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In vitro competitive binding index using fluorochrome-labelled spermatozoa for predicting bull fertility

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Summary

This work evaluated if an *in vitro* test, with the combined power of the statistical evaluation of spermatozoa and zona pellucida (ZP) competitive binding ability and a rapid method for accessory sperm counts, could predict the bull fertility. Ten Holstein Friesian bulls of known field fertility (five of high and five of low fertility) were selected. An *in vitro* heterospermic insemination approach, based on differential staining, was tested on 45 possible pairs of bulls (two batches per bull). Motility and quality (abnormalities and membrane status) seminal characteristics and estimated relative conception rates (ERCR) highlighted only one association between membrane integrity and ERCR ($p = 0.007$). Differences in ZP binding allowed us to rank bulls into two categories based on low and high binding ability. For eight bulls, this classification reflected the ERCR. Differences between batches were reported for two bulls, in which the effect of heterospermic insemination (the number of sperm binding to ZP from different bulls not in a 1:1 ratio) showed a significant bull-related effect ($p < 0.001$) in the first batch and no effect ($p > 0.05$) in the second batch for both bulls. Reduction of the number of oocytes per assay from 25 to 5 had no effect ($p > 0.5$) on the bulls' ranking. Our results suggest that *in vitro* competitive binding is a promising approach for estimating bull fertility and support concepts for further implementation, e.g. drastic reduction of oocyte number in a single pair assay and larger scale testing for batches.

Keywords: Bull fertility, Fluorochrome-labelled spermatozoa, Heterospermic insemination, *In vitro*, Zona pellucida

Introduction

The decline in dairy herd fertility is virtually worldwide (Lucy, 2001; Bousquet *et al.*, 2004; Evans *et al.*, 2006; Royal *et al.*, 2008; Yaniz *et al.*, 2008; Kerby, 2009; Maas *et al.*, 2009), with a negative association between the level of milk production and fertility (Harrison *et al.*, 1990; Butler 1998), as well as a negative genetic correlation between the level of milk production and fertility (Boichard *et al.*, 2002; Wall *et al.*, 2003; Winding *et al.*, 2006; Flint, 2009). Excess inbreeding due to selective pressure may contribute to the decline in fertility (Wall *et al.*, 2005; Pollot & Coffey, 2008).

Fertility is a complex parameter that depends on physiological and environmental factors, such as nutrition and management. Environmental factors are easy to control, while physiological factors are more complex and can depend on the female, male or both. Problems of the female are well known while male hypofertility is more subtle, is difficult to define and measure, and it can have a huge impact on the population due to artificial insemination (AI).

Fertility of the bull may depend on the quality of semen and on the level of intrinsic fertility of the bull itself. Reduced fertility due to poor semen quality can, in some way, be controlled and improved by acting on certain seminal features (concentration, motility, morphology, membrane integrity) (Pace *et al.*, 1981; Den Daas, 1992; Saacke *et al.*, 1994, 2000; Hammerstedt, 1996; Mehmood *et al.*, 2009), while it can not be improved if it is associated with the intrinsic factors of the bull.

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In order to make optimal use of semen, a production centre must rationalize the dosage of insemination straws, keeping into consideration the fertility of a bull (Galli *et al.*, 1990). Despite this need, no single measurement or *in vitro* based test is currently available that provides a reliable estimate of bull fertility, although a combination of several sperm trait assessments seems to be more informative (Amann, 1989; Farrell *et al.*, 1998; Zhang *et al.*, 1999; Januskauskas *et al.*, 2003; Pillips *et al.*, 2004; Giritharan *et al.*, 2005; Hallap *et al.*, 2006; Gillan *et al.*, 2008; Kastelic & Thundathil 2008).

At present, the most popular method worldwide for fertility estimation is related to non-return rate (NR), and is accurate only if a vast amount of data, randomized and corrected for main factors of environmental variability, is available. Therefore, with the NR it is possible to estimate the level of fertility only after the widespread use of a bull, and the need to rapidly acquire information on fertility remains unresolved.

Over a large series of inconclusive approaches, a promising way to provide a proper estimation of fertility is the use of an index of heterospermic fertility, proposed by Beatty *et al.* back in 1969. This approach includes the use of inseminating doses containing populations of sperm of two different bulls, placed directly in competition in the female genital tract. Using appropriate experimental designs, all pairs of bulls are compared and the number of offspring from each bull is compared with all the other (Martin & Dziuk, 1977). This competitive index eliminates factors related to the environment, operators and sperm number (Berger 1995), and is not influenced by male–female interaction (Berger & Dally, 2001). The limit is the complexity and the cost of experimental design in the field. Sperm oocyte binding *in vivo* assessed by collection of zygotes soon after fertilization (Saacke *et al.*, 1994) showed an important association with the fertility of the bull, but this experimental approach was too complex to allow actual application.

