

## Relationship Between Sperm Viability as Determined by Flow Cytometry and Nonreturn Rate of Dairy Bulls

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**ABSTRACT:** A newly developed flow cytometric method for determination of sperm concentration and viability was tested in an insemination trial with cryopreserved bull sperm to establish the relationship between sperm viability and nonreturn rates. Semen for experimental inseminations was produced from 157 young sires (114 Holstein and 43 Jersey), each contributing 4 experimental semen collections. Straws containing approximately  $15 \times 10^6$  motile sperm before freezing were used in 118 680 experimental inseminations performed by 254 artificial insemination technicians in 6352 Danish herds. Statistical analysis based on 44 946 experimental first inseminations showed that the major part (95.4%) of variation in the 56-day nonreturn rate (NRR56) was residual. Only 0.38% of the total variation in NRR56 was due to bulls and differences between ejaculate within bull. However, bulls were preselected, and a relatively

high insemination dose was used. Correlations between sperm viability as assessed by flow cytometry and NRR56 was slightly lower than observed for microscopic assessment of sperm motility. However, flow cytometry makes it possible to achieve an objective and precise determination of sperm viability. It was therefore possible to calculate the effect on NRR56 provided selection of semen is based on the flow cytometric method. Three freezing extenders were used in this experiment, but a significant difference in NRR56 was not observed. Flow cytometric results for 1 extender (Biociphos Plus) indicated poorer sperm survival during postthaw incubation compared with Triladyl extender with whole and with clarified egg yolk.

Key words: Spermatozoa, quality, insemination, fertility, precision, accuracy.

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Since the start of artificial insemination (AI) in cattle, the primary method for evaluation of semen quality has been a visual assessment of sperm motility under phase contrast microscopy (Saacke and White, 1972). This method is inexpensive and rapid but is also subjective and imprecise (Saacke et al, 1991; Stålhammar et al, 1994; Garner, 1997; Christensen et al, 1999). A more objective and precise assessment of sperm motility can be achieved with a computer-assisted semen analyzer (CASA), but bias from program settings and differences between systems, as well as the speed of the analysis, makes this method impractical for routine use (Davis and Katz, 1992; Holt et al, 1994). Traditional assessment of sperm morphology (Barth and Oko, 1989) is time consuming and subjective and appears to be of limited value for assessment of potential bull fertility (Elliot, 1978;

Whitfield and Parkinson, 1995; Garner, 1997). In recent years, new methods have been based on assessment of sperm functions, such as the ability of sperm to acrosome react, the ability of sperm to bind selectively to the zona pellucida, or the outcome of in vitro fertilization (Whitfield and Parkinson, 1995; Fazeli et al, 1997; Zhang et al, 1998). Although some of these methods have been correlated with fertility, they are also technically demanding, and the availability of materials such as oocytes are likely to limit their use to research purposes. A simpler approach is evaluation of plasma membrane integrity, which can be assessed with various fluorescent probes (Evenson et al, 1982; Garner et al, 1986; Ericsson et al, 1989; Garner et al, 1994; Januskauskas et al, 1996). The combination of a suitable method for fluorescent staining with flow cytometry makes it possible to analyze thousands of sperm per sample and achieve a much higher precision than is possible with microscopic assessment or CASA systems. The drawback of this technology is the price of equipment and the need for a skilled operator. Recently, the FACS-Count, a small stand-alone flow cytometer that provides fully automated analyses, was developed by BD Biosciences Immunocytometry Systems (San Jose, Calif; Strauss et al, 1996). This automated system is easy to use and requires no expert user intervention for analysis.

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Christensen et al (2004a) have developed a new method<sup>1</sup> based on staining with SYBR-14 plus propidium iodide (PI; Garner et al, 1994), making it possible to determine sperm viability and concentration simultaneously on a modified version of the FACSCount. This method was developed for routine use by AI stations and is rapid, precise, and objective.

Fertility data presented in most publications have severe limitations when used for the validation of a diagnostic assay (Amann and Hammerstedt, 2002). Often fertility studies are based on a limited number of males, too few females are inseminated per semen sample, or only 1 sample was used per male (Amann, 1989). To be successful, a sperm needs to express a number of attributes to achieve fertilization (Amann and Hammerstedt, 1993), and causes of subfertility are likely to vary from male to male. Thus, a relatively high number of males should be included in a fertility trial to draw a valid conclusion regarding the utility of a new sperm assay. The ideal fertility trial should be performed “on the edge of the dose-response curve” because the effect of a compensable sperm trait can be masked by use of a high number of sperm per insemination dose (Saacke et al, 1994; Shannon and Vishwanath, 1995; den Daas et al, 1998; Amann and Hammerstedt, 2002). In the study presented here, the flow cytometric method described by Christensen et al (2004a) was tested in a large insemination trial to describe the relationship between sperm viability and nonreturn rate of dairy bulls. The trial also included a comparison of the Biociphos Plus extender (IMV, Cedex, France) against the Triladyl extender (Minitüb, Tifenbach, Germany) which contained whole or clarified egg yolk.

## Materials and Methods

### Preparation of Bulls

Over a period of 7 months, 157 dairy bulls (aged 13 to 18 months) were used in semen production at 2 bull stations. The bulls were either Holstein (114) or Jersey (43). In the experiment, each bull was collected once weekly for 4 weeks. Prior to these collections, the bulls passed a clinical andrological examination and were prepared by 1 semen collection weekly until sperm motility after freezing and thawing was determined to be acceptable for insemination (>50% progressively motile sperm after thawing). Microscopic assessments of sperm motility for raw and frozen-thawed semen were carried out by a single observer in each of the 2 laboratories. Both observers were experienced and participated in an annual course regarding this procedure. No additional step regarding standardization of the motility assessments was taken prior to the trial. The number of bulls and inseminations in this trial was based on previous observations (Christensen et al, 1999) and a calculation of the num-

ber of inseminations necessary to detect a difference of 2 percentage units in the 56 days nonreturn rate (NRR56) between the 3 freezing extenders used.

