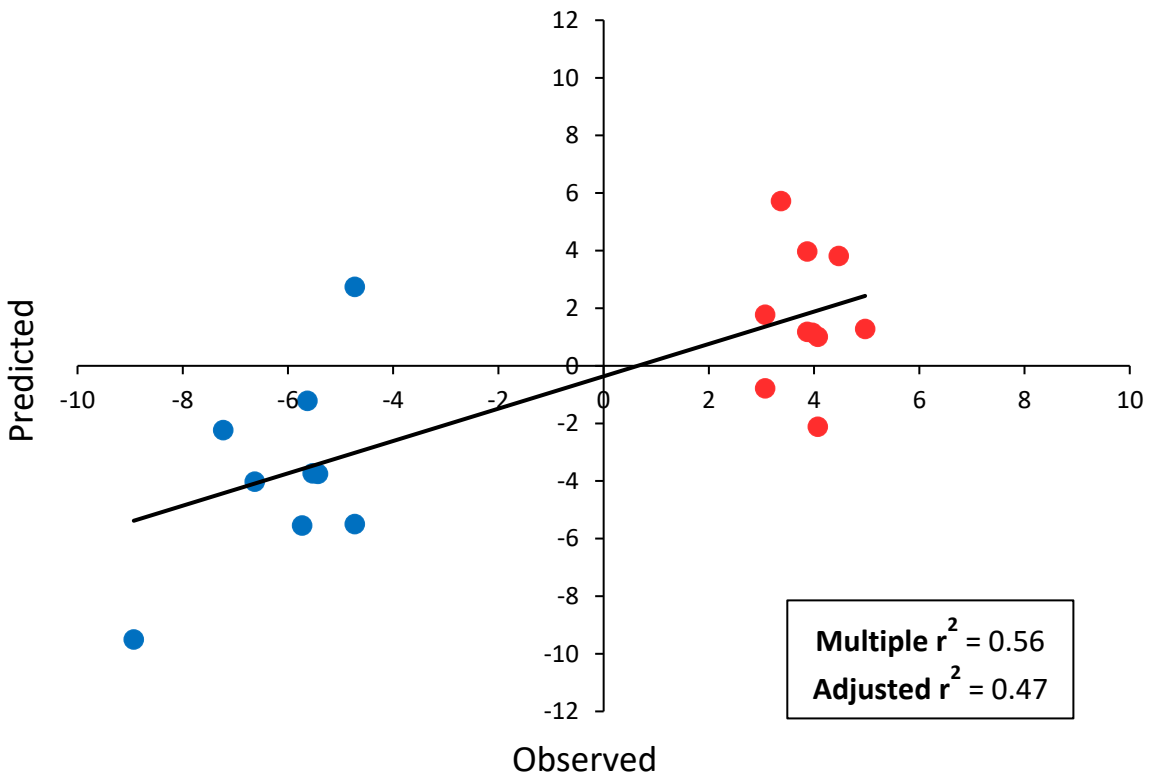

Figure 5. Relationship between the observed adjusted pregnancy rates (%) of the high and low fertility bulls and the predicted adjusted pregnancy rates (%) based on the final linear model. Bulls with high or low fertility are denoted by the red or blue dots, respectively (Experiment 2; n = 19; one low fertility bull removed during analysis).

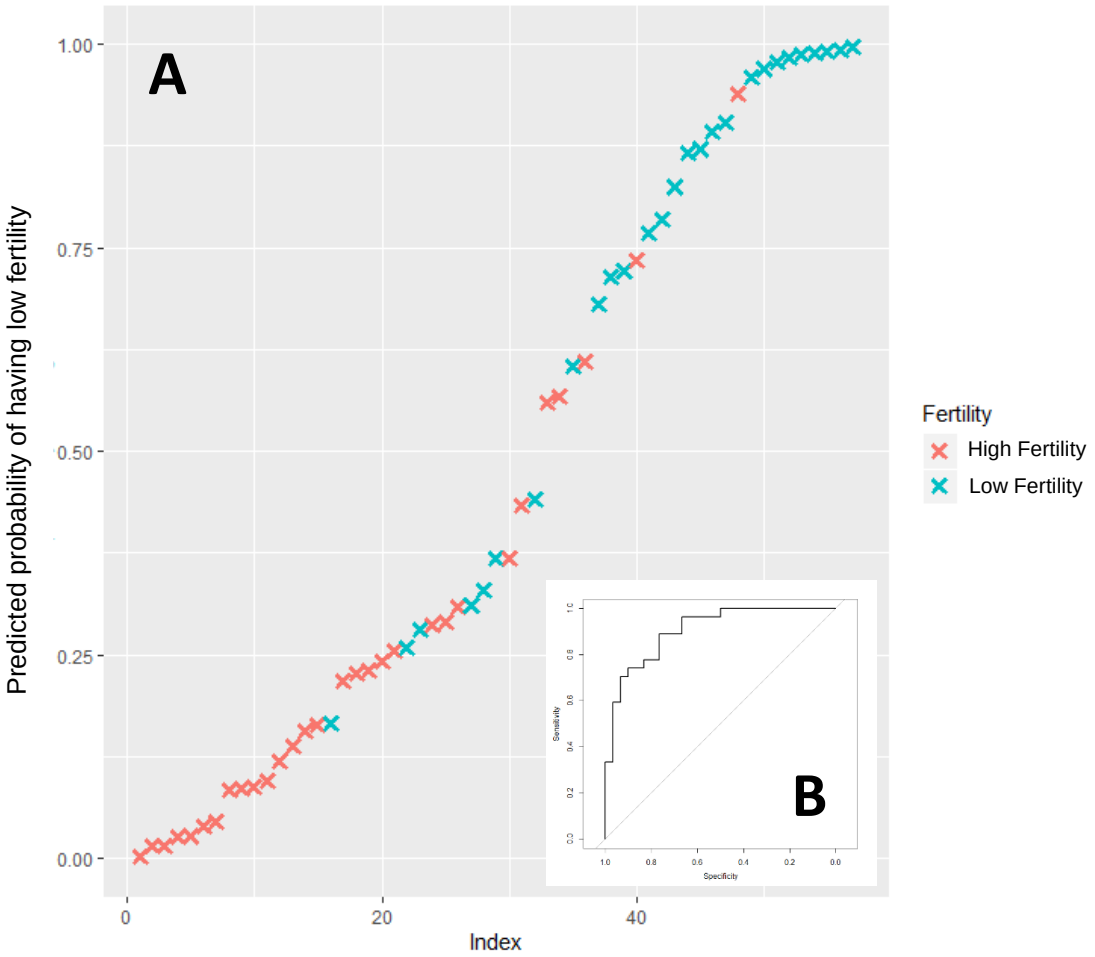

Figure 6. Plot presenting the predicted probability of a bull having low fertility based on the final logistic model including the most significant predictors related to the categorical response (**A**; n = 57 ejaculates; 3 ejaculates removed during analysis; Experiment 2). The associated receiver operating characteristic (ROC) curve for this model is inset within the plot (**B**), where the solid line denotes the ROC for the current model and the angled, dashed line denotes the ROC for a model with no discriminating capacity between fertility groups.