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Relationship among seminal quality measures and field fertility of young dairy bulls using low-dose inseminations

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ABSTRACT

Optimal use of genetically superior bulls through artificial insemination (AI) is highly dependent on precise assessment of seminal quality which allows for reasonable estimations of field fertility with normal or low-dose inseminations. In the present study, seminal measures such as sperm motility and morphology, sperm viability, sperm DNA fragmentation, and the ability of the sperm to display an acrosome reaction were tested. The relationships between field fertility and the seminal measures were investigated using 3 ejaculates from each of 195 bulls (156 Holstein and 39 Jersey) participating in a progeny test program. A range of AI doses, varying from 2×10^6 to 15×10^6 sperm/straw, was obtained by a controlled dilution process applied to each ejaculate. The different AI doses were distributed at random among 75,610 experimental first inseminations in 4,721 herds and 208 AI technicians. Most of the seminal measures appeared to contain a predictive value for the nonreturn to estrus at 56 d post-AI (NRR56) regardless of the number of sperm per AI dose and can be regarded as noncompensable sperm traits. But, due to correlations between the individual measures, the best model for describing (and predicting) NRR56 was based on sperm concentration and viability in the neat (raw) semen, and post-thaw sperm viability. The statistical models for describing NRR56 included the following explanatory variables: strength of the estrus, number of sperm per AI dose, breed, parity, and random components representing herds and AI technicians. The present results show that the most precise estimation of a bull's NRR56 can be achieved through flow cytometric detection of sperm concentration and viability in neat semen as well as flow cytometric detection of post-thaw sperm viability.
Key words: flow cytometry, DNA fragmentation, acrosome reaction, sperm viability

INTRODUCTION

Optimal use of genetically superior bulls through AI is highly dependent on precise assessment of seminal quality which allows reasonably accurate estimations of field fertility with normal or low-dose inseminations. Since the beginning of AI, the overall goal has been to maximize the semen production from the best males, which implies that the number of sperm per straw should be reduced to a minimum without lowering field fertility. A major obstacle in achieving this goal has been the limitation in the methods used for evaluation of semen. Furthermore, the use of sexed semen routinely in cattle AI is only possible using low-dose inseminations (2 million sperm/straw; Garner and Seidel, 2008), and, therefore, the need for precise and accurate methods for assessment of seminal quality has increased. Since the start of AI in cattle, the primary method for assessment of seminal quality has been a microscopic assessment of sperm motility using phase contrast (Saacke and White, 1972). Although this method is inexpensive, it is highly subjective and imprecise (Stålhammar et al., 1994; Garner, 1997). Computer-assisted analysis of sperm motility appeared more objective and precise, but suffered from bias due to program settings, differences between different systems and is a method too slow for routine use (Davis and Katz, 1993; Holt et al., 1994). An essential step in the production of AI doses with a desired number of sperm is that the assessment of sperm concentration in the neat (raw) semen be highly precise and accurate (Fenton et al., 1990; Woelders, 1991; Evenson et al., 1993). Christensen et al. (2004) developed a flow cytometric method that allowed accurate and simultaneous determination of sperm concentration and viability. Staining of sperm DNA with propidium iodide (**PI**, dead sperm) or SYBR-14 (live sperm) en-

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sured that other particulate matter or debris, without DNA, is not counted and that sperm concentration can be determined in both neat and frozen-thawed samples. This method was implemented at Danish AI stations and because of the high precision in determinations, smaller variations in semen packaging were observed for the AI doses produced as compared with those of traditional methods (Christensen et al., 2005b).

Schenk et al. (1987) reported that reduced fertility was observed if the AI dose contained less than 11×10^6 sperm per straw. Den Daas et al. (1998) used 20 mature, evaluated bulls in an insemination trial with AI doses that ranged from 2.5 to 15×10^6 sperm and concluded that no decrease in fertility was observed for 5 of the 20 bulls in the trial. Prior to the development of high-speed flow cytometric sorting of X- or Y-chromosome-bearing sperm (Garner, 2006), the use of AI doses with as little as 2.5×10^6 sperm was only of value for a few top bulls with very high genetic merit. Flow cytometric sexing of sperm has made it routine to use AI doses with a very low number of sperm (2×10^6 sperm/straw) and enhanced the ability to identify the bulls where this is possible without reducing fertility beyond the generally-accepted fertility level of 80% of conventional semen (DeJarnette et al., 2009).

The aim was to examine several measures of seminal quality as descriptors and predictors of field fertility in cattle when utilizing a minimal to normal range of AI doses. To achieve this goal, an insemination trial with 195 young bulls was performed with AI doses ranging from 2 to 15×10^6 sperm/straw. In addition to classical seminal measures, such as sperm motility and morphology, determinations of and concentration for the neat and frozen-thawed semen were conducted as described by Christensen et al. (2004). Furthermore, the ability for sperm to display an acrosome reaction was tested according to the protocol by Birck et al. (2010) and DNA integrity was assessed using the sperm chromatin structure assay (**SCSA**, Evenson and Jost, 2000).

MATERIALS AND METHODS

Preparation of Bulls

Semen for AI was produced from 195 young bulls (156 Holstein and 39 Jersey bulls, aged 12 to 17 mo at the time of semen collections). Production of semen took place during a period of 1 yr at 2 AI stations (Assentoft station and Bovlund station, both under Viking Genetics, Denmark). For practical reasons, the preparation of the bulls and experimental collections were carried out with groups of 12 to 16 bulls. Each bull was prepared for the trial by 1 seminal collection weekly until sperm viability and motility after freezing and thawing were found acceptable (>40% viable sperm and >50% progressively motile sperm). After this start-up phase, 3 ejaculates were collected from each bull with an interval of 1 wk between collections. In the rare case in which 1 of the ejaculates was rejected, seminal collections were continued for the particular bull until 3 ejaculates had been produced and approved for AI. For each seminal collection, only a single ejaculate was collected and all collections were done using an artificial vagina. For each bull the time in the collection room and number of false mounts were varied to ensure optimal sexual preparation of that particular bull. In total, 660 ejaculates were collected. Due to low sperm concentration in the neat semen ($<250 \times 10^6$ sperm/mL), 10 ejaculates were rejected. Post-thaw evaluations resulted in rejection of 65 ejaculates due to low sperm viability (<40%) or low motility (<50%). These selection criteria were standard practice in the evaluation of semen collected from bulls in the AI centers at Viking Genetics.