### Semen Production and Routine Evaluation

For practical reasons, groups of 10 to 18 bulls were collected each day for production of experimental semen. A total of 628 experimental ejaculates were collected over 44 days and 623 ejaculates were processed for freezing. For all ejaculates, the volume of the raw semen was assessed and sperm concentration was determined with a Sysmex F-820 particle counter (Sysmex GmbH, Hamburg, Germany). Wave motion of the raw semen was assessed by phase contrast microscopy (100× magnification) and scored subjectively on a scale from 1 to 6. Sperm motility was assessed at 200× magnification after a 10-fold dilution of the raw semen in Triladyl extender (Minitüb) containing 20% (vol/vol) egg yolk. All microscopic assessments were performed with a heated stage at 37°C. Following the routine assessments, ejaculates were split into 3 aliquots and diluted in Biociphos Plus (IMV) or Triladyl extender with either whole or clarified egg yolk (debris was removed by centrifugation at  $10\,000 \times g$  for 30 min). The number of sperm per dose was adjusted for differences in sperm motility in the raw semen and each 0.25-mL straw contained approximately  $15 \times 10^6$  motile sperm. Straws were printed with a 2-figure experimental code that indicated the number of the ejaculate as well as the type of extender. After packing and 3 to 5 hours equilibration, straws were frozen in a programmable freezer (IMV). After freezing, 2 straws frozen in Triladyl extender (whole egg yolk) was thawed and assessed for sperm motility as described previously. Semen was used for inseminations if at least 50% of the sperm were motile 1 to 2 minutes after thawing.

### Flow Cytometry

For flow cytometry, 20  $\mu\text{L}$  of the raw semen was diluted to 5 mL with a Hamilton Microlab A503 autodilutor (Struers KEBO Lab, Albertslund, Denmark). Dilution medium was phosphate-buffered saline, pH 7.4, containing 0.1% bovine serum albumin (Sigma-Aldrich Chemie GmbH, Germany). A 50- $\mu\text{L}$  aliquot of the dilution was withdrawn and added to a counting tube containing approximately 100 000 fluorescent microspheres in 400  $\mu\text{L}$  FACSCount diluent (BD Biosciences). Before use, 50 nM of SYBR-14 and 12  $\mu\text{M}$  propidium iodide (PI, Molecular Probes, Eugene, Ore) were added per counting tube. Incubations were for 4 minutes at room temperature ( $\sim 20^\circ\text{C}$ ), and the tubes were subsequently analyzed on a modified FACSCount flow cytometer (BD Biosciences) with a 488-nm air-cooled argon laser. A total of 5000 sperm were analyzed for the log of their fluorescence for each sample. Emission signals were separated by a 620-nm short-pass dichroic mirror. The green fluorescence was collected through a band-pass filter (515 to 545 nm), and the red fluorescence was collected through a 645-nm long-pass filter. No compensation was used. Subsequent data analysis was performed with Attractors Software (BD Biosciences) on a Macintosh Quadra 650 computer. Each analysis gave an estimate of the percentage of membrane-intact sperm (only stained with SYBR-14), as well as the total sperm concentration (sperm stained with

<sup>1</sup> Patent Pending, Int Publ No WO/00/54026.

SYBR-14, PI, or both and calculated relatively to the fluorescent microspheres).

For each combination of ejaculate and extender, 2 straws were thawed in a waterbath at 37°C for 30 seconds, pooled, and diluted 1:10 in Thyrode's medium with albumin, lactate, and pyruvate (TALP, Parrish et al, 1989). Diluted samples were incubated at 37°C for 30 minutes before a 10- $\mu$ L aliquot was withdrawn and added to a counting tube. Incubations and flow cytometric analyses were performed as described previously and resulted in determination of sperm concentration and viability. On the basis of an average content in straws of 0.2 mL, the dilution rate, and the flow cytometric determination of sperm concentration, the number of sperm per straw was calculated.

The longevity of sperm viability was assessed for both raw and thawed semen. A sample of the raw semen was diluted to a concentration of  $12 \times 10^6$  sperm/mL in TALP, incubated at 37°C, and analyzed 6 hours after semen collection ( $T = 6$ ). The diluted samples of thawed semen (mentioned previously) were analyzed 6 hours after thawing ( $T = 6$ ). All analyses of raw semen or thawed semen were performed in duplicate at each time point (after collection or thawing [ $T = 0$ ] or at 6 hours [ $T = 6$ ]). A total of 9988 flow cytometric analyses were done.

### Experimental Inseminations and Field Data

Inseminations were carried out at random in 6352 Danish herds by 254 AI technicians from December 1, 1998, to December 31, 1999. The total number of inseminations performed was 118 680, but only 51 307 were first inseminations, making more than 50% of the inseminations second, third, or subsequent inseminations. For each insemination, the identity of the bull and cow/heifer, the code for type of extender, and the semen production date were recorded. A "heat score," which was an evaluation of the strength of the signs of heat on a scale from 1 (weak) to 4 (strong) was also recorded. The recording of heat score was introduced as a part of the study, and we did not attempt to standardize the subjective assessment made by individual AI technicians. All information regarding experimental inseminations, other inseminations, and the outcome (measured as NRR56) were extracted from the national Danish cattle database. To estimate the fixed effects, all inseminations in the herds, regardless of whether the bulls were experimental or non-experimental, were included in the data set. The following editing rules were applied: double registrations were deleted and only 5 breeds were included (Danish Red and White, Danish Holstein, Danish Jersey, Danish Red, and crossbreds). Insemination records more than 300 days after calving, or in the case of heifers, more than 800 days after birth, were deleted. The number of inseminations was set to a minimum of 5 per herd and 15 per bull. Only cows with 5 or fewer parities were considered. In accordance with the general editing rules in Denmark, the minimum calving interval was 200 days, accounting for abortions, and minimum age at first calving was not allowed to be below 18 months for Jerseys and 20 months for the other breeds. During editing of first inseminations, 6361 experimental inseminations were lost; the data set contained 44 946 experimental inseminations and 450 225 first inseminations by non-experimental bulls. Only first inseminations were used in the analyses because software was not available for a proper (bivar-