An interesting *in vitro* alternative was proposed for the rabbit (Parrish & Foote, 1985) and cat species (Niu *et al.*, 2006). Using these methods, fluorochrome-labelled spermatozoa from two bulls are simultaneously exposed to oocyte zona pellucida (ZP) *in vitro*, and allow immediate evaluation of sperm binding capacity. Application of this approach to bovine has given controversial results (Henault & Killian, 1994; Braundmeier *et al.*, 2002) but may still allow *in vitro* evaluation of fertility.

The aim of this work was to evaluate an *in vitro* test that combined the power of a statistical evaluation of competitive ZP-binding ability of spermatozoa with a simple and accurate method for sperm counts, and then to compare this to field fertility (ERCR).

Table 1 Estimated relative conception rates (ERCR) of sample bulls

High fertility		Low fertility	
ID Bull	ERCR	ID Bull	ERCR
1	+3.95	6	−2.30
2	+2.62	7	−3.11
3	+2.39	8	−3.52
4	+2.26	9	−3.93
5	+2.20	10	−4.74

Materials and methods

Experimental design

Ten Holstein Friesian bulls were identified on the base of their estimated field fertility and assigned to two groups of high and low fertility (Table 1). Semen quality and kinetic parameters were assessed.

Semen samples were randomly assigned with an internal code so that blind procedures could be used, and an *in vitro* heterospermic insemination approach based on ZP-binding by differentially stained sperm was applied to 45 pairs of bull semen. The test was then repeated using a second batch of semen samples. To minimize any effect of the stain the fluorochromes used for each of the two sperm samples were switched between batches.

Finally, an index of competitive binding ability was developed using the data obtained *in vitro* and the results were compared with values of fertility estimated *in vivo*.

Calculation of fertility reference in the field

An updated file reporting the 56-day non-return rate in lactating cows in service in Italy (data and data processing provided by Associazione Nazionale Allevatori di Frisona Italiana 'ANAFI', Italy) was used to calculate the field fertility index of bulls, using a modification of the model designed by Clay & McDaniel (2001). Briefly, the model is described as follows:

$$NR56 = FYM + ECM + P + DOI + ERCR + OSIRE + AIS + PE + e$$

where: NR56 = 56-day non-return status after AI (1 no return; 0 re-inseminated); FYM = farm–year–month of insemination; ECM = energy-corrected milk production adjusted to 3.5% fat and 3.2% protein content; five classes (<5.5 to 9, 10 to 14, 15 to 19 and >20 kg). P = parity (1, 2, 3); DOI = days open at first mating; 6 classes (<42, 42 to 62, 63 to 83, 84 to 104, 105 to 126, >126 days); ERCR = random effect of service sire estimated

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using a complete data set consisting of 1,091,377 records (771,235 cows and 2450 bulls); OSIRE = origin of the service sire (1, foreign; 2 progeny; 3 proved); AIS = AI centre (a number of 14 AI centres were used); PE = permanent cow effect; e = residual.

As indicated by Clay & McDaniel (2001), only bulls with an adequate number of inseminations (range from 938 to 9769) were used in this work.

In summary, the model assigned the bulls a value of fertility measured as a percentage of non return cows inseminated with their semen, purified of the main factors of variability.

Semen analysis

Two batches of frozen semen of the 10 selected bulls were evaluated for quality parameters. Kinetic parameters (total motility, progressive motility and mean velocity) were assessed by a computerized image analyser (CASA system, HTM-IVOS version 12; Hamilton Thorne). Spermatozoa with an average path velocity (VAP) > 25 μm/s were defined as motile (MT), while the sperm with a straight-line velocity/path velocity > 0.8 were defined as progressively motile (MP). Sperm integrity (IS), with particular regard to abnormalities (acrosome anomalies, total sperm anomalies and proximal cytoplasmic drops) was evaluated by visual estimation under interferential–differential contrast microscope (magnification: ×1250) after fixation in 0.2% glutaraldehyde solution in PBS without calcium and magnesium (1:2 v/v). Furthermore, concentration (CONC) analysis and a viability test to assess membrane integrity (IM) were performed using NucleoCounter SP-100 (ChemoMetec A/S).

Oocyte recovery and maturation

Ovaries were collected from a local abattoir and transported to the laboratory in PBS supplemented with penicillin and streptomycin, at 20–25°C. Follicles of 2–8 mm in diameter were aspirated with an 18-gauge needle connected to a vacuum pump and oocytes were selected on the basis of morphology and the presence of homogeneous layers of cumulus cells in HEPES-buffered tissue culture medium (TCM199, Sigma-Aldrich) supplemented with 0.1% bovine serum albumin (BSA, no. A9418, fraction V, Sigma). Groups of a 100 to 120 selected oocytes were then matured for up to 24 h in 2 ml bicarbonate-buffered TCM199 supplemented with follicle stimulating hormone (FSH)/luteinizing hormone (LH) (0.05 IU/ml, Pergovet) and 10% fetal bovine serum (FBS, Sigma), in 5% CO_2 and 95% humidified air at 38.5°C.