Assessment of Neat Semen

Seminal volume was estimated from the weight of the ejaculate using a density of 1.08 g/mL. Assessment of sperm viability and concentration was performed according to the method described by Christensen et al. (2004) using a FACSCount AF flow cytometer equipped with a 488 nm air-cooled laser and the BD Sperm Counting Reagent (BD Biosciences, San Jose, CA). A 50-μL sample of the neat semen was diluted 250-fold using a Sysmex 270AD autodilutor (Sysmex, GmbH, Hamburg, Germany). From that, a 50-μL subsample was withdrawn and added to a counting tube containing approximately 100,000 fluorescent microspheres in 400 μL of FACSCount diluent (BD Biosciences). Prior to use, the counting tubes were prepared by vortexing and adding SYBR-14 and PI to final concentrations of 50 nM and 12 μM, respectively (fluorochromes from Invitrogen, Eugene, OR). Samples were stained for 4 min at room temperature (20 to 22°C) and analyzed in the FACSCount AF flow cytometer. For each sample, 15,000 events were analyzed using the logs of green and red fluorescence. Emission signals were separated by a 620-nm short-pass dichroic mirror and green fluorescence was collected through a 515 to 545 nm band-pass (**BP**) filter, whereas the red fluorescence was collected through a 645-nm long-pass (**LP**) filter. No compensation was used and following acquisition, the data were analyzed automatically using Attractors software (BD Biosciences). All flow cytometric analyses of neat semen were carried out in duplicate.

Production of Experimental Semen and Post-Thaw Evaluation

Dilution of the ejaculate was performed according to the result of the FACSCount AF determination of sperm concentration. First, a basic dilution with a concentration of 68.2×10^6 sperm/mL was prepared using a freezing extender containing Triladyl (Minitüb, Tiefenbach, Germany) with 20% of egg yolk. Then, neat semen dilutions containing 45, 34, 23, 16, and 9×10^6 sperm/mL were made by addition of an appropriate volume of freezing extender to the basic dilution. Within 30 min of the dilution procedure, the diluted semen was put into 0.22-mL plastic straws (Minitüb) and the straws were packaged to provide approximate AI doses of 15, 10, 7.5, 5.0, 3.5, and 2.0×10^6 sperm/straw. Straws were subsequently cooled to 5°C over a period of 60 min. After 5 h of equilibration, straws were frozen in a programmable freezer (IMV, Cedex, France).

After freezing, 2 straws (containing 15×10^6 sperm/straw) were thawed at 37°C for 30 s and used for post-thaw evaluation of sperm motility at 200 × magnification using phase contrast microscopy with a heated stage (37°C). Microscopic assessments were performed in each of the 2 laboratories by an experienced technician and 2 assessments per sample were performed in a blinded fashion to ensure that the identity of the sample was unknown to the technician. For flow cytometric assessment of sperm viability and concentration, a subsample of the thawed semen was diluted 20-fold in a PBS medium (CellWash, BD Biosciences, Broendby, Denmark), and a 50-μL aliquot was analyzed in the FACSCount AF flow cytometer as described above. In addition to this determination, the concentration of sperm for each of the dilutions of each ejaculate was analyzed in the FACSCount AF. Two straws were thawed and analyzed per dilution and the results revealed that the actual AI doses applied were distributed in the interval between 1.1 and 20×10^6 sperm/straw. The actual AI doses determined by this flow cytometric analysis were used in the subsequent statistical analyses.

Evaluation of Sperm Morphology

For each ejaculate, a sample of the neat semen was diluted to 36×10^6 sperm/mL in a 3.8% (wt/vol) sodium citrate solution (pH = 6.8). Subsequently, 5 μL of this dilution was placed on a prewarmed (37°C) microscope slide and after addition of 5 μL of eosin-nigrosin, the 2 solutions were mixed, smeared across the slide and air-dried within 1 min. Two stained smears were made per ejaculate.

Evaluation of sperm morphology was performed using bright field microscopy at 1,000 × magnification and oil immersion. The assessment of a slide was continued until a total of 100 unstained sperm were evaluated and registered in the sperm morphology program (TopData, Frederiksberg, Denmark). During the assessment, some sperm stained by Eosin were recorded in the program, but this information was not included in the calculation of morphology. Sperm defects were classified according to Blom (1973), but for statistical analyses 4 morphology groups were formed: normal sperm (**Morf1**), defects in the sperm head (**Morf2**), defects in the midpiece (**Morf3**), and defects in the tail (**Morf4**).

Sperm Chromatin Structure Assay

In order to analyze sperm DNA fragmentation, the SCSA was performed according to Evenson and Jost (2000). For each ejaculate, 2 straws were thawed at 37°C for 30 s and emptied into a microcentrifuge tube which was placed on ice. Aliquots were diluted to a total volume of 200 μL and a concentration of 2×10^6 sperm/mL with TNE buffer (0.01 *M* TrisCl, 0.15 *M* NaCl, 1 m*M* EDTA disodium, pH 7.4). Subsequently, 0.4 mL of acid detergent solution [0.08 *M* HCl, 0.15 *M* NaCl, 0.1% (vol/vol) Triton X-100, pH 1.2] was added. After exactly 30 s, 1.20 mL of an acridine orange (**AO**) staining solution was added. The staining solution, which contained 6 μg/mL of chromatographically purified AO (Polysciences Inc., Warrington, PA), was added per 1 mL of buffer (0.037 *M* citric acid, 0.126 *M* Na_2HPO_4, 1.1 m*M* EDTA disodium, 0.15 *M* NaCl, pH 6.0). For each ejaculate, 2 measurements were processed after incubation of the thawed sample on ice for 5 and 10 min, respectively. Frozen-thawed reference samples from a bull with a DNA fragmentation index (**DFI**) of 10.5% were analyzed for every 6 samples throughout the experiment to ensure instrument stability.