iate) analysis of all inseminations jointly with laboratory data. Forty bulls did not produce enough insemination doses for progeny testing during the 4 experimental semen collections and were therefore also used for nonexperimental inseminations during the period of the trial.

### Statistical Analyses

*Preparation of Laboratory Data*—Data for the different laboratory methods were not normally distributed; therefore, they were transformed to approximate normal distributions by Box-Cox power transformation (Box and Cox, 1964; Handelsman, 2002). Transformed values ( $z_i$ ,  $i = 1, \dots, n$ ;  $n =$  number of observations) were centered and scaled to approximately the same scale as the raw data (mean and variance did not change).

For the transformed values, apply

$$z_i = \begin{cases} gy + \frac{y_i^\lambda - gy^\lambda}{\lambda \cdot gy^{\lambda-1}} & \text{if } \lambda \neq 0 \text{ or} \\ gy + (\log y_i - \log gy)gy & \text{if } \lambda \sim 0, \end{cases}$$

where  $gy$  is the geometric mean of  $y$  and

$$gy = \sqrt[n]{\prod_{i=1}^n y_i}.$$

Only the transformed data were used for the statistical analyses.

*Insemination Data Set*—Variance components were estimated with DMU-AI (DIAS Multivariate analysis by restricted maximum likelihood; Jensen et al, 1996–97). The trait NRR56, though scored on a binary scale, was analyzed as if normally distributed by the following model:

$$y_{ijklmnopqrt} = YM_i + SR_j + E_k + P \cdot B_l + P \cdot ES_m + C_n + h_o + t_p + b_q + tb_r + tb \cdot SR_t + e_{ijklmnopqrt}$$

where  $y_{ijklmnopqrt}$  is the observation of NRR56. The model contained the following fixed effects: year-month of insemination ( $YM_i$ ,  $i = 1, \dots, 13$ ), sampling round (the ejaculate number for the bull;  $SR_j$ ,  $j = 1, \dots, 4$ ), extender used to dilute the ejaculate ( $E_k$ ,  $k = 1, 2, 3$ ), parity  $\times$  breed ( $P \times B_l$ ) and parity  $\times$  strength of estrus ( $P \times ES_m$ ) interactions, and AI-company ( $C_n$ ,  $n = 1, \dots, 7$ ). Herd ( $h_o$ ), AI technician ( $t_p$ ), and nonexperimental bull ( $b_q$ ) were included as random effects. Further random effects were test bull ( $tb_r$ ) and the interaction of test bull  $\times$  sampling round, that is, ejaculate ( $tb \times SR_t$ ) and the residual ( $e_{ijklmnopqrt}$ ). Variance components were estimated for the random effects.

*Laboratory Data Sets*—Models were developed individually for each trait after data transformation. The initial model contained the fixed effects of breed, group of bulls, sampling round, person, sample (and for frozen semen, extender), and all their possible 2- and 3-way interactions. These models were analyzed in SAS GLM (Statistical Analysis Systems Inc, Cary, NC), and nonsignificant effects were stepwise deleted, beginning with the least significant. This procedure was repeated, until a "compact" model was found, including all significant 3-way interactions and all 2-way interactions, unless they were included in the 3-way combinations. All main effects were part of the model anyway. Finally, the random effects of bull and bull  $\times$  sampling round (= ejaculate) were added, and these models were reanalyzed in DMU-AI.

*Bivariate Analyses*—The 2 models for NRR56 and for the laboratory data were combined to give the following bivariate model:

$$\begin{bmatrix} \mathbf{Y}_{\text{NRR56}} \\ \mathbf{Y}_{\text{LAB}} \end{bmatrix} = \begin{bmatrix} \mathbf{X}_{\text{NRR56}} & 0 \\ 0 & \mathbf{X}_{\text{LAB}} \end{bmatrix} \begin{bmatrix} \mathbf{b}_{\text{NRR56}} \\ \mathbf{b}_{\text{LAB}} \end{bmatrix} + \begin{bmatrix} \mathbf{Z}_{\text{NRR56}} & 0 \\ 0 & \mathbf{Z}_{\text{LAB}} \end{bmatrix} \begin{bmatrix} \mathbf{u}_{\text{NRR56}} \\ \mathbf{u}_{\text{LAB}} \end{bmatrix} + \begin{bmatrix} \mathbf{e}_{\text{NRR56}} \\ \mathbf{e}_{\text{LAB}} \end{bmatrix},$$

where  $\mathbf{Y}_{\text{NRR56}}$  and  $\mathbf{Y}_{\text{LAB}}$  are vectors of observations on NRR56 or laboratory trait (LAB), respectively;  $\mathbf{b}_{\text{NRR56}}$  and  $\mathbf{b}_{\text{LAB}}$  are vectors of fixed effects;  $\mathbf{u}_{\text{NRR56}}$  and  $\mathbf{u}_{\text{LAB}}$  are the random effects; and  $\mathbf{X}_{\text{NRR56}}$ ,  $\mathbf{X}_{\text{LAB}}$ ,  $\mathbf{Z}_{\text{NRR56}}$ , and  $\mathbf{Z}_{\text{LAB}}$  denote corresponding incidence matrices. The random residuals are denoted by  $\mathbf{e}_{\text{NRR56}}$  and  $\mathbf{e}_{\text{LAB}}$ . Random effects of bull of the 2 traits were assumed to be correlated, as well as ejaculate effects (nested within bull) of the 2 traits. The residuals of NRR56 and the laboratory trait were assumed to be independent because they were generated in entirely different environments.

