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Effect of inbreeding depression on bull sperm quality and field fertility

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Abstract. The present study investigated the effect of inbreeding depression on sperm quality using automated and objective methods and subsequent effects on beef bull field fertility. Individual inbreeding coefficient (F) values and field fertility data were determined using a dataset of AI bulls belonging to the Spanish Retinta Breeders Association (Asociación Nacional de Criadores de Ganado Vacuno Selecto de Raza Retinta (ANCRE)). Animals were clustered in two groups according to the F values as follows: (1) a high inbreeding group (HI; $F \geq 13.5\%$, mean 16.3); and (2) a non-inbreeding group (NI; $F = 0\%$). In total, 17 different assessments were performed in both experimental groups, including evaluation of sperm morphology, acrosomal and DNA status, sperm plasma membrane integrity and function (hypo-osmotic swelling test), 10 kinetic parameters and the structure of sperm subpopulations. Sperm morphology, acrosomal and DNA status and osmotic tolerance were similar in both groups. Three velocity parameters (curvilinear velocity, straight line velocity and average path velocity) and the amplitude of lateral head displacement were higher in HI ($P < 0.05$). Cluster analysis of kinematic parameters revealed three different sperm subpopulations (sP1, sP2 and sP3), with the proportion of the sP1 population (highly active but non-progressive spermatozoa) being significantly ($P < 0.05$) higher in the HI group. Field fertility was assessed using two calving record datasets. In a smaller database including only bulls evaluated in the present study, there was a significant increase in the calving interval of cows sired with HI bulls. Conversely, in an extended genetic analysis of the ANCRE database, inbreeding only explained a small part of the variation in calving interval, and the results of regression analysis were not significant among bulls. The findings of the present study suggest that high inbreeding levels have a moderate effect on bull semen quality, with an increased percentage of highly active but non-progressive spermatozoa, but only when F values reached a certain threshold. This motility pattern could explain, in part, the higher calving interval produced by inbred bulls under field conditions.

Additional keywords: calving interval, cattle, computer-aided sperm analysis (CASA), DNA fragmentation, genetic trait, sperm function, sperm morphology, sperm subpopulations.

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Introduction

Sperm quality is one of the most important traits defining the reproductive potential of bulls. It is normally determined by a group of characteristics including morphology, motility, viability and DNA function (Söderquist *et al.* 1996; Gil *et al.* 2000). It is well known that sperm quality is affected by environmental factors, such as the age of the animal, fitness and absence of diseases (Brito *et al.* 2002). However, sperm quality is also affected by the genetic pool of the individual, but shows only low to moderate heritability (Mathevon *et al.* 1998), which is why it is rarely included in cattle selection schemes (Karoui *et al.* 2011). In practice, candidate sires are evaluated before they are included in AI programs, discarding those that do not meet certain minimum sperm quality standards because of genetic causes (Rodriguez-Martinez and Larsson 1998).

Inbreeding depression, a consequence of breeding related individuals, results in reduced viability and adaptability in a given population (Kristensen *et al.* 2010). According to

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Charlesworth and Willis (2009), the genetic causes of inbreeding depression may be due to three different possibilities: (1) increased expression of deleterious recessive alleles (partial dominance); (2) fitness superiority of heterozygotes over homozygotes (overdominance); or (3) increased possibility of favourable gene combinations in heterozygotes (epistasis). Although the relative contribution of each cause to inbreeding depression remains unclear, it is well known that inbred animals show reduced flexibility in coping with environmental challenges (Lacy 1997; Leberg and Firmin 2008), decreased fitness and mean phenotypic traits and impaired reproductive success across wild, captive, domestic and experimental populations (O'Grady *et al.* 2006; Leroy 2014). This phenomenon is particularly important in cattle, where the high intensity of current selection programs and the use of autochthonous populations with reduced census have brought about a rapid increase in genetic relationships among individuals (Hansen 2000; Rodero-Serrano *et al.* 2013). In these animals, inbreeding depression is expressed in different ways, such as reduced viability and lifetime performance in beef cattle (Smith *et al.* 1998) and decreased lifetime milk production in dairy cattle (Thompson *et al.* 2000). Nevertheless, this genetic trait has not been considered to any great extent in selection schemes, which primarily focus on improving production traits (Gandini *et al.* 2014), leading to a slow but steady increase in inbreeding depression over time (Sørensen *et al.* 2005; Stachowicz *et al.* 2011).