Flow cytometric measurements were performed using a FACScan (BD Biosciences, San Jose, CA). This flow cytometer was equipped with an air-cooled argon laser and operated at 15 mW and 488 nm. Emission signals were separated by a 560-nm, short-pass dichroic mirror and green fluorescence (**FL1**) was collected thorough a 515 to 545 nm BP filter. After passing a 640 LP dichroic mirror, red fluorescence was collected through a 650 nm LP filter. The sheath/sample was set on "low" and adjusted to a flow rate of 200 events/s when samples with a concentration of 2×10^6 sperm/mL were analyzed. Immediately after addition of the AO staining solution, the sample was placed in the flow cytometer and was run through the system. After 3 min from initiation of the acid detergent treatment, 5,000 events were collected in list mode using BD CellQuest Pro version 4.0 (BD Biosciences). A manual recording of X-mean (red fluorescence) and Y-mean (green fluorescence)

were performed for each analysis. Analysis of the list mode files was performed using the SCSAsoft program (SCSA Diagnostics Inc., Brookings, SD). This software automatically calculated the percentage of sperm with an abnormally high DNA stainability (**HDS**), the DFI, and the standard deviation of DFI (**SD-DFI**).

In Vitro Induction of the Acrosome Reaction and Assessment of Acrosome Reactions

For each ejaculate, the thawing, washing, incubation procedures, and subsequent flow cytometric analysis were conducted in duplicate to yield 2 independent analyses. For each replicate, 3 straws were thawed in a water bath (30 s at 37°C) and pooled. Egg yolk, debris and decapacitating factors were removed with a dual washing procedure (Whitfield and Parkinson, 1995). Approximately 0.6 mL of thawed extended semen was diluted in 4 mL of prewarmed H-TLPP (38.5°C) and centrifuged (300 × *g*, 5 min, 38.5°C). The H-TLPP medium was based upon Tyrode's buffer with the modifications by Whitfield and Parkinson (1995) and contained 114 m*M* NaCl, 3.16 m*M* KCl, 9 m*M* lactic acid, 0.35 m*M* NaH_2PO_4, 3 m*M* $CaCl_2$, 2 m*M* $MgCl_2$, 20 m*M* HEPES acid, 20 m*M* HEPES sodium salt, 5.6 m*M* D-glucose, 1 mg of cold water-soluble polyvinyl chloride (PVA)/mL, 0.09 m*M* sodium pyruvate, 50 μg of gentamicin sulfate/mL, and 10 m*M* $NaHCO_3$. The final H-TLPP solution had a pH of 7.40 at 38.5°C and an osmolarity of 340 mOsm. The medium was stored in the dark at 4°C for no longer than 2 wk and aliquots were preheated to 38.5°C before use. The washing procedure was repeated and finally the pellet was resuspended in 150 μL of H-TLPP. The concentration of the washed sperm was measured using the NucleoCounter SP-100 (ChemoMetec A/S, Alleroed, Denmark). Based on the measured sperm concentration, a dilution was made to a final concentration of 5×10^6 sperm/mL.

The washed sperm were incubated at 38.5°C for 40 min in H-TLPP medium with addition of 1 μ*M* ionophore (calcium-ionophore A23187, Invitrogen, Eugene, OR). Viable (plasma membrane intact) sperm were stained by 2 μ*M* SYTO-17; sperm with leaking or disrupted plasma membranes were stained by PI (12 μ*M*), and sperm that were acrosome-reacted were stained by peanut (*Arachis hypogaea*) agglutinin fluorescein isothiocyanate (PNA-FITC, 1 μg/mL; Thomas et al., 1997). After addition of dyes, tubes were capped, gently mixed, and incubated for 10 min at 38.5°C. Just before analysis, the tubes were gently remixed.

A FACScan flow cytometer (BD Biosciences) equipped with a 488-nm argon excitation laser was used for analysis. The SYTO-17, which emits red fluorescence with low intensity compared with that of PI, was detected through photomultiplier tube 3 (**PMT** 3) using a 650-nm LP filter. The log of PI fluorescence was detected through PMT 3. The log of PNA-FITC fluorescence was detected through PMT 1 using a 515 to 545-nm BP filter. During the period of the experiment only minor adjustments of the PMT were made and compensation measures were kept unchanged during the entire experiment. Scatter detection was done in linear mode, whereas all fluorescence detectors were used in log mode. Temperature-calibrated sterile-filtered PBS was used as sheath fluid. The flow cytometer was used in "low" flow rate mode giving an event rate of approximately 300 to 600 events/s. Data were acquired using the CellQuest software (version 3.2.1f1, BD Biosciences). For each analysis, the recording of scatter and fluorescence properties was stopped when 10,000 double-gated events were obtained.

The data acquired from the flow cytometer were analyzed by quadrant statistics. On a forward versus side scatter plot (linear mode), each data file was analyzed by applying a scatter gate around the single-sperm events to exclude debris and double-sperm events from further analysis. Second, on a green (PNA-FITC) versus red fluorescence (SYTO-17/PI) dot plot (log mode) displaying the scatter-gated events only, a second gate was set around the fluorescent populations to omit debris not initially excluded by the scatter gate. Finally, these double-gated sperm events were quantified using quadrant statistics giving simultaneous information on sperm viability and acrosomal status. The fraction of live acrosome-reacted sperm in the live sperm population (**AR.live.live)** index was calculated (Birck et al., 2010).

Inseminations and Field Data

Inseminations were carried out from April 2004 to June 2005 in 4,721 Danish Holstein or Jersey herds by 208 AI technicians. Experimental AI doses were randomly distributed among all AI technicians. A total of 78,964 experimental first inseminations were subsequently recorded. For each insemination, the AI technician recorded the identity of the bull and cow along with the code on the straw. Information regarding the dilution step of the semen and the production date was included in the code on the straws, but this information was not known by the AI technician at the time of AI. An evaluation of the strength of the signs of estrus was scored on a scale from 1 (weak) to 4 (strong) by the AI technicians. This evaluation was a subjective impression of the cow or heifer which was obtained during AI. All information regarding the AI and the outcome (measured as the nonreturn rate at d 56, **NRR56**) were extracted from the national Danish cattle database.