*Rejection Values*—Assuming that NRR56,  $\mathbf{Y}_{\text{NRR56}}$ , and the 2 laboratory traits of viability in raw semen ( $\mathbf{Y}_{\text{LAB1}}$ ) and viability in postthaw semen ( $\mathbf{Y}_{\text{LAB2}}$ ) follow a trivariate normal distribution,

$$\begin{bmatrix} \mathbf{Y}_{\text{NRR56}} \\ \mathbf{Y}_{\text{LAB1}} \\ \mathbf{Y}_{\text{LAB2}} \end{bmatrix} = N_3 \left( \begin{bmatrix} \mu_{\text{NRR56}} \\ \mu_{\text{LAB1}} \\ \mu_{\text{LAB2}} \end{bmatrix}, \begin{bmatrix} \sigma_{\text{NRR56}}^2 & \sigma_{\text{NRR56,LAB1}} & \sigma_{\text{NRR56,LAB2}} \\ \sigma_{\text{LAB1,NRR56}}^2 & \sigma_{\text{LAB1}}^2 & \sigma_{\text{LAB1,LAB2}} \\ \sigma_{\text{LAB2,NRR56}}^2 & \sigma_{\text{LAB2,LAB1}}^2 & \sigma_{\text{LAB2}}^2 \end{bmatrix} \right)$$

then the expected increase in NRR56 using only ejaculates with sperm viability of raw semen above the rejection value  $\tau_1$  and postthaw semen with sperm viability above the rejection value  $\tau_2$  (ie, expected NRR56 given selection on ejaculates [across bulls] minus expected NRR56 without selection) is given by the formula

$$\begin{aligned} & E(\mathbf{Y}_{\text{NRR56}} | \mathbf{Y}_{\text{LAB1}} \geq \tau_1, \mathbf{Y}_{\text{LAB2}} \geq \tau_2) - E(\mathbf{Y}_{\text{NRR56}}) \\ &= \left\{ \left[ \int_{\tau_2}^{\infty} \int_{\tau_1}^{\infty} \int_{-\infty}^{\infty} y_{\text{NRR56}} p(y_{\text{NRR56}} | y_{\text{LAB1}}, y_{\text{LAB2}}) \right. \right. \\ &\quad \left. \left. \times p(y_{\text{LAB1}}, y_{\text{LAB2}}) dy_{\text{NRR56}} dy_{\text{LAB1}} dy_{\text{LAB2}} \right] \right. \\ &\quad \left. \div \left[ \int_{\tau_2}^{\infty} \int_{\tau_1}^{\infty} p(y_{\text{LAB1}}, y_{\text{LAB2}}) dy_{\text{LAB1}} dy_{\text{LAB2}} \right] \right\} - \mu_{\text{NRR56}}, \end{aligned}$$

where  $p(y_{\text{NRR56}} | y_{\text{LAB1}}, y_{\text{LAB2}})$  is the conditional density of  $\mathbf{Y}_{\text{NRR56}}$  given  $\mathbf{Y}_{\text{LAB1}}$  and  $\mathbf{Y}_{\text{LAB2}}$ , and  $p(y_{\text{LAB1}}, y_{\text{LAB2}})$  is the joint density of  $\mathbf{Y}_{\text{LAB1}}$  and  $\mathbf{Y}_{\text{LAB2}}$ .

Expected increase in NRR56 was calculated for extender 1 (first inseminations). The covariance matrix was based on 2 bivariate analyses (with the model described in “Bivariate Analyses” section) of NRR56 and LAB1 and NRR56 and LAB2, respectively, and on 1 bivariate analysis of the 2 laboratory measurements. In the bivariate analysis of the 2 laboratory measurements, random effects of bull and ejaculate (nested within bull) of the 2 traits were correlated, as well as residuals of the 2 laboratory traits.

Table 1. Semen parameters from routine evaluation expressed as mean and SD (in parentheses) for the 2 artificial insemination (AI) stations. The number of bulls collected were 71 at station A (25 Jersey and 46 Holstein) and 86 at station B (18 Jersey and 68 Holstein). In total, 628 ejaculates were collected (station A: 284; station B: 344) and 623 were frozen

| AI station  | A          | B          |
|---|------------|------------|
| Initial sperm motility (%)*                           | 74.4 (4.6) | 68.6 (5.0) |
| Postthaw sperm motility (%)                           | 62.4 (8.9) | 61.9 (9.6) |
| Sperm concentration (raw semen, 10 <sup>6</sup> /mL)* | 1006 (792) | 1271 (861) |
| Volume of ejaculate (mL)*                             | 6.4 (2.2)  | 4.9 (2.4)  |
| Wave motion*  | 4.5 (0.7)  | 5.9 (0.7)  |
| No. of doses per ejaculate†                           | 315 (199)  | 278 (215)  |

\* The means differed significantly ( $P < .0001$ ) between stations.

† The means differed significantly ( $P = .0269$ ) between stations.