There is general agreement that fertility is negatively affected by inbreeding (Keller and Waller 2002). This impairment is particularly important in endangered wild animals, for which inbreeding levels are very high (Cassinello *et al.* 1998; Ruiz-Lopez *et al.* 2010). However, only a few studies have analysed the effect of inbreeding on bull sperm quality and contradictory results have been reported (Losdat *et al.* 2014). For example, Flade and Zeller (1992) demonstrated that sperm quality was not affected in high inbreeding bulls (inbreeding coefficient (F) = 25%, produced experimentally) compared with their non-inbred half-brothers (F = 0%). In contrast, Maximini *et al.* (2011) more recently described a low negative correlation between inbreeding and sperm quality in Simmental bulls.

On the basis of this background information, the aim of the present study was to perform a comprehensive evaluation of the effects of inbreeding on bull sperm motility (using computer-aided sperm analysis (CASA)) and DNA integrity (using flow cytometry). Concurrently, we assessed sperm viability and the status of both acrosomal and plasma membranes. Finally, we determined the effects of inbreeding on *in vivo* fertility in our experimental animals as well as in a reproductive dataset of AI commercial beef bulls with broad genealogy and reproductive records over several seasons.

Materials and methods

Animals

Genealogical data from 159 sperm donor bulls belonging to the Spanish Retinta Breeders Association (Asociación Nacional de Criadores de Ganado Vacuno Selecto de Raza Retinta (ANCRE)) AI program were analysed. Individual F values (Wright 1931) and the number of fully traced, maximum number of generations traced and the equivalent complete generations (ECG) for each animal were estimated by ENDOG (v4.8; Gutierrez and Goyache 2005) using all known generations. Thereafter, 11 bulls with at least two complete generations and 2.5 ECG and available frozen semen doses were selected and classified into two groups based on F values as follows: (1) animals with at least 13.5% consanguinity (F = 16.3 ± 0.9% (mean ± s.e.m.); minimum 13.5%, maximum 18.7%), which were classified as the high inbreeding (HI) group (*n* = 6); and (2) animals without consanguinity (F = 0%), which were classified as the non-inbreeding (NI) group (*n* = 5). The mean (± s.e.m.) ECG for the HI and LE groups were 5.2 ± 0.6 and 4.1 ± 0.6, respectively. Minimum consanguinity in the HI group (F = 13.5%) was higher than the equivalent to a half-sibling cross (12.5%), which is considered the threshold value from which serious fitness problems derived from inbreeding depression appear (Sewalem *et al.* 2006).

Semen samples

In all, 55 frozen semen samples (five per bull) were analysed. Semen was collected from individuals between 20 and 30 months of age with a positive breeding soundness examination score and was frozen at the Centro de Selección y Reproducción Animal (CENSYRA; Badajoz, Spain) following the procedures of ANCRE's AI program. Frozen semen was thawed at 37°C in a water bath for 1 min and diluted in Biladyl A (Minitüb, Tiefenbach, Germany) to reach a working concentration of 25×10^6 spermatozoa mL^{-1}. Only samples with acceptable post-thawing sperm membrane integrity and motility (>40% sperm membrane integrity and >50% total motility) were used.

Semen evaluation

Sperm morphology

The percentage of spermatozoa with abnormal morphology was estimated on Diff-Quik-stained smears (Baxter DADE Diagnostics, Düdingen, Switzerland), as described previously (Hidalgo and Dorado 2009). The proportion of spermatozoa with normal morphology (NSM) and abnormal morphology (ASM) was recorded. Two independent replicates of at least 200 spermatozoa were assessed for each sample.

Plasma membrane integrity

Sperm plasma membrane integrity was assessed using a Vital-Test kit (Halotech, Madrid, Spain) according to the manufacturer's instructions under a fluorescence microscope (BX40; Olympus, Tokyo, Japan) using a 460–490-nm excitation filter. Two staining patterns were discerned: (1) spermatozoa with a green-stained head were determined to be viable spermatozoa (VS); and (2) spermatozoa with red-stained heads were recorded as dead spermatozoa (DS). Two independent replicates of at least 200 spermatozoa were assessed for each sample.

Plasma membrane function

The function of the sperm plasma membrane was assessed using the hypo-osmotic swelling (HOS) test (Revell and Mrode 1994). Samples were incubated for 1 h at 38.5°C in

hypo-osmotic solution (100 mOsmol mL^{-1}) containing 1 : 1 (v/v) fructose : sodium citrate. Thereafter, 20 μL solution was smeared on a clean slide, dried and evaluated at a magnification of ×400. Spermatozoa with unaltered tail morphology were classified as negative (HOS−), whereas those showing a coiled tail were classified as positive (HOS+). Two independent replicates of at least 200 spermatozoa were assessed for each sample.