The following editing rules were applied: double registrations were deleted and only data from AI of Danish Holstein and Jersey cows with <11 parities were used. Insemination records more than 300 d after calving were deleted. If a second AI was performed within 8 d of the first AI, the record was deleted. According to Danish rules, abortions that occur before 200 d after the last calving are not recorded. Therefore, calving intervals of <200 d were automatically excluded. After applying these editing rules, 75,610 experimental first AI were kept in the data set.

Statistical Analyses

The predictive power of each seminal quality measure (***S***) was studied by fitting suitable semiparametric generalized additive models (Hastie, 1990; Hastie and Tibshirani, 1990) where the *NRR56* was entered as a Bernoulli binary response variable (successful or not). The seminal quality measure ***S*** entered as a continuous explanatory variable and adjustments for the effect of breed (***B***), parity (***Pa***), interaction of breed by parity ($B \times Pa$), intensity of estrus (***H***), the estimated number of sperm in the AI dose (***D***), viability of neat (raw) semen (***NV***), sperm concentration of neat semen (***NC***), herd (***He***), and AI technicians (***AIT***) were performed. More precisely, the following generalized additive model was fitted:

$$\begin{aligned}\text{logit}\ (\Pr[NRR56 = \text{success}]) &= \mu + B + Pa \\ &+ B \times Pa + H + f(D) + h(NV) + u(NC) \\ &+ \text{random}(He) + \text{random}(AIT) + g(S). \qquad [1]\end{aligned}$$

Here, logit $(\Pr[NRR56 = \text{success}]) = \log\{\Pr[NRR56 = \text{success}]/(1 - \Pr[NRR56 = \text{success}])\}$ is the logit-transformed probability (Pr) of a successful result of the current insemination; f, g, u, and h are nonspecified smooth functions; and S is the index under evaluation. Factors representing herd and *AIT* are entered in the model as random factors. Note that no specific form for the response to the continuous explanatory variables *D, NV, NC*, and S is assumed because they enter the model as the image of the nonspecified smooth functions, f, g, u, and h (the nonparametric part of the model).

The predictive value of each seminal quality measure S was evaluated by comparison of the model specified by [1] and the following model (not containing the effect of the explanatory variable S):

$$\begin{aligned}\text{logit}\ (\Pr[NRR56 = \text{success}]) &= \mu + B + Pa \\ &+ B \times Pa + H + f(D) + h(NV) + u(NC) \\ &+ \text{random}(He) + \text{random}(AIT). \qquad [2]\end{aligned}$$

Models [1] and [2] were compared by a deviance difference test (equivalent to a likelihood ratio test), and P-values of the test below 0.05 were interpreted as an indication that the seminal measure S under evaluation carries significant information on field fertility.

The generalized additive models described by [1] and [2] were extended to a model that allowed the fertility (represented by μ) and the smooth function that represented the effect of the dose on field fertility (represented by the function f) both to depend on the bull. These models were used to study the amount of information that a seminal measure S carries on the field fertility that is not due to variation on individual bull differences on general fertility or characteristic response of fertility to the AI dose. More precisely, the extended models were given by

$$\begin{aligned}\text{logit}\ (\Pr[NRR56 = \text{success}]) &= \mu_b + B + Pa \\ &+ B \times Pa + H + f_b(D) + h(NV) + u(NC) \\ &+ \text{random}(He) + \text{random}(AIT) + g(S). \qquad [3]\end{aligned}$$

Here sub-index b in μ_b and f_b indicate that these quantities depend now on the bull used in the AI. Analogously, the model was defined (not containing the effect of the explanatory variable S) by

$$\begin{aligned}\text{logit}\ (\Pr[NRR56 = \text{success}]) &= \mu_b + B + Pa \\ &+ B \times Pa + H + f_b(D) + h(NV) + u(NC) \\ &+ \text{random}(He) + \text{random}(AIT). \qquad [4]\end{aligned}$$

Testing the possible reduction of model [3] to model [4] allowed for the determination of whether the seminal measure S under evaluation carried significant information on field fertility that cannot be explained by differences in individual bull fertility or response to the applied AI dose.

Additionally, the following cross-validation test was performed to further characterize the predictive power of each of the seminal indices. The data were randomly split into 2 parts by sampling the combinations of bulls and ejaculates. A model, as defined by [1], was then fit using one-half of the data and used to predict the *NRR56* for each combination of bull and ejaculate in the other half of the data. The Pearson correlation between the observed and the predicted *NRR56* in the half of the data used to predict was then calculated. This process was repeated 1,000 times with independent random splits of the data and a bootstrap estimate, together with a 95% confidence interval, was obtained for the correlation between observed and the predicted *NRR56*. A similar procedure was then applied to the

model defined by [2], yielding a bootstrap estimate of 0.422 (95% CI from 0.405 to 0.442) and the difference between the estimate of the correlation between the observed and the predicted *NRR56* with the models defined by [1], and this estimate was reported as the improvement in the prediction power.

Some of the seminal measures considered may carry the same, or partially the same, information as other seminal measures studied here. To disentangle the information contents of these measures, a detailed analysis of the distribution of the information was performed using graphical models (i.e., models in which the multivariate structure of the data are coded by a graph; Lauritzen, 1996; Labouriau and Amorim, 2008a,b) following the construction described below: Each seminal measure was represented by a point (vertex) in the graph. Pairs of variables for which the conditional correlation given the other variables is significantly different from zero are connected by a line (edge). The absence of an edge joining 2 variables indicated that the 2 variables in question were not significantly correlated, given the other variables. According to the theory of graphical models (Whittaker, 1990), if 2 variables represented in the graph were directly connected, then the variables carry new information on each other that was not already contained in the other variables present in the graph. If 2 variables were only connected indirectly, then they might be correlated, but this correlation is spurious, in the sense that it could be completely explained by correlations with other variables. Another general result of the theory of graphical models is the separation theorem (Whittaker, 1990; Lauritzen, 1996), which stated that if 2 groups of variables, A and B, are separated by a third group of variables, C, then A and B are conditionally noncorrelated, given C. Here the expression "group C separates A and B" means that every path connecting an element of A with an element of B necessarily contains an element of C. The graphical model with minimum Bayesian information criterion (BIC) was selected for the seminal measures using the R package gRapHD (Abreu et al., 2010), which yields the graphical model which best represents the data (Haughton, 1988).