## Results

### Semen Production and Routine Evaluation

The 157 bulls were collected at 2 AI stations. Station A collected 71 bulls (25 Jersey + 46 Holstein) and station B collected 86 bulls (18 Jersey + 68 Holstein). Four ejaculates were collected from each bull (628 ejaculates), and 623 ejaculates were diluted, packaged in straws, and frozen. Five semen collections resulted in low sperm concentration or low semen volume and were therefore not processed. Postthaw sperm motility was below 50% for 9 ejaculates, and only 614 ejaculates were used for experimental inseminations. Data from routine evaluation at the 2 AI stations are shown in Table 1. It appears that station A collected ejaculates with a larger volume and lower concentration than station B (on average 6.4 mL and  $1006 \times 10^6$  sperm/mL vs 4.9 mL and  $1271 \times 10^6$  sperm/mL, respectively, both  $P < .0001$ ). Wave motion also differed significantly between stations (4.5 vs 5.9,  $P < .0001$ ). The subjective assessment of sperm motility differed significantly for raw semen (on average 74.4% vs 68.6%,  $P < .0001$ ), but for thawed semen, no significant difference was detected (62.4% vs 61.9%,  $P > .05$ ). The number of insemination doses produced per ejaculate differed slightly between stations (on average 315 doses vs 278 doses,  $P = .0269$ ).

When Jersey bulls ( $n = 43$ ) were compared with Holstein bulls ( $n = 114$ ), the average volume of the ejaculates appeared to be significantly lower ( $P < .0001$ ) for Jersey (5.2 vs 6.1 mL). On the other hand, Jersey bulls had a higher sperm concentration ( $1199 \times 10^6$  vs  $1079 \times 10^6$  sperm/mL), but this difference was not significant ( $P > .05$ ). Motility of the raw semen or wave motion did not differ significantly between Jersey and Holstein bulls (71.4% vs 71.7% and 5.2 vs 5.3, respectively). Postthaw motility was only assessed for semen frozen in the Triladyl extender with whole egg yolk and was significantly higher for Holstein bulls (63.8% vs 60.5%,  $P < .0001$ ).

Table 2. Flow cytometric analyses of raw and thawed semen. Values represent mean and SD (in parentheses) for the percentage of viable sperm. Raw semen was analyzed after semen collection, and frozen semen was analyzed 30 minutes after thawing ( $T = 0$ ). Both types of semen were also analyzed after dilution in TALP and 6 hours of incubation at 37°C ( $T = 6$ )

| Semen Type                                      | % Viable Sperm |             |
|---|----------------|-------------|
|   | $T = 0$        | $T = 6$     |
| Raw semen                                       | 82.1 (7.1)     | 80.5 (6.6)  |
| Thawed semen, Triladyl with whole egg yolk*     | 55.1 (11.1)    | 45.3 (11.0) |
| Thawed semen, Triladyl with clarified egg yolk† | 53.1 (11.4)    | 43.5 (11.3) |
| Thawed semen, Biociphos Plus                    | 50.0 (11.2)    | 35.6 (9.5)  |

\* Triladyl extender with 20% (vol/vol) egg yolk.

† Egg yolk was centrifugated for 30 minutes at 10 000 × *g*. Supernatant (20% vol/vol) was used in the extender.

### Flow Cytometry

Results of the flow cytometric analyses are shown in Table 2. For raw semen, a small decrease in sperm viability occurred from semen collection to 6 hours after collection. Although this decrease was significant ( $P < .0001$ ), the measurements at the 2 time points were highly correlated ( $r = .85$ ). With frozen-thawed sperm in each extender, the decrease in viability was significant between the 2 time points ( $P < .0001$ ). The sperm viability for semen frozen in Triladyl extender with whole egg yolk vs clarified egg yolk differed significantly ( $P < .01$ ) at both time points. Postthaw viability of sperm frozen in Biociphos Plus was lower ( $P < .0001$ ) than for sperm frozen in either type of Triladyl extender at both time points. Although sperm viability appeared to decrease more over the 6 hours of incubation when semen was frozen in Biociphos Plus, the difference was not statistically significant ( $P > .05$ ) compared with the 2 Triladyl extenders. Results for the 2 time points were highly correlated for the 3 extenders ( $r = .89$ ). From the flow cytometric determination of sperm concentration in the diluted thawed samples, the average number of sperm was calculated as  $20.4 \times 10^6$  sperm/straw with a standard deviation of  $6.3 \times 10^6$  sperm/straw (effective straw volume = 0.20 mL). Viability of raw or thawed semen did not differ significantly between Holstein and Jersey bulls ( $P > .05$ ).

### Insemination Data

After editing the insemination data, the total number of experimental first inseminations were 44 946. Differences in NRR56 between the 3 extenders are shown in Table 3. The results indicate that NRR56 was highest for Triladyl with whole egg yolk, 0.38 percentage units lower for Triladyl with clarified egg yolk, and 0.68 percentage units lower for Biociphos Plus. However, these results were not

Table 3. Fertility level for the 3 freezing extenders used in the experiment. Values are percentage of 56 days nonreturn rate (NRR56) relative to Triladyl extender with whole egg yolk. The standard error was 0.55%, and differences between the extenders were not statistically significant ( $P > .05$ )

| Extender                          | No. of First Inseminations | Fertility Level, % |
|-----------------------------------|----------------------------|--------------------|
| Triladyl with whole egg yolk*     | 14 738                     | 0.00               |
| Triladyl with clarified egg yolk† | 14 984                     | -0.38              |
| Biociphos Plus                    | 15 224                     | -0.68              |

\* Triladyl extender with 20% (vol/vol) egg yolk.

† Egg yolk was centrifugated for 30 minutes at 10 000 × *g*. Supernatant (20% vol/vol) was used in the extender.

significantly different ( $P > .05$ ) because the overall standard error was 0.55 percentage units. No significant difference was observed in average NRR56 for the 2 breeds of bulls (Jersey + 0.52% compared with Holstein, SE = 0.78). NRR56 did not differ for the 2 AI stations (station A + 0.24% compared with station B, SE = 2.1%). An overall effect of sample round (ejaculate) was not found to be significant ( $P > .05$ ).