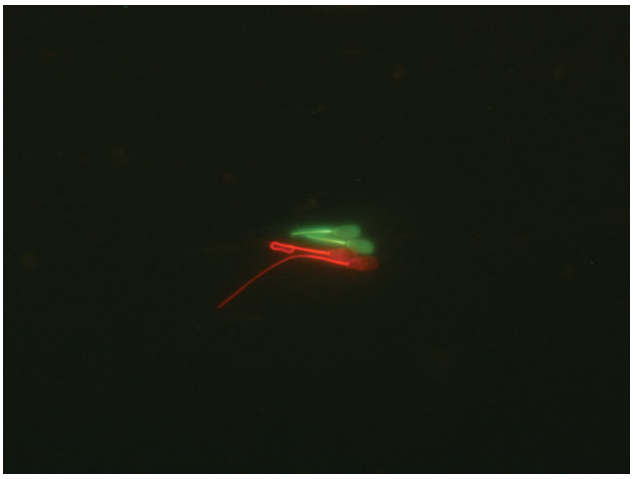

Figure 1 Fluorescence microscopy (×60) showing spermatozoa stained with red (CellTracker™ Red) or green (Fluo-4 AM and CellTracker™ Green) dyes.

Sperm staining

Frozen semen samples were thawed at room temperature for 30 s in water bath and washed by 10 min centrifugation at 500 *g* in 10 ml HEPES-buffered Ca^{2+}-free TALP medium (H-TALP) (pH 7.4) supplemented with 0.6% BSA. Sperm pellets were then differentially resuspended in 1 ml H-TALP medium containing either CellTracker(tm) Red (10 μM) (Invitrogen Ltd), or a combination of CellTracker(tm) Green (25 μM) (Invitrogen) and Fluo-4 AM (2.5 μM) (Invitrogen) and incubated for 1 h at room temperature in a non-static system. CellTracker(tm) is a supravital stain that reacts with thiols and is transformed into a cell-impermeant fluorescent dye-thioether. Green (CMFDA) and red (CMTPX) fluorescence in the cells is reasonably photostable during microscopic examination. CMFDA and CMTMX probes were brightly fluorescent for at least 72 h after incubation. Fluo-4 AM is a fluorescent Ca^{2+} indicator dye that offers a bright fluorescence emission in response to Ca^{2+} binding. Concentration of stains was empirically determined to ensure that the spermatozoa are visible and that the populations can be well discriminated (Fig. 1). The dyes were dissolved in DMSO and subsequently diluted in H-TALP. In order to eliminate possible differences due to the potential toxicity of DMSO, the diluent in all working solutions was adjusted to the same concentration.

After staining, motile sperm were selected by 20 min centrifugation at 500 *g* on discontinuous Percoll gradients (45–90%). The 90% Percoll fraction was obtained combining 9 ml of Percoll (no. P1644, Sigma) and 1 ml of 10 × Tyrode's solution (46.75 g NaCl/l, 2.3 g

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KCl/l, 0.4 g NaH_2PO_4/l, 20.9 g HEPES/l; pH 7.4). In the isotonic 90% Percoll was then added 2.1 g $NaHCO_3$/l, 3.7 ml 60% Na-lactate/l and 7.8 mg $MgCl_2.6H_2O$/l. For Percoll gradients preparation, 2 ml of Percoll 90% was carefully pipetted under 2 ml of Percoll 45%, which was obtained by mixing 1 ml of Percoll 90% with 1 ml of H-TALP. After centrifugation, sperm pellets were resuspended in 5 ml H-TALP medium and washed by 10 min centrifugation at 500 *g*. Finally the sperm concentration of the pellet was determined using a haemocytometer.

In vitro heterospermic insemination

For each competitive assay the semen of the two bulls to be tested was differentially stained and then suspended in 1 ml of IVF medium containing 10 µg/ml heparin and 10 µl penicillamine, hypotaurine and epinephrine (PHE, 100×) (Rosenkrans *et al.*, 1993), at a concentration of 0.15×10^6 sperm/ml per bull. The total concentration of semen in IVF was 0.3×10^6 sperm/ml. The sperm suspension was poured into wells in 300 µl volumes and 25 matured oocytes (1500 sperms/oocyte) were added and co incubated in 5% CO_2 and 5% O_2 in humidified air at 38.5°C (Galli *et al.*, 2001) over a period of 1 h. Prior to insemination, mature oocytes were vortexed to completely deprive them of granulosa cells.

According to the scheme, each bull was tested nine times for each of the two batches, using different harvests of oocytes.

Slide preparation

At the end of co-incubation, using a plastic pipette, groups of 25 oocytes per pair tested were harvested and washed three times in TALP-Wash Medium, in order to remove loosely attached sperm. The last step involved a double wash in distilled water containing 0.6% BSA. With a glass Pasteur pipette pulled on the flame, the oocytes were then individually collected and placed on slides in single drops and air dried at room temperature in the dark. The drops of distilled water containing single oocytes were identified under a stereo microscope and marked, to facilitate their identification.