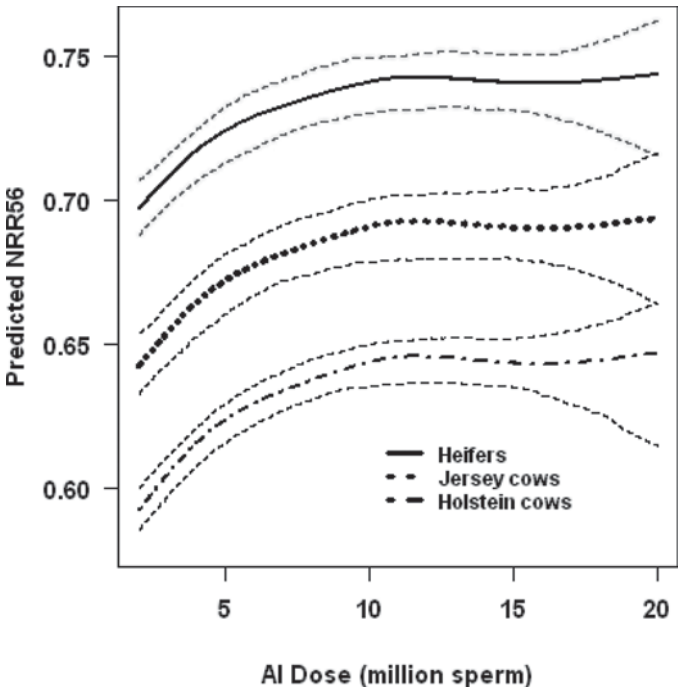

Figure 1. Relationship between the number of bovine sperm/AI dose ($\times 10^6$ sperm/dose) and the estimated nonreturn to estrus at d 56 post-AI (NRR56, %) for Holstein cows (n = 54,314), Jersey cows (n = 9,654), and heifers (Holstein or Jersey, n = 11,842). Estimates are based on a post-thaw viability of 56% (median of the observed viability) and 95% confidence interval (limits marked with dashed lines) obtained with parametric bootstrap (500 bootstrap samples).

RESULTS

Initial statistical analysis revealed that the dose-response curves (Figure 1) did not follow the anticipated exponential curve described previously (den Daas et al., 1998), because the *P*-value for a goodness of fit test by comparing the exponential model against a free curve model via deviance difference test was below 0.001. The dose-response curves were modeled via generalized additive models using a regression model transformed through a smooth continuous unspecified function (f). The dose-response curves were at different levels ($P < 0.001$) for AI in Holstein cows, Jersey cows, and heifers (coinciding for Holstein and Jersey heifers, $P > 0.20$), but follow the same shape, which can be seen from the almost parallel curves represented in Figure 1 ($P > 0.20$ for a formal test of effect modification). The dose-response curves were formed by a steep part at the low doses where the NRR56 increases with increasing AI dose and a plateau part where NRR56 remains essentially constant regardless of increase in the AI dose. Note that this behavior of the dose-response curve cannot be described by an exponential curve.

The seminal measures were analyzed individually using the models given by equations [1] and [2] to determine if they carried information regarding NRR56. No statistically significant effect modifications (or statistical interaction) between the seminal measures studied and parity/breed were found ($P > 0.20$). Whether different seminal measures affected only the steep part of the dose-response curve (a compensable sperm trait) or if the measures studied affected the maximal NRR56 (a noncompensable sperm trait) was explored. As shown in Table 1, almost all seminal measures were able to

Table 1. Range of variation, mean, SE, and significance of individual bovine seminal traits for prediction of nonreturn to estrus at d 56 post-AI (NRR56) adjusted for differences in bull individual fertility and response to doses (third column) and not adjusted for differences in bull fertility (fourth column)

Seminal trait	Range of variation	Mean (SE)	*P*-value (adjusted for bull effects)	*P*-value (not adjusted)
Post-thaw viability[1]	35.9–75.6	56 (7.4)	<0.0001	<0.0001
Diff-Viab[2]	8.6–49.8	27.8 (6.4)	0.7234	0.6720
Post-thaw motility[3]	17.5–74.5	57.8 (6.5)	<0.0001	<0.0001
AR.live.live[4]	0.22–0.83	0.61 (0.11)	<0.0001	<0.0001
Morf1[5]	16–97	77.1 (12.5)	0.0002	<0.0001
Morf2[6]	0–57	10.5 (7.9)	0.0001	<0.0001
Morf3[7]	0–72	9.3 (10.6)	0.0003	<0.0001
DFI0[8]	91.4–99.3	97.5 (10.6)	<0.0001	<0.0001
DFI1[9]	0.6–7.8	2.4 (1.1)	<0.0001	<0.0001
DFI2[10]	0.7–8.8	0.2 (0.1)	<0.0001	0.0003
SD-DFI[11]	17.4–71.4	33.3 (8.4)	<0.0001	<0.0001
HDS[12]	0.7–9.5	2.8 (1.2)	0.0004	<0.0001

[1]The seminal viability was assessed flow cytometrically using SYBR-14/propidium iodide staining in AI doses containing 15×10^6 sperm/straw.

[2]The measure is the calculated difference in post-thaw sperm viability in AI doses containing 15×10^6 sperm/straw versus that in doses with 2×10^6 sperm/straw.

[3]Post-thaw sperm motility was assessed in AI doses containing 15×10^6 sperm/straw.

[4]The percentage of acrosome-reacted live sperm among the population of live sperm after a 40-min challenge with 1 μM calcium ionophore A23187.

[5]The group of sperm without any morphological defects.

[6]The group of sperm with defects in the sperm head.

[7]The group of sperm with defects in the midpiece.

[8]Sperm without detectable DNA fragmentation using the sperm chromatin structure assay (SCSA).