The effect of heat score is shown in Table 4. No heat score was recorded for 334 first inseminations (0.7%). A strong or normal heat was recorded for 86.6% of the experimental inseminations, moderate heat was observed for 11.7% of the inseminations, and a weak heat was observed in the remaining 1%. Compared with the average NRR56, cows or heifers with a normal or strong heat were 4% to 7% above the average for cows and heifers. In contrast, cows or heifers with a moderate or weak heat were 5% to 15% below their average NRR56.

Estimated variance components for the random effects of herd, AI technician, nonexperimental bulls and experimental bulls, and ejaculate × extender are shown in Table 5. The largest proportion of the variation in the NRR56 was residual (95.4% of the total variation). Differences between herds accounted for 3.78% of the variation and differences between AI technicians accounted for 0.25%. The proportion of variation in NRR56 explained by experimental bulls was 0.17% (nonexperimental bulls, 0.18%), and the combination of ejaculate × ex-

Table 4. Effect of heat score at the time of artificial insemination on the 56 days nonreturn rate (NRR56) for 44 946 experimental first inseminations. Effect is expressed as percentage units relative to the average NRR56 in the experiment of 61%

| Heat Score | Description of Heat | No. of Observations* | Effect on NRR56, % |      |
|------------|---------------------|----------------------|--------------------|------|
|            |                     |                      | Heifers            | Cows |
| 1          | Weak                | 449                  | -15                | -9   |
| 2          | Moderate            | 5239                 | -10                | -5   |
| 3          | Normal              | 32 309               | 4                  | 5    |
| 4          | Strong              | 6615                 | 6                  | 7    |

\* Heat score was not recorded for 334 first inseminations.

Table 5. Variance components for the random effects of herd, artificial insemination (AI) technician, nonexperimental bulls, experimental bulls, ejaculate  $\times$  extender on 56 days nonreturn rate (NRR56) based on 44 946 first inseminations. Variance, percentage of total variance, standard deviation (SD) in percent, and 99% confidence interval (CI) for NRR56 are shown. Average NRR56 was 61.0%

| Effect of                   | Variance | % of Total Variance | SD  | 99% CI for NRR56 |
|-----------------------------|----------|---------------------|-----|------------------|
| Herd                        | .0086774 | 3.78                | 9.3 | 33.1–88.9        |
| AI technician               | .0005798 | 0.25                | 2.4 | 53.8–68.2        |
| Nonexperimental bulls       | .0004101 | 0.18                | 2.0 | 55.0–67.0        |
| Experimental bulls          | .0003824 | 0.17                | 2.0 | 55.0–67.0        |
| Ejaculate $\times$ extender | .0004749 | 0.21                | 2.2 | 52.2–69.7*       |
| Residual                    | .2192628 | 95.41               | ... | ...              |
| Total                       | .2297874 | ...                 | ... | ...              |

\* The 99% CI for NRR56 is calculated for bull  $\times$  ejaculate  $\times$  extender.

tender accounted for 0.21% of the total variation. The overall variation in NRR56 was large, and the 99% confidence interval (CI) for different herds was from 33.1% to 88.9%. In comparison, the 99% CI for NRR56 for experimental bulls varied from 55% to 67% and for individual ejaculates (bull  $\times$  ejaculate  $\times$  extender) from 52.2% to 69.7%.

#### Correlation Between NRR56 and Seminal Traits

The total number of sperm per straw, as well as the number of motile or viable sperm per straw, did not correlate with NRR56 ( $P > .05$ ). Routine assessments of ejaculate volume, sperm concentration of the raw semen, wave motion, or number of doses per ejaculate did not correlate with NRR56 ( $P > .05$ ).

Microscopic assessment of sperm motility of the raw and thawed semen correlated with NRR56 ( $r = .477$  and  $.553$ , respectively) as indicated in Table 6. Because only 1 assessment of sperm motility was performed for each ejaculate or batch of thawed semen, it was not possible to separate bull and bull  $\times$  ejaculate effects for sperm motility. Sperm viability assessed through flow cytometry also correlated significantly with NRR56. Raw semen cor-

Table 7. Rejection values are based on 14 738 first inseminations with semen extended in Triladyl with whole egg yolk and assessment of sperm viability by the FACSCount AF system. Numbers indicate the increase (percentage units) in 56 days nonreturn rate (NRR56), and numbers in parentheses indicate the percentage of ejaculates above the different rejection points

| Viable sperm Post-thaw, % | Raw semen      |                |                |                |                |                |
|---------------------------|----------------|----------------|----------------|----------------|----------------|----------------|
|                           | 65%            | 70%            | 72.5%          | 75%            | 77.5%          | 80%            |
| 30                        | 0.04<br>(98.4) | 0.09<br>(95.6) | 0.15<br>(91.7) | 0.24<br>(84.9) | 0.36<br>(75.0) | 0.52<br>(62.1) |
| 35                        | 0.09<br>(96.2) | 0.13<br>(93.8) | 0.18<br>(90.3) | 0.26<br>(84.1) | 0.38<br>(74.5) | 0.53<br>(61.9) |
| 40                        | 0.19<br>(91.1) | 0.22<br>(89.5) | 0.26<br>(86.7) | 0.32<br>(81.3) | 0.42<br>(72.7) | 0.56<br>(60.9) |
| 45                        | 0.36<br>(81.7) | 0.37<br>(80.8) | 0.39<br>(79.0) | 0.44<br>(75.1) | 0.52<br>(68.2) | 0.63<br>(58.1) |
| 50                        | 0.58<br>(67.7) | 0.59<br>(67.2) | 0.60<br>(66.3) | 0.63<br>(63.9) | 0.69<br>(59.4) | 0.78<br>(51.9) |

relations were 0.323 at the bull level and 0.409 at the ejaculate level. For thawed semen, correlations were 0.412 (bull level) and 0.506 (ejaculate level), respectively. On the ejaculate level, the standard error for the correlation estimate could not be obtained but is assumed to be the same magnitude as at the bull level. Correlations for sperm viability on ejaculate level for raw or thawed semen appeared slightly lower than for the corresponding correlations for sperm motility.