Acrosome membrane integrity

Acrosome integrity was assessed using a standard protocol (Demyda-Peyras *et al.* 2012). A droplet of diluted sample was smeared onto a microscopic slide, air dried, fixed and permeabilised with 70% (v/v) ethanol for 30 s. Thereafter, 30 μL of a staining mixture (1 part propidium iodide (PI; 0.1 mg mL^{-1}) and 2 parts isothiocyanate-labelled peanut (*Arachis hypogaea*) agglutinin (0.1 mg mL^{-1})) was spread over each smear and samples were incubated in a dark and humid chamber at 4°C for 30 min. Preparations were subsequently washed, mounted with Vectashield (Vector Laboratories, Peterborough, UK) and scored under an epifluorescence microscope (BX40; Olympus) at ×400 magnification. Two sperm subpopulations were identified: (1) spermatozoa with a uniform green fluorescence of the acrosomal cap (acrosome-intact spermatozoa (AIS)); and (2) spermatozoa with a disrupted, patch-like, green fluorescence staining in the acrosomal cap, a fluorescent band at the equatorial segment or no fluorescence (acrosome-reacted spermatozoa (ARS)). All spermatozoa showed red fluorescence because of counterstaining with PI. Two independent replicates of at least 200 spermatozoa were assessed for each sample.

Chromatin integrity

The percentage of altered DNA (strand breaks) was assessed using the acridine orange (AO) sperm chromosome structure assay (SCSA; Evenson and Jost 2000). Immediately after thawing, samples were placed on ice and diluted separately in TNE buffer (0.015 M NaCl, 0.01 M Tris and 0.001 M EDTA, pH 6.8) to a final concentration of 1×10^6 spermatozoa mL^{-1}. A 200-μL aliquot of sperm solution was treated with 400 μL acid detergent solution (0.08 M HCl, 0.15 M NaCl and 0.1% (w/v) Triton X-100, pH 1.2) and then after exactly 30 s 1.2 mL AO staining solution was added (6 mg AO per mL buffer (0.037 M citric acid, 0.12 M Na_2PO_4, 1.1 mM disodium EDTA and 0.15 M NaCl), pH 6.0). Samples were analysed in an EPICS XL cytometer (Beckmann Coulter, Miami, FL, USA) containing a 488-nm dichroic long-pass filter, a 488-nm blocking filter, a 550-nm dichroic long-pass filter and a 525-nm band pass filter for the first fluorescent channel (FL1), a 600-nm dichroic long-pass filter and a 575-nm band pass filter for the second fluorescent channel (FL2), a 645-nm long-pass filter and a 620-nm band pass filter for the third fluorescent (FL3) channel and a 675-nm long-pass filter for the fourth fluorescent channel (FL4). Fluorescence was recorded at FL1 and FL4. Two independent replicates per bull of at least 20000 spermatozoa were assessed for each sample at an average flow rate of 200 spermatozoa per second. Data were collected individually in list mode and transformed to plain text using MFI software (Martz 1992–2001). The chromatin damage (DNA fragmentation index; DFI)) and high DNA stainability (HDS), associated to the percentage of immature sperm were determined by analysing the ratio of red : green fluorescence, as described by Rybar *et al.* (2010).

CASA

Sperm motility was assessed using a CASA system (Sperm Class Analyzer; Microptic, Barcelona, Spain). Three consecutive 5-μL drops of each semen working sample were evaluated using a phase contrast microscope (Eclipse 50i; Nikon, Tokyo, Japan) with a prewarmed stage at 37°C (×100 magnification). Three drops and two microscopic fields per drop were randomly analysed, including a minimum number of 200 spermatozoa. The analysis was performed on 25 consecutive digital images captured in 1 s from a single microscope field.