[9]Sperm with moderate DNA fragmentation index (DFI) with the SCSA.

[10]Sperm with high DNA fragmentation index (DFI) with the SCSA.

[11]Standard deviation of DFI with the SCSA.

[12]Sperm with high DNA stainability with the SCSA.

contribute to differences in NRR56 when considered individually. This was the case for analyses when no adjustments were made for differences in individual bull fertility (models [1] and [2]) and for models [3] and [4] in which adjustments to possible differences in bull fertility and the bull characteristic dose response curve were made. The 2 types of tests, although of different nature, produced virtually the same results. The only seminal measure that did not show statistically significant predictive power was the difference in post-thaw viability in AI doses with 15×10^6 sperm/straw versus 2×10^6 sperm/straw (Diff-viab, Table 1). Because no statistically significant evidence of modification of the effect of seminal measures for different AI doses by changes in the seminal dosage were found (i.e., no significant statistical interaction between the seminal measures and the dosage), all of the seminal measures studied were classified as noncompensable sperm traits. Therefore, the seminal measures studied affected both the steep part of the dose-response curve as well as the maximal NRR56 that can be achieved.

Some seminal measures may carry the same information or partially the same information regarding NRR56. Therefore, graphical models were used to disentangle the information that the different seminal measures might carry on the NRR56 and the other seminal measures. Figure 2 displays the graph that best represents the seminal measures in terms of the BIC criterion. The graph is connected, indicating that all of the seminal measures considered were directly or indirectly associated with each other, although some of the directly observed correlations might be spurious. The only seminal measures present in the graph that were directly connected to NRR56 were NC, NV, and post-thaw viability. Moreover, post-thaw motility, AR.live.live, DFI, Morf, and HDS were not directly connected to NRR56, indicating that associations between these measures and NRR56 were necessarily spurious. More precisely, the variables NC, NV, and post-thaw viability separate NRR56 from the other seminal measures. This implies, according to the separation theorem, that the conditional correlation between NRR56 and any of the other seminal measures (i.e., post-thaw motility, AR.live.live, DFI, Morf, and HDS) are zero if we simultaneously condition on NC, NV, and post-thaw viability. This indicated that all of the information that

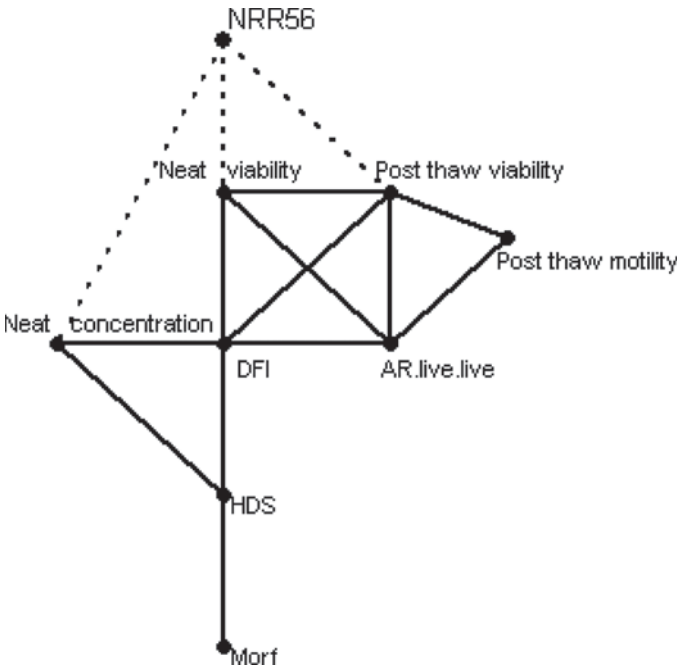

Figure 2. The graphical model with minimal Bayesian information criterion for the relevant seminal measures affecting nonreturn rates. The seminal measures are represented by points (circles); 2 points are connected by a continuous line when the conditional correlation between them, given the other measures, is statistically significant. The variables Morf1 (normal), Morf2 (defects in the sperm head), Morf3 (defects in the midpiece), and Morf4 (defects in the tail), which describe the morphologic defects, are represented by Morf because they are all connected between each other; for the same reason, the variables DFI0 (sperm without detectable DNA fragmentation), DFI1 (sperm with moderate DNA fragmentation index), and DFI2 (sperm with high DNA fragmentation index) are represented by DFI; HDS is the percentage of sperm with an abnormally high DNA stainability and AR.live.live is the percentage of acrosome-reacted live sperm among the population of live sperm after a 40-min challenge with 1 μM calcium ionophore A23187. Only post-thaw viability, neat viability, and neat concentration are genuinely associated to nonreturn to estrus at 56 d post-AI (NRR56), because they are the only variables directly connected to NRR56 in the graph.

post-thaw motility, AR.live.live, DFI, Morf, and HDS might carry on NRR56 was entirely contained in NC, NV, and post-thaw viability. Moreover, including in the graphical model described above a discrete variable representing the bull identity in the model and, for technical reasons, transforming the continuous variables in discrete variables by splitting them in disjoint categories (Whittaker, 1990), a graph was obtained that was similar to that presented in Figure 2, with the only difference being edges connecting the vertex representing the bull identity and the vertices representing NRR56, NC, NV, and post-thaw viability and some other seminal measures.

A forward sequential stepwise analysis was carried out to identify the most important seminal measures to improve the predictive power of the parameters already included in model 1. Starting with a model similar to the model defined by [1], but without including the effects of NV and NC, the stepwise procedure first incorporated the effects of NC, NV, and post-thaw viability ($P < 0.001$). When NV, NC, and post-thaw viability were included in the model, none of the other seminal measures appeared to improve the power for estimation of NRR56, which is in agreement with the analysis based on graphical models. Analogous results were obtained when using a similar stepwise procedure based on the model defined by [3] (i.e., when adjusting for possible differences in bulls regarding their general fertility or their response to changes in the AI doses).