During this drying phase, the oocytes suffered a gradual flattening, which led the entire surface of the ZP to adhere to the slide, forming a thin film. Stored in the dark, spermatozoa were clearly visible for up to three days after preparation.

Slide analysis

Oocytes were analysed under a fluorescence microscope using a filter set for green (excitation 465–495; emission 515–555) and red (excitation 540–580; emission 600–660) fluorescence, at ×40 magnification. When necessary, a ×60 immersion objective lens was also used.

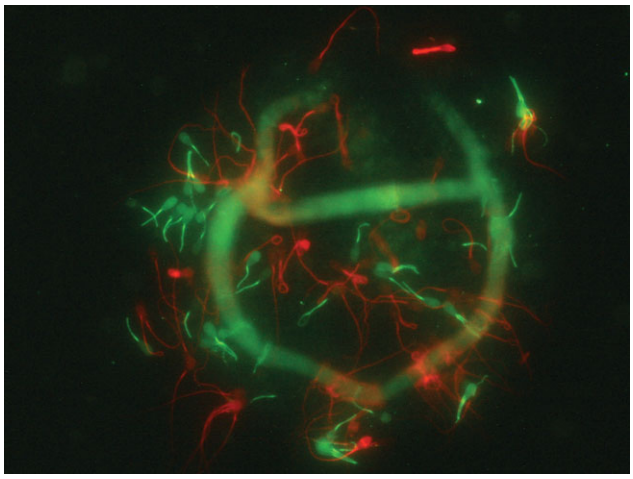

Figure 2 Fluorescence microscopy (×40) showing spermatozoa stained with red (CellTracker™ Red) or green (Fluo-4 AM and CellTracker™ Green) dyes bound to the zona pellucida. Spermatozoa are displayed on a single focus plane after air drying.

Sperms stained with each fluorochrome bound to the ZP were counted (Fig. 2).

Evaluation of stain toxicity

A preliminary test was conducted to assess whether the different stains could interfere in the sperm/ZP bond. The evaluation was conducted using the protocol described, as briefly specified below:

Semen of control bulls was thawed and after the first wash was divided into two equal volumes. The two aliquots were stained with green and red dye respectively, and after the staining procedure, motile sperm were selected using Percoll gradients. In addition, this procedure was effective for the removal of excess dye. The two populations, red and green, were adjusted to the same concentration and used for insemination. Spermatozoa attached to the ZP were counted. The trial was performed using three different bulls and a total of 79 oocytes were used.

Calculation of competitive binding index (CBI)

The index of *in vitro* competitive binding was calculated based on the relative frequencies for each bull, considering all the confrontations using the following model:

$$CBI_i = \sum_{j=1}^{N} p_{ij} / \left(\sum_{j=1}^{N} p_{ij} + \sum_{j=1}^{N} p_{ji} \right)$$

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Table 2 Descriptive statistics of quality parameters of two semen batches of bulls used in heterospermic insemination trials

	Quality parameters					
Variables	CONC (no. × 10^6)	MT (%)	MP (%)	VAP (μm/s)	IM (%)	IS (%)
Average	37.27	51.55	40.95	92.8	53.95	66.25
Standard deviation	8.15	8.45	7.8	8.6	8.68	10.54
Variance	0.22	0.16	0.19	0.09	0.16	0.16
Min	18.6	37.0	26.0	76.0	40.0	40.5
Max	51.9	70.0	57.0	108.0	70.5	87.5

CONC, concentration per paillette; IM, membrane integrity; IS, sperm integrity; MP, progressive motility; MT, total motility; VAP, average path velocity.

where: CBIi = CBI of i-th bull; Pij = absolute frequency of sperm of the i-th bull in the competition with the j-th bull by the N competitions; Pji = absolute frequency of sperm of the j-th bull in the competition with the i-th bull by the N competitions. Bulls were then classified according to the CBI.

Statistical analyses

Descriptive statistics of quality seminal parameters was calculated and variables were subjected to Pearson linear regression analyses using R programming and statistical packages (Crawley, 2007). Statistical analysis of stain toxicity results was performed using the chi-squared and the Fisher tests.

In order to evaluate any effect that number of oocytes could have on ranking of bulls, a dataset was organized by creating for each bull and for each of the two batches, five classes of oocytes (OOCYTE) of increasing group size (5, 10, 15, 20 and 25 oocytes, respectively classes 1 to 5). The five classes were generated by extrapolating from the data obtained with 25 oocytes in the competitive assays conducted on each batch. The CBI for the 10 bulls was then calculated for the five oocyte classes on batch 1 and 2 (BATCH), and the dataset was subjected to statistical analysis using the following general linear model:

$$CBIijk = m + OOCYTEi + BATCHj + eijk$$

where: CBIijk = ijk-th data value; OOCYTEi = differential effect of the i-th level of factor OOCYTE (i = 1–5); BATCHj = differential effect of the j-th level of factor BATCH (j = 1, 2); eijk = error associated with the ijk-th data value with N (0, σ).