Parameters of the analysis software were set according to Kathiravan *et al.* (2011). Briefly, spermatozoa with mean average path velocity (VAP) <10 μm s^{-1} were considered immotile. Spermatozoa with a VAP >90 μm s^{-1} were considered rapid, and spermatozoa deviating <25% from a straight line were designated as linear motile. The following kinetic traits were assessed: curvilinear velocity (VCL), the total distance travelled by the sperm head per unit time; straight line velocity (VSL), the net distance gain of the sperm head per unit time; VAP, the length of a derived 'average' path of sperm head movement per unit time; wobble (WOB), calculated as (VAP/VCL) × 100; linearity (LIN), calculated as (VSL/VCL) × 100; straightness (STR), calculated as (VSL/VAP) × 100; beat cross frequency (BCF), the number of times the curvilinear path crosses the average path per unit time; approximation of the flagellar beat frequency for seminal sperm (in Hz); and amplitude of lateral head displacement (ALH), the width of the head movement envelope.

Classification, ordination and identification of sperm subpopulations

Motility data from all individual spermatozoa assessed were included in this analysis and initially grouped in two categories based on F values (22 190 spermatozoa from HI bulls and 23 097 spermatozoa from NI bulls). A four-step clustering procedure was used to classify the spermatozoa in the dataset (45 287 in total) into a reduced number of subpopulations according to their motility patterns as described previously by Martinez-Pastor *et al.* (2005). All determinations were performed using SAS/STAT software package release 9.0 (SAS Institute, Cary, NC, USA). First, the PRINCOMP procedure was used for principal component analysis (PCA). Thereafter, a non-hierarchical cluster analysis (FASTCLUS procedure) was performed using the selected principal components as variables. Then, the processed data were reclustered by hierarchical methods (CLUSTER procedure) using the average linkage method (AVERAGE) for joining clusters. To determine the final number of subpopulations (sP; Step 4), we studied the evolution along the clustering process of three statistics provided by CLUSTER (pseudo-t2, pseudo-F and cubic clustering criterion) looking for certain types of consensus among them, specifically local peaks of the cubic clustering criterion

and pseudo-F statistics combined with a small pseudo-t2 value and a larger pseudo-t2 for the next cluster fusion.

Statistical analysis

All statistical analyses were performed using SAS/STAT (SAS Institute). Results are expressed as the mean ± s.e.m. Sperm parameters were compared between groups (HI vs NI) using a nested general linear model (GLM) with group (fixed factor) and bull (nested in group) as the random factor. The parameter SGoF+ (Carvajal-Rodriguez and de Uña-Alvarez 2011) was used to control the false discovery rate (FDR) of the *P*-values at a significance level of 5% and an FDR of 5%. The percentage of sperm subpopulations in the HI and LE groups was compared by Fisher's exact test (FREQ procedure).

Assessment of bull fertility under field conditions

Calving intervals (CI) were estimated based on mating records of the ANCRE. Records from 824 cows inseminated or mated by HI ($n = 490$) and NI ($n = 334$) bulls were compared using GLM. Group (HI or NI) and bull (nested in group) were included as fixed and random factors, respectively. Age of the cow at delivery was included as a covariate.

Genetic analysis

A subsequent broader genetic analysis included the CI of 5230 cows inseminated or mated by 743 bulls (159 semen donors) belonging to the ANCRE dataset. The effects of several factors and the genetic component of CI were analysed with an animal model using Bayesian methodology as follows:

$$y = Xb + Z_1a_1 + Z_2a_2 + Wp + e$$

where y is observed CI, b is systematic fixed effects, a_1 and a_2 are cow and bull additive genetic effects, respectively (11 445 levels), p is bull permanent environmental effects (743 levels), e is residual effects and X, Z and W are incidence matrices.

The systematic fixed effects (b) included a random contemporary group effect (herd × year × breeding season; 1323 levels) with four different seasons (December–March, April–June, July + August and September–November) defined according to the weather characteristics of the region, and the fixed age effect of the cow. Inbreeding coefficients for both cow and bull were obtained by the tabular method (Tier 1990) and included as covariates.

The prior distributions of $a = (a_1$ and $a_2)$, p and e were $N(0, A \otimes G_o)$, $N(0, I\sigma^2_p)$ and $N(0, I\sigma^2_e)$, respectively, where G_o is a (co)variance matrix for the additive genetic components on CI_{12} of cow and bull. Gibbs sampling algorithm was used to make Bayesian inferences for the parameters of interest. Data analysis consisted of a long chain of 10^6 iterations, a burn-in of 500 000 rounds and a thin of 10 iterations using TM software (Legarra *et al.* 2008).

Results

Sperm morphology and function

Inbreeding did not induce differences ($P > 0.05$) in sperm morphology, acrosomal and plasma membrane integrity or results of the HOS test between the HI and NI groups (Table 1). In addition, inbreeding had no significant effect on DNA structure (DFI), despite the large number of spermatozoa analysed ($P > 0.05$; Table 2), or on the percentage of immature spermatozoa (HDS). The variability in both these parameters was similar between the HI and LE groups.