#### Effect of Semen Rejection Based on Sperm Viability

Calculated rejection values for 14 738 first inseminations indicated that rejection of semen with less than 75% viable sperm in the raw semen and less than 30% viable sperm postthaw will result in an increase in NRR56 by 0.24% (Table 7). For this rejection point, 15.1% of the semen batches in the present experiment would have to be rejected (84.9% of the ejaculates were above both rejection values). If, for instance, the combination of rejection values was 75% viability for raw semen and 45% for postthaw, the corresponding increase in NRR56 would be 0.44%, and 24.9% of the semen batches would have to be rejected.

Table 6. Correlations to 56 days nonreturn rate (NRR56), for sperm viability assessed by the FACSCount AF flow cytometer (after semen collection or 30 minutes postthaw) and microscopic assessment of sperm motility (after semen collection or 1 to 2 minutes postthaw). Correlations are based on 44 946 first inseminations. Standard errors (parentheses) could not be obtained at bull  $\times$  ejaculate level in this study

| Parameter       | Raw Semen     |                         | Thawed Semen  |                         |
|-----------------|---------------|-------------------------|---------------|-------------------------|
|                 | Bull          | Bull $\times$ Ejaculate | Bull          | Bull $\times$ Ejaculate |
| Sperm viability | 0.323 (0.157) | 0.409 (—)               | 0.412 (0.186) | 0.506 (—)               |
| Sperm motility* |               | 0.477 (0.196)           |               | 0.553 (0.220)           |

\* Only one assessment of motility was carried out per sample and it was therefore not possible to separate bull and ejaculate (bull  $\times$  ejaculate) effects.

## Discussion

More than 95% of the variation in NRR56 in the present trial was residual and was not explained by the effects of herd, AI technician, bull, or ejaculate. To describe the potential sources of variation as precisely as possible, AI technicians were asked to score subjectively the signs of heat on a scale from 1 (weak heat) to 4 (strong heat). This information was clearly valuable because large differences in fertility were observed for the different groups. The bivariate model made it possible to obtain a correlation between a seminal trait and NRR56 for bulls and ejaculates, with an exclusion of all variation that could not be accounted for by bull or ejaculate. Together, bull and ejaculate effects only accounted for approximately 0.4% of the random variation in NRR56. This is in agreement with Stålhammar et al (1994), who observed that motility of semen before and after freezing accounted for less than 1% of the variation in NRR56. The average number of sperm per insemination dose in the study by Stålhammar et al (1994) was  $20 \times 10^6$  motile sperm, and semen was only used for inseminations if postthaw motility was above 45% to 50%. In this study, the average number of sperm per straw was  $20.4 \times 10^6$  (SD =  $6.3 \times 10^6$ ), and straws were only used for inseminations if postthaw motility was above 50%. Preselection of semen used for inseminations and a high number of sperm per dose are likely to have reduced the potential differences between bulls and ejaculates. Saacke and White (1972) observed that the greater portion of variation in fertility was due to differences between bulls rather than among ejaculates within bulls. In this study, the standard deviation for NRR56 between bulls was 2.0%. The extenders tested in this trial interacted differently with the individual ejaculates, and it was therefore not possible to separate extender and ejaculate variation. However, because the overall effect of extender was insignificant, it is assumed that the standard deviation for ejaculates was roughly 2.2% and thus similar to the variation for bulls. The standard deviations for both bulls and ejaculates were smaller than reported previously (Saacke and White, 1972; Foote and Oltenacu, 1980; Stålhammar et al, 1994) but represents the real situation in Denmark, in which young bulls for progeny testing are preselected and semen is not used for inseminations unless postthaw sperm motility is above 50%. Standard deviations for NRR56 in this trial were not different from that of the nonexperimental bulls used in Denmark during the same period.

The present trial included a comparison of the Biociphos Plus compared with the Triladyl extender with whole egg yolk, which is used routinely in Denmark. Flow cytometric assessment of viability indicated poorer sperm survival and longevity in the Biociphos Plus than the Tri-

ladyl extender, but the difference between extenders was not significant ( $P > .05$ ). Insemination data indicated a tendency toward a lower NRR56 for the Biociphos Plus extender, but differences between extenders were not statistically significant on the basis of approximately 15 000 inseminations performed per extender. Neither Gil et al (2000) nor Thun et al (2002) observed significantly lower nonreturn rates for the Biociphos Plus compared with Triladyl and a Tris egg yolk extender, respectively. In these trials, the total number of sperm per dose was  $17 \times 10^6$  (Gil et al, 2000) and  $20 \times 10^6$  (Thun et al, 2002). In contrast, van Wagtenonk-de Leeuw et al (2000) found a significantly lower NRR56 for Biociphos Plus compared with a TRIS extender but used a total number of sperm per dose that varied from 2.5 to  $20 \times 10^6$ . A large-scale low-dose insemination trial is a laborious and expensive way to test new extenders. A simpler approach would be to use flow cytometry to screen extenders for postthaw viability and longevity for a number of different bulls and ejaculates. An insemination trial could then be carried out if initial results are positive.