Table 2. Examples of correlations (Spearman correlations) between some of the individual seminal traits (the correlations are between the 2 seminal measures in the first and second columns; $P < 0.001$ for all of the correlations in the table)

Seminal trait	Seminal parameter	Spearman correlation
Post-thaw viability[1]	Neat viability[2]	0.58
Post-thaw viability	Post-thaw motility[3]	0.43
Post-thaw viability	AR.live.live[4]	0.22
Post-thaw viability	DFI0[5]	0.48
Neat viability	Morf2[6]	−0.18
Neat concentration[7]	DFI0	0.18

[1]Sperm viability was assessed by flow cytometry using SYBR-14/propidium iodide staining in AI doses containing 15×10^6 sperm/straw.

[2]Sperm viability was assessed by flow cytometry using SYBR-14/propidium iodide staining in neat (raw) semen samples.

[3]Post-thaw sperm motility was assessed in AI doses containing 15×10^6 sperm/straw.

[4]The percentage of acrosome-reacted live sperm among the population of live sperm after a 40-min challenge with 1 μM calcium ionophore A23187.

[5]Sperm without detectable DNA fragmentation using the sperm chromatin structure assay (SCSA).

[6]The group of sperm with defects in the sperm head.

[7]The concentration of sperm in neat (raw) semen ($\times 10^6$ sperm/mL).

The results of the additional cross-validation study are in Table 2. The only seminal measure that had a statistically significant increase in the correlation between the observed and the predicted NRR56 was the post-thaw viability, which showed an improvement of 9.1% (95% bootstrap CI from 0.083 to 0.103). This confirmed the results of the forward selection and the graphical models above.

The relationship between the NRR56 and the percentage of viable sperm in the neat semen is in Figure 3. According to the additive model used, the NRR56 was predicted to increase from approximately 73.6 to 74.3% when the viability of the neat semen increases from 80% to 95%. Analogously, Figure 4 shows that when the post-thaw sperm viability varied from 40 to 65%, the NRR56 was predicted to increase from 71.65 to 74.45%. Figures 3 and 4 show predictions of the

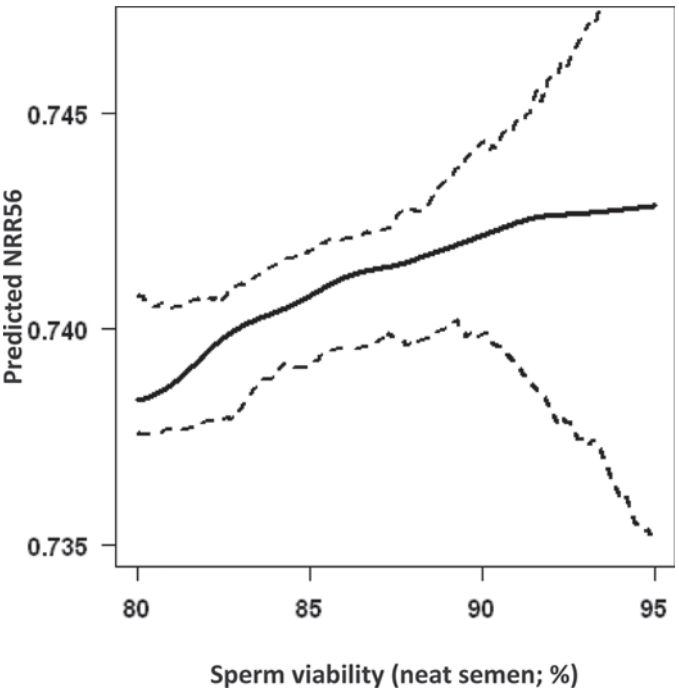

Figure 3. Relationship between the percentage of viable (SYBR-14 positive/propidium iodide negative) bovine sperm in the neat (raw) semen and the estimated nonreturn to estrus rate at 56 d post-AI (NRR56, solid line for Holstein and Jersey heifers; n = 11,842). Estimates are based on AI with an intermediate intensity of estrus, an AI dose of 14×10^6 sperm/straw, and a post-thaw viability of 56% (the relationship was not confounded by the number of viable sperm/straw); 95% confidence interval (dashed line) was obtained via parametric bootstrap (500 bootstrap repetitions). Similar graphs were obtained for Holstein cows and Jersey cows.

NRR56 for an AI dose of 14×10^6 sperm/straw and post-thaw sperm viability of 56%. When the concentration of sperm in the neat semen increased from $1{,}000 \times 10^6$ to approximately $1{,}600 \times 10^6$ sperm/mL, the NRR56 increased from 73.5 to 74.5%.

DISCUSSION

Our results demonstrated that almost all of the seminal measures studied carried information on the NRR56. This was done by using 2 different types of generalized additive models: the models described by equations [1] and [2], which did not directly account for possible variations on the bulls, and the models given by equations [3] and [4], which incorporate additionally adjustments for differences in individual bull fertility and for differences between the individual response curves relating NRR56 to semen dosage of each bull. The results obtained with those 2 types of models have qualitatively different interpretations. When using the first type of model, the differences on the NRR56 detected by a seminal measure can be attributed to differences among the bulls and to differences due to other undetermined causes. On the other hand, when using the second type of model (i.e., models defined by [3] and [4]), the differences on the NRR56 detected by a seminal measure can be attributed to causes not related to differences among the bulls. Because essentially the same conclusions with respect to the predictive power of the seminal measures were obtained when using both types of models, the seminal quality measures considered here, except the Diff-viab, can detect differences in NRR56 that are not due to differences in the individual fertility of the bulls studied. Moreover, those seminal quantities can detect differences in NRR56 in a scenario where the information on individual differences among bulls is not available. This ensures that the conclusions were not invalidated by a partial confounding due to differences among the bulls on the different seminal quality measures.

Because the seminal measures were correlated, there might be an overlap in the information they carry on NRR56. Indeed, the analysis based on graphical models revealed that the association between NC, NV, and post-thaw viability and the NRR56 could completely explain the reported associations between NRR56 and the other seminal measures. Knowing simultaneously the values of these 3 key seminal measures renders the other seminal measures noninformative with respect to the NRR56. The theory of graphical models (specifically the separation theorem) says more: any system of predictors of the NRR56 involving the seminal measures studied here should necessarily contain these 3 key seminal measures; otherwise, the system would lose information on the NRR56. This conclusion is confirmed by a stepwise selection procedure using additive generalized linear models.