The effect of the bull on CBI within the same class of oocytes was also evaluated using the following linear model:

$$CBIijk = m + OOCYTEi + BULLj(i) + eijk$$

where: CBIijk = ijk-th data value; OOCYTEi = differential effect of the i-th level of factor OOCYTE (i = 1–5); BULLj(i) = differential effect of the j-th level of factor BULL (j = 1–10) nested to i-th level of OOCYTE; eijk = error associated with the ijk-th data value with N (0, σ).

To assess the extend of influence of semen batches, bull effect was evaluated within single batches using the following model:

$$CBIijk = m + BATCHi + BULLj(i) + eijk$$

where: CBIijk = ijk-th data value; BATCHi = differential effect of the i-th level of factor BATCH (i = 1, 2); BULLj(i) = differential effect of the j-th level of factor BULL nested to i-th level of BATCH; eijk = error associated with the ijk-th data value with N (0, σ).

The effect of oocytes number on the final ranking of bulls calculated on pooled data of the two batches was finally statistically evaluated with the following:

$$CBIij = m + OOCYTESi + eij$$

where: CBIij = ij-th data value; eij = error associated with the ij-th data value with N (0, σ). In all the models m = overall mean.

Results

Semen analysis

Descriptive statistics of seminal quality of the 10 bulls is reported in Table 2. The results of linear regression analyses between the individual seminal variables or their linear combination and ERCR highlighted only one notable association between IM and ERCR (adjusted $R^2 = 0.2955$; F = 8.97; DF = 1.18; $p = 0.007769$). These data alone could not predict bull fertility.

Evaluation of stain toxicity

The total number of spermatozoa differentially stained (red vs. green) counted on the surface of the oocytes was 1890 vs. 1984 (total oocytes no. = 79; three control bulls).

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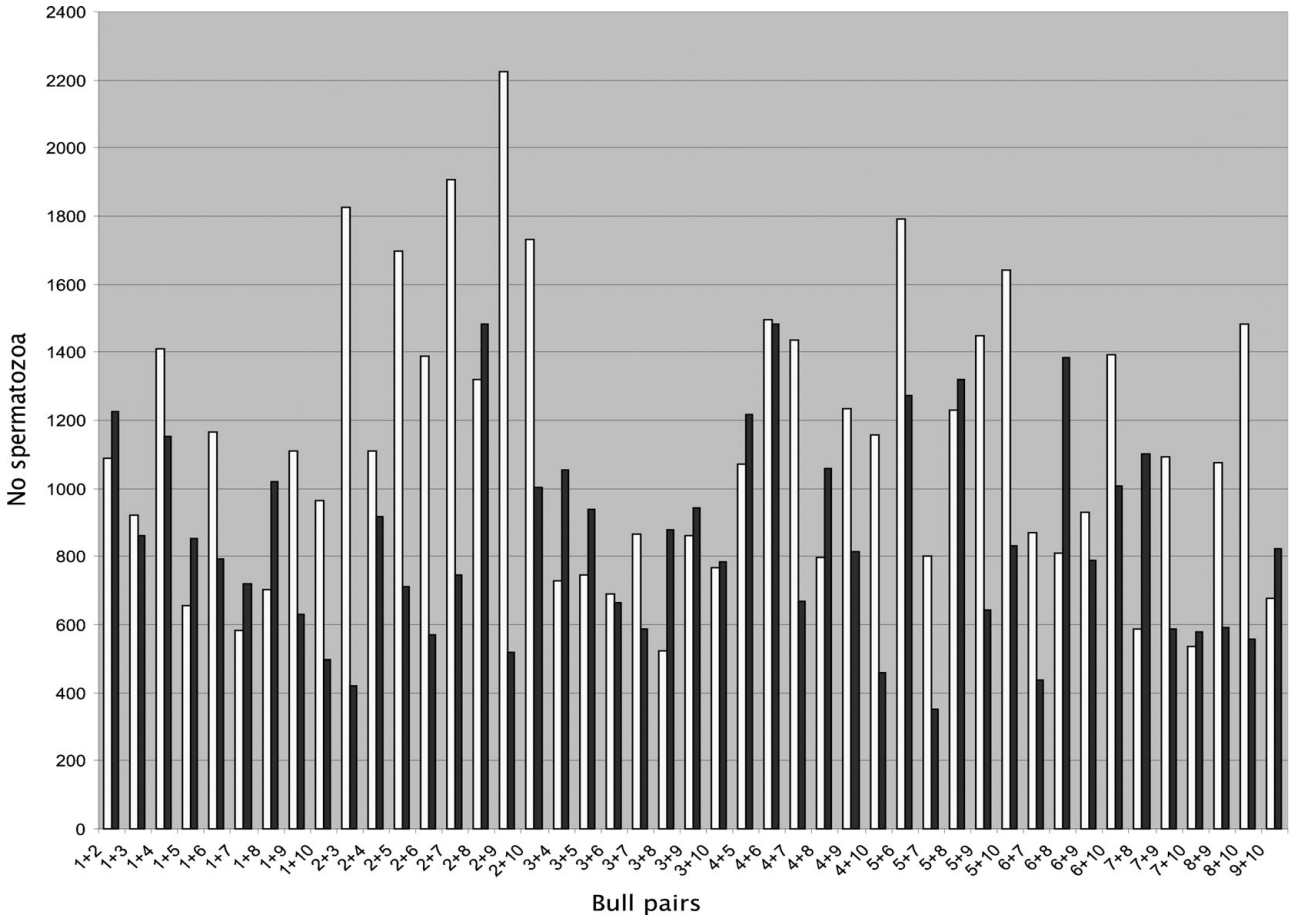