Table 1. Morphological and functional assessment of sperm samples from inbred (HI; *n* = 30) and non-inbred (NI; *n* = 25) bulls

Results are expressed as the mean ± s.e.m. *$P < 0.05$ compared with HI bulls. ASM, abnormal sperm morphology; VS, viable spermatozoa; AIS, acrosome-intact spermatozoa; HOS+, hypo-osmotic swelling test positive

Sperm parameters	HI	NI
% ASM	33.15 ± 2.68	36.07 ± 2.23
% VS	47.70 ± 1.70	50.85 ± 1.94
% AIS	82.97 ± 1.68	80.15 ± 2.09
% HOS+ spermatozoa	42.20 ± 2.07	47.23 ± 3.07*

Table 2. Sperm chromatin structure assay results from inbred (HI; *n* = 30) and non-inbred (NI; *n* = 25) bulls

	DNA fragmentation index (%)			High DNA stainability (%)		
	Mean	s.e.m.	Range	Mean	s.e.m.	Range
HI bulls	2.99	1.48×10^{-6}	2.01–5.36	2.09	2.26×10^{-6}	0.17–4.95
NI bulls	3.01	2.43×10^{-6}	0.98–6.02	1.82	2.48×10^{-6}	0.28–3.47

Table 3. Motility analysis of sperm samples from inbred (HI; *n* = 30) and non-inbred (NI; *n* = 25) bulls

Results are expressed as the mean ± s.e.m. Within rows, values with different superscript letters differ significantly at $P < 0.05$ after control of the false discovery rate at 5%. VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency

Sperm parameters	HI bulls	NI bulls
MOT[A] (%)	76.68 ± 1.97	79.77 ± 2.10
PMOT[B] (%)	53.43 ± 1.93	50.86 ± 2.58
VCL (μm s^{-1})	77.73 ± 1.69^a	69.64 ± 2.74^b
VSL (μm s^{-1})	40.60 ± 0.99^a	37.34 ± 2.11^b
VAP (μm s^{-1})	55.93 ± 1.41^a	50.46 ± 2.29^b
Linearity (%)	45.04 ± 0.89	44.48 ± 1.06
Straightness (%)	62.73 ± 0.81	62.34 ± 0.84
Wobble (%)	67.60 ± 1.10	66.64 ± 0.80
ALH (μm)	2.81 ± 0.08^a	2.61 ± 0.07^b
BCF (Hz)	6.81 ± 0.14	6.46 ± 0.16

[A]Total motility (MOT) was defined as spermatozoa with a mean VAP >15 mm s^{-1}.
[B]Progressive motility (PMOT) was defined as spermatozoa with a VAP >50 mm s^{-1} and straightness >75%.

Motility analysis

Only four CASA-derived parameters were affected by inbreeding (Table 3). VCL, VAP, VSL and ALH were higher ($P < 0.05$) in HI compared with NI bulls. Two principal

Table 4. Motility parameters for the three sperm subpopulations (sP1, sP2 and sP3) defined after pattern analysis in semen samples from inbred (HI; *n* = 30) and non-inbred (NI; *n* = 25) bulls

Results are expressed as the mean ± s.e.m. Within rows, values with different superscript letters differ significantly ($P < 0.05$). The total number of spermatozoa analysed was 45 287. VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency

Sperm parameter	sP1	sP2	sP3
No. spermatozoa	19 942	7720	17625
% Spermatozoa	44.05	17.05	38.9
VCL (μm s^{-1})	109.19 ± 0.17^{a}	62.42 ± 0.26^{b}	29.81 ± 0.12^{c}
VSL (μm s^{-1})	63.90 ± 0.21^{a}	44.21 ± 0.24^{b}	6.06 ± 0.04^{c}
VAP (μm s^{-1})	84.07 ± 0.18^{a}	53.64 ± 0.27^{b}	15.46 ± 0.09^{c}
Linearity (%)	56.84 ± 0.15^{b}	68.16 ± 0.15^{a}	20.14 ± 0.09^{c}
Straightness (%)	74.30 ± 0.15^{b}	81.48 ± 0.14^{a}	39.30 ± 0.16^{c}
Wobble (%)	76.04 ± 0.09^{b}	83.71 ± 0.12^{a}	51.00 ± 0.12^{c}
ALH (μm)	3.62 ± 0.01^{a}	1.97 ± 0.01^{b}	1.68 ± 0.01^{c}
BCF (Hz)	9.16 ± 0.02^{a}	6.71 ± 0.04^{b}	3.25 ± 0.02^{c}

components with eigenvalues >1 were identified by the PCA, accounting for 84.05% of the variance. Considering the scores of CASA parameters, the first principal component was related to fast linear movement, whereas the second was related to fast but irregular movement.