Decreasing the number of sperm per dose would result in more males with relative low fertility and would provide a better basis for testing a diagnostic assay for a compensable sperm defect (Amann, 1989; Amann and Hammerstedt, 2002). To perform an insemination trial on the edge of the dose-response curve requires the consent of the participating farmers, as well as financial compensation, depending on trial results. It was therefore not realistic to perform a low-dose insemination trial; rather, a large-scale trial with 157 bulls, 2 AI stations, and 254 AI technicians was carried out. This involved no consent from the farmers, and data could be collected through the Danish cattle database. The total number of experimental inseminations were 118 680, but only 51 307 were first inseminations (numbers refer to unedited data). For detection of differences in male fertility, observations based on first inseminations are ideal (Amann, 1989). Den Daas et al (1998) used both first and second inseminations, but did not discuss how information from second inseminations can be included in the statistical analysis in a proper way. The problem is that second, third, or subsequent inseminations always are carried out after an insemination (or a number of inseminations) that did not lead to pregnancy. Therefore, second, third, or subsequent inseminations are likely to include a higher proportion of cows with compromised reproduction and which furthermore might be unevenly distributed among the farms (ie, more inseminations in farms with poor management). Decuadro-Hansen et al (2002) performed a low-dose trial to test the FACSCount AF flow cytometer on frozen semen from top bulls. The number of viable sperm per dose in that trial was from 2.5 to  $7.5 \times 10^6$  sperm/dose, with a correlation ( $r = .24$ ) between the nonreturn rate and sperm

motility, whereas the correlation ( $r = .69$ ) to sperm viability was higher ( $P < .01$ ). Both the number of motile sperm per dose as well as the number of viable sperm per dose correlated significantly with nonreturn rates ( $r = .49$ ,  $P < .05$ ;  $r = .83$ ,  $P < .01$ , respectively), indicating that inseminations were on the dose-response rather than the asymptotic part of the curve (Amann and Hammerstedt, 2002). Pace et al (1981) reported an insemination trial in which insemination doses of 2 to  $8 \times 10^6$  motile sperm per dose were used and concluded that the number of motile sperm per dose rather than the percentage of motile sperm correlated with nonreturn rates. In this study, neither the total number of motile nor the total number viable sperm per dose correlated significantly with nonreturn rates. This indicates that when insemination doses of approximately  $15 \times 10^6$  motile sperm is used, differences in motility or viability are largely compensated. The relatively low correlations for both sperm motility and viability should be viewed on this background, as well as the preselection of bulls and ejaculates. In addition to the flow cytometric determination of sperm viability of raw semen just after semen collection and frozen semen 30 minutes postthaw, all flow cytometric measures were repeated after 6 hours of incubation at 37°C. However, these analyses did not provide more information regarding the semen quality because the results after incubation were highly correlated with initial results after semen collection or thawing.

In this trial, the correlation between sperm motility and NRR56 appeared to be slightly higher than for sperm viability against NRR56. In a recent trial with boar semen (Christensen et al, 2004b), we found that the correlation between sperm viability and litter size was higher than between sperm motility and litter size. The boar trial was also carried out with relatively large insemination doses ( $2.3 \times 10^9$  motile sperm per dose) and in agreement with this study, neither the total number of motile nor the total number of viable sperm correlated with litter size. The studies by Decuadro-Hansen et al (2002) and Christensen et al (2004b), as well as this study, show that sperm viability and sperm motility correlates with the fertilizing ability of a particular sample of semen. Although sperm motility is applied most commonly for routine evaluations, it should be kept in mind that this method is subjective and is relatively imprecise because of the evaluation of a limited number of sperm (Christensen et al, 2005). In contrast, flow cytometric determination of sperm viability can be performed objectively with high precision (Christensen et al, 2004a). Although the correlation between sperm viability and nonreturn rates appears to be slightly lower than the corresponding correlation for sperm motility, the flow cytometric method appears to be a better tool to reject semen of poor quality because precision is much higher than for sperm motility

(Christensen et al, 2005). Potential increases in overall NRR56 at given thresholds for sperm viability for raw semen and postthaw sperm viability (assessed by flow cytometry) are shown in Table 7. The first impression from this table is that only relatively small increases in NRR56 are possible if, for instance, a threshold of 75% viability for raw semen is used in combination with a postthaw threshold of 40%. At these thresholds, the increase in NRR56 would be approximately 0.32 percentage units, and 18.7% of the semen batches would be rejected. This corresponds quite well to the calculations by Foote and Oltenacu (1980), who predicted that if a correlation was 0.40 and 20% of the semen was rejected, average NRR56 would increase by 0.4 percentage units. An increase in 0.4 percentage units in NRR56 appears small but should be judged from the observation that a correlation of 1.0 and use of the same thresholds would increase NRR56 by only 0.9 percentage units because bulls and ejaculates account for only 0.4% of the total variation in NRR56. The benefit becomes more evident if the fertility of the rejected semen is considered. If a threshold for raw semen of 70% is used for sperm viability, 96% of all semen batches will be above this threshold and NRR56 would increase by 0.09 percentage units. However, semen below this threshold would have a NRR56 (if used) that would be 1.8 percentage units lower (not shown in Table 7). Use of sperm motility in the same manner would not be possible because this method is subjective and imprecise (Christensen et al, 2005).

Flow cytometry for assessment of sperm viability appears to be a valuable tool for the AI industry. When a high number of sperm is packed in each insemination dose, the effect of selecting the best ejaculates according to sperm viability has a relatively limited effect on NRR56. However, sperm viability might be more important when combined with low-dose inseminations. The FACSCount AF flow cytometer also determines sperm concentration accurately and precisely during the same analysis (Christensen et al, 2004a). The combination of assessment of sperm viability and concentration appears to be useful in the improvement of quality control at AI stations. Because of the results of this trial, this method has been implemented by Danish AI stations (Christensen et al, 2005).

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