Figure 3 Number of spermatozoa counted in the 45 combinations of bull pairs. Each combination was tested on two semen batches and spermatozoa were counted on 50 oocytes (25 oocytes per batch).

The chi-squared test ($\chi^2 = 3.7808$, $p = 0.151$) and Fisher test ($p = 0.1506$) showed no significant statistical difference between the two stains.

In vitro heterospermic insemination

The absolute number of spermatozoa counted in the 45 combinations of bull pairs is reported in Fig. 3.

The number of spermatozoa bound to a single oocyte for each bull, regardless of the stain, ranged from 0 to 60. Thus, for each competitive assay (25 oocytes per batch, 50 oocytes per assay) the total number of sperms counted per bull showed a wide distribution.

Calculation of the competitive binding index

The frequencies of the 10 bulls and the deriving values of the CBI are presented in Table 3. The classification of the 10 bulls on the basis of CBI and their classification according to ERCR are reported in Table 4.

The CBI rank was subdivided into two classes of high (CBI > 0.50) and low (CBI < 0.50) binding ability. The comparison between CBI and ERCR shows only two bulls with a different classification in the two ranks: bull 3 with low score in CBI and high in ERCR and bull

Table 3 Frequencies of bulls and deriving values of competitive binding index (CBI)

	Frequency (no. spermatozoa)		
ID Bull	BULL	TOTAL	CBI
1	8602	16350	0.526116
2	14431	21890	0.659251
3	6459	15058	0.428941
4	10315	19264	0.535455
5	10634	19221	0.553249
6	8787	18935	0.464061
7	5725	14455	0.396057
8	10807	17930	0.602733
9	6189	16988	0.364316
10	6539	16885	0.387267

Frequencies are expressed as number of spermatozoa counted on the surface of oocytes. BULL represents the sum of spermatozoa of a single bull in all its combinations, TOTAL is the sum of BULL and the number of spermatozoa of all the bulls confronted with it.

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Table 4 Competitive binding index (CBI) classification of the 10 bulls calculated on two semen batches in confront with classification according to ERCR

ID Bull	CBI	ID Bull	ERCR
9[a]	0.364316	10	−4.74
10[a]	0.387267	9	−3.93
7[a]	0.396057	8	−3.52
3[a]	0.428941	7	−3.11
6[a]	0.464061	6	−2.30
1[b]	0.526116	5	+2.20
4[b]	0.535455	4	+2.26
5[b]	0.553249	3	+2.39
8[b]	0.602733	2	+2.62
2[b]	0.659251	1	+3.95

[a]Bulls (CBI < 0.50; ERCR <0) are classified respectively as low binding ability and low fertility bulls.
[b]Bulls (CBI > 0.50; ERCR > 0) are classified as high binding ability and high fertility bulls, respectively.

Table 6 Competitive binding index (CBI) of the 10 bulls calculated with data pooled from two semen batches using data from 25, 20, 15, 10 and five oocytes per single competitive assay

ID Bull	CBI-25	CBI-20	CBI-15	CBI-10	CBI-5
1	0.53	0.53	0.53	0.54	0.53
2	0.66	0.66	0.65	0.66	0.64
3	0.43	0.43	0.44	0.44	0.45
4	0.54	0.53	0.53	0.52	0.53
5	0.55	0.54	0.56	0.56	0.54
6	0.46	0.47	0.46	0.46	0.47
7	0.40	0.40	0.41	0.41	0.41
8	0.60	0.60	0.60	0.60	0.60
9	0.36	0.37	0.36	0.35	0.35
10	0.39	0.39	0.39	0.39	0.39

For each bull, values in different columns are not statistically different ($p > 0.5$).

8 with the opposite situation, high score in CBI and low in ERCR.

After statistical analysis by general linear model, the number of oocytes per assay did not affect the computing of CBI for none of the bulls when associated to each of the two batches ($p > 0.5$) (Table 5). Similarly, the effect of bull expressed as level of significance, remained completely unvaried with decreasing the number of oocytes for all the 10 bulls.