Three sperm subpopulations were defined using non-hierarchical and subsequent hierarchical classification of 45 287 individual motile spermatozoa and the eight motility parameters (VCL, VSL, VAP, LIN, STR, WOB, ALH and BCF). Summary statistics for the motility characteristics of the subpopulations are given in Table 4.

Subpopulation 1 (sP1) included highly active but non-progressive spermatozoa (highest VCL, ALH and BCF values, together with low LIN and STR values), accounting for 44.05% of the total motile population. Subpopulation 2 (sP2) contained the lowest number of spermatozoa (17.05%) and included spermatozoa with relatively low velocity (medium VCL, VSL and VAP) but high progressiveness (high LIN, STR, WOB and low ALH). Subpopulation 3 (sP3) included spermatozoa with less vigorous movements (low VCL, VAP, ALH and BCF) and less progressiveness (low VSL, LIN and STR) than all other groups and 38.9% of the population consisted of total motile spermatozoa. The proportion of spermatozoa assigned to sP2 (moderately slow but progressive sperm) and sP3 (slow and non-progressive sperm) was significantly lower in the HI group ($P < 0.05$; Table 5), whereas the HI group had a significantly higher proportion of sP1 spermatozoa (highly active but non-progressive).

Bull fertility under field conditions

Analysis of the mating records of the 11 bulls studied showed a CI significantly higher in the HI compared with NI group (15.07 vs 14.44 months, respectively; $P < 0.05$). Bull effect and age of the cow (included as a covariate) were not significant, suggesting that the differences between groups could be explained, in part, by inbreeding.

Table 5. Changes in the frequency of sperm subpopulations sP1, sP2 and sP3 in semen samples from inbred (HI; *n* = 30) and non-inbred (NI; *n* = 25) bulls

*$P < 0.05$ compared with HI bulls within columns (Chi-squared test)

	sP1 (%)	sP2 (%)	sP3 (%)
HI bulls	48.2	16.6	35.2
NI bulls	40.0*	17.4*	42.5*

Table 6. Assessment of field fertility of inbred bulls: differences between the best and the worst solutions for each genetic and non-genetic effect studied

*$P < 0.05$. HYS, herd–year–season of first calving; PEE, permanent environmental effect

Effect	Range solutions (months)	Variance components ratio (%)
HYS	17.89	39.83
Cow age	2.63	
Cow genetic	2.43	4.98
Bull PEE	7.53	2.432
Bull genetic	1.48	0.839
	Regression coefficient	
Fi[A] cow	0.176*	
Fi[A] bull	0.089	

[A]Inbreeding depression effect (months/percentage increase of inbreeding).

Results of extended genetic analysis, showing differences between the best and worst solutions for each effect in our animal model are given in Table 6. The largest difference, accounting for nearly 40% of the CI variability, was determined by the contemporary group. This effect is produced by the combination of herd, year, mating time of the year and therefore, environmental effects. A marked influence was also found for cow genetics (4.98% of CI variability) and permanent environmental effect of the bull (PEE) (2.4% of CI variability but with differences up to 7.53 months among bulls). In contrast, the genetic effect of the bull (F) was very limited (0.839%, with differences of 1.48 among bulls). Finally, regression analysis of the effects of inbreeding on CI was significant for cows ($P < 0.05$) but not for bulls.

Discussion

Inbreeding has been widely described as a genetic trait that could adversely affect sperm quality in wild mammals (Shivaji *et al.* 1998; Asa *et al.* 2007; Ruiz-Lopez *et al.* 2010), domestic cats (Pukazhenthi *et al.* 2006), horses (van Eldik *et al.* 2006) and even cattle (Maximini *et al.* 2011). However, to our knowledge, the present study is the first comprehensive analysis assessing the effect of high inbreeding values (mean F = 16.3%) on sperm motility traits using computerised methods. In cattle, results from previous studies evaluating the inbreeding effect on spermatozoa are not consistent. For example, Flade and Zeller (1992) evaluated semen of experimentally produced