The predictive value of a seminal measure depends not only on the correlation to NRR56, but also on the precision of the determination of the parameter. A good example is post-thaw sperm viability, which was assessed by microscopy. The inherent drawbacks are that this seminal measure is imprecise and subjective. In support of our previous study (Christensen et al., 2005a) post-thaw sperm motility carried predictive information regarding NRR56. But, when the post-thaw sperm viability was included in the model, the post-thaw sperm motility became not statistically significant. The reason for this might be that the precision in the determination of sperm motility is much lower than flow cytometric determination of sperm viability, because these 2 traits measure essentially the same quantity.

A concern in relation to low-dose AI is that a high dilution rate could affect sperm survival (Garner et al., 2001). The difference in post-thaw sperm viability in AI doses containing 15×10^6 sperm/straw compared

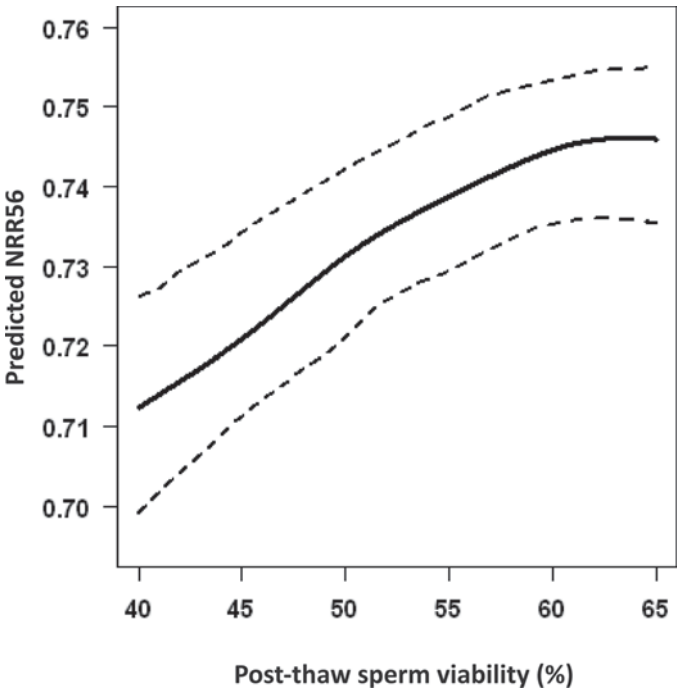

Figure 4. Relationship between the percentage of viable (SYBR-14 positive/propidium iodide negative) bovine sperm post-thaw and the estimated nonreturn to estrus at 56 d post-AI (NRR56, solid line for Holstein and Jersey heifers; n = 11,842). Estimates are based on AI with an intermediate intensity of estrus, an AI dose of 14×10^6 sperm/straw, and a post-thaw sperm viability of 56% (the relationship was not confounded by the number of viable sperm per straw); 95% confidence interval (dashed line) was obtained via parametric bootstrap (500 bootstrap repetitions). Similar graphs were obtained for Holstein cows and Jersey cows.

with that in doses with 2×10^6 sperm/straw was calculated. As shown in Table 1 it had no predictive value for NRR56.

A flow cytometric method for detection of acrosome-reacted sperm was recently reported by Birck et al. (2010) and was combined with a 40-min induction of the acrosome reaction with calcium ionophore A23187. They showed that the most precise determination of acrosome reactions occurred when only the proportion of live acrosome-reacted sperm in the live sperm population was considered (AR.live.live). Furthermore, this index appeared to describe the biological variation among the bulls studied and appeared to have the highest predictive value for determination of NRR56. The AR.live.live index was included in the present study and appears useful for estimation of NRR56 when considered individually (Table 1). This index did not have an (additional) statistically significant predictive value when considered together with post-thaw viability and, therefore, was not included in the final model for describing the NRR56.

Defects in morphology of the sperm head (Morf2) or midpiece (Morf3) appeared to have a negative predictive value for NRR56, whereas the percentage of normal sperm (Morf1) had a positive predictive value (Table 1). None of the morphology measures presented a significant predictive effect in the presence of post-thaw viability and were not included in the final model. One explanation for this could be that morphological defects were observed with a very low frequency for most of the bulls used and that the biological variation in these measures is very low. Second, sperm morphology was assessed on a maximum of 200 sperm, which yields low precision, although manual classification of larger numbers of sperm would not be feasible in large-scale production processes.

The results for the 195 bulls showed that the biological variation in the DFI among bulls was low. None of the bulls had a DFI value >10% or a HDS value >4%. When considered individually, all of the SCSA measures contained information for the estimation of NRR56, but none of the measures presented a significant predictive effect in the presence of post-thaw viability, and were not included in the final model for describing the NRR56.

In the present study, the fertility data was analyzed using a generalized additive model with a nonspecified smooth continuous regression function. The data did not fit the previous functions described for the dose-response curve (Pace et al., 1981; den Daas et al., 1998; Fearon and Wegener, 2000). Although it was not the original aim to characterize the dose-response curve, it was essential that analyses use a curve that fits the data adequately. Therefore, a semiparametric model was used that makes less restrictive assumptions on the form of the dose-response curve.

Although seminal measures such as sperm motility, sperm morphology, sperm DNA fragmentation, or the ability to acrosome react were not included in the final model for estimation of NRR56, it is not recommend that these measures be eliminated from the screening routine of new bulls entering an AI station. Individual bulls may very well have a high percentage of sperm with a certain morphologic defect or have low motility.

In conclusion, the present study provides evidence that the most precise estimation of a bull's nonreturn rate can be achieved through flow cytometric detection of sperm concentration and viability in neat semen combined with flow cytometric detection of post-thaw sperm viability, although the effects of those seminal measures are of moderate magnitude. Sperm morphology, acrosome reaction and the DNA fragmentation did not appear to improve the estimation of nonreturn rate. This does not exclude that these measures may still be useful, because they could be important in establishing a precise diagnosis for some sires or for special purposes such as development of new extenders.

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