Considering the estimate of variability among batches, when effect of bull was associated to the batch a high significance ($p > 0.001$) was reported equally in batch 1 and batch 2 for eight bulls, whereas two bulls (bull 3 and 6) highlighted a difference between the two batches showing a high significant effect in batch 1 ($p > 0.001$) and a no statistically significant effect ($p > 0.05$) in batch 2.

For ranking the bulls CBI was therefore calculated by pooling data from the two batches, and the statistical evaluation of the influence of oocytes number on the CBI was confirmed not to be significant ($p > 0.5$) for all the bulls, even if number of oocytes per assay is reduced to 5 (Table 6).

Discussion

As heterospermic insemination was proposed as a tool for predicting male fertility, numerous researches have been aimed at simplifying its use for a more practical application. When experiments were conducted *in vivo*, a good consistency was generally evidenced (Parrish & Foote 1985; Stahlberg *et al.*, 2000; Flint *et al.*, 2003). Alternatively, *in vitro* redefinition of the approach resulted in more conflicting outcomes. A test based

Table 5 Competitive binding index (CBI) values of the 10 bulls calculated separately for batch 1 and batch 2, using data relative at 25, 20, 15, 10 and five oocytes per single competitive assay

	CBI-25		CBI-20		CBI-15		CBI-10		CBI-5	
ID Bull	1	2	1	2	1	2	1	2	1	2
1	0.53	0.52	0.53	0.52	0.53	0.53	0.54	0.53	0.54	0.53
2	0.63	0.68	0.64	0.67	0.63	0.67	0.63	0.67	0.62	0.66
3	0.43	0.43	0.43	0.43	0.44	0.43	0.45	0.44	0.47	0.44
4	0.50	0.56	0.50	0.55	0.50	0.55	0.49	0.54	0.49	0.55
5	0.60	0.51	0.58	0.51	0.61	0.51	0.61	0.51	0.60	0.48
6	0.51	0.42	0.51	0.43	0.50	0.43	0.50	0.43	0.50	0.44
7	0.46	0.35	0.46	0.36	0.46	0.37	0.46	0.37	0.46	0.37
8	0.63	0.68	0.64	0.67	0.63	0.67	0.63	0.67	0.62	0.66
9	0.35	0.37	0.37	0.37	0.35	0.37	0.34	0.36	0.32	0.37
10	0.34	0.42	0.34	0.43	0.35	0.43	0.35	0.43	0.36	0.43

For each bull, values in different columns show no statistical difference ($p > 0.5$) within the same batch.

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on different fluorochrome-labelled spermatozoa gave a positive correlation between *in vivo* fertility and penetration of zona-free bovine oocytes (Henault & Killian 1994). By contrast Braundmeier and colleagues (2002) *in vitro* competitive zona binding assay could rank the bulls, but did not correlate with the 56-day no return rates, zona binding *in vivo*, or the number of accessory spermatozoa detected on *in vivo* produced embryos. Their contrasting findings are probably due to the different experimental designs and data interpretation.

Generally, a main notable difference between reports is the source of oocytes. Especially in non-domestic species, in which a sufficient number of oocytes can not be easily recruited at once, several regimens for storing oocytes have been adopted. As a consequence, ZP-binding tests include the use of immature, metaphase II-arrested, chilled overnight or even fixed oocytes, in which the success of these storing procedures seems to vary between species. In our study the use of fresh *in vitro* matured oocytes was adopted, as the beneficial use of IVM oocytes for application of ZP-binding assay has been reported in different species (Marco-Jiménez & Vicente 2004; Hermansson *et al.*, 2007) as also supported by the fact that mature and immature oocytes differ in the glycoproteins of the zona pellucida, along with the number of sperm receptors (Lucas *et al.*, 2003). In these studies the authors also thought that the damage from extensive artificial manipulation to be the cause of drastic reduction in sperm binding capacity.

A large variability in response to *in vitro* tests between different ejaculates of the same bull has been generally reported (Otoi *et al.*, 1993; Zhang *et al.*, 1997, 1998b). Variation in sperm-binding capacity between single oocytes and between oocyte batches was also reported using homospermic ZP-binding assays in canine (Ström Holst *et al.*, 2000) and bovine (Zang *et al.*, 1995) species. Successful application of homospermic ZP-binding tests were therefore applicable only when large numbers of oocytes per batch and several batches per test were used (Zang *et al.*, 1995; 1998a) or by performing hemi-zona assays (Franken *et al.*, 1993; Fazeli *et al.*, 1997), in which both the halves of microsurgically bisected ZPs were used to compare control sperm and test sperm populations. Whilst ZP dissection minimizes the variability related to oocytes its application imposes a precise microdissecting-based and time-consuming technique and its use in humans has recently been debated (Magerkurth *et al.*, 1999).

Considering the variability due to ejaculates and oocytes, in the present work the information for calculating the CBI of each bull was drawn from data obtained using two semen batches and, within each batch, using nine independent tests of the bull throughout the combinations with all the others. While the competitive approach overcame the variability in oocyte quality within single pair tests, variations in oocyte batches had to be randomized to ensure that the same bull was tested on different days on different oocyte batches. Overall, the experimental design included extensive testing of all subjects, thus giving a reasonably accurate characterization of ZP binding ability.

The range of number of spermatozoa bound to oocyte per bull reported in this work is compatible with those reported by other authors: e.g. from 0 to more than 200 spermatozoa (Fazeli *et al.*, 1997), and 26.9–141.9 (73.2 $\pm$ 31.3, Zhang *et al.*, 1999). The number of sperms/oocyte is principally due to the concentrations of sperm, insemination duration and method of removing loosely attached semen before counting. This last step, which includes manual washing of oocytes by pipetting, is subjectively performed in the laboratories. When assessments are based on the absolute number of sperms bound to the ZP, such differences among oocytes may have a great impact on the outcome of the test and therefore require the use of a large number of oocytes. In this work we clearly showed that through the heterospermic approach, which is based on evaluation of relative differences, this problem is completely overcome so that the same result using a very large number of oocytes (2250 oocytes) could be obtained using only a fifth of this number. This finding provides suggestions for possible future review of the experimental design that could be addressed by a drastic scaling at single pair assay level with the benefit of allowing the implementation of alternatives, e.g. a larger scale testing for batches.

Quality assessment of seminal parameters confirms the lack of significant correlation between field fertility and seminal features. Even though this observation is traditionally accepted, any investigation of intrinsic sperm fertility should not leave out the consideration of the possible pathological status of sperm samples that are used as a model. In this work, neither the high nor the low fertility sperm samples could reveal their fertility potential after traditional screening for quality parameters. However, when samples were compared with the same oocytes, differences in functional traits became evident. In particular, ZP-binding ability of high fertility bulls was generally superior to that of low fertility ones. These differences were sufficient to rank the bulls into two categories and, for eight bulls out of 10, their classification were in accordance with the *in vivo* estimates.

The discrepancy in the classification reported for two bulls deserves some consideration: firstly, regarding the bull with low ERCR and high binding ability as determined *in vitro*, the simple explanation could be that, for this bull, the ability to bind with the ZP is not the limiting trait responsible for its hypo-fertility,

nor can this trait (or even more traits) be identified among those seminal parameters that we have screened in this work. Of interest is the low sperm membrane integrity of both semen batches for this bull (46% and 49%, data not presented) as compared with the average for all the other bulls (53.95 ± 8.68). By contrast, the other bull with defective ZP-binding ability performed normally when was used in AI. An explanation could be hypothesized due to current of AI practice, which proposes the use of straws that are generally packaged with a sperm content that exceeds that needed (Pace *et al*., 1981). In this study the authors have identified that the best range is 2–8 million spermatozoa, beyond which there is no significant increase in field fertility, while more recent works (Den Daas, 1997; Seidel *et al*., 1999) have shown that this number can be reduced even more without affecting the fertility. In this regard, the low semen concentration used in the present work to perform *in vitro* tests seems to be adequate for detecting a sperm functional deficiency that paradoxically, for some subjects, can be compensated by the AI use of straws produced with a non-rational and excessive number of spermatozoa. This observation is supported by the high semen concentration per paillette reported on average for the bulls used in this work.

Some technical observations concern the need to run an accurate count of spermatozoa over the entire ZP surface, given the spherical shape of the oocyte. The only method proposed in the literature, which includes fixation and crushing of oocytes with a coverslip, is in our view not fit for our purpose. Although flattened, such oocytes are still so thick that several focal planes must be counted, making this operation onerous and it did not allow the use of immersion objectives. Furthermore, sperm attached on the lower surface had a loss of fluorescence due to the presence of cytoplasm. Drying eliminated completely the three-dimensional effect of oocytes and displayed all the spermatozoa in one single plane. Use of distilled water prevented crystal formation during evaporation, while the BSA allowed the manipulation of oocytes that was otherwise impossible in this medium.

In conclusion, the significant congruency that appeared after comparing the ERCR and CBI ranking allows consideration of competitive binding as a valid support in estimating the fertility of young bulls and, if confirmed in other species, could lead to a drastic reduction of the number of oocytes required when their availability is a limiting factor.

Given the complex nature of fertility and the argumentation discussed about the possible occurrence of some 'false positives' or 'false negatives', it shows how integrated use of different approaches while attempting to estimate fertility is increasingly important. The characterization of each subject, which should include systematic screening for both qualitative and functional traits, could enable users to better compensate some minor functional reproductive deficits or, where not possible, could provide early identification and removal of those subfertile sires before entering a breeding programme. CBI could provide useful information to support the optimization in semen production centres for a more rational use of semen straws in AI. However before this protocol can be widely accepted it should be validated on a more extensive group of specimens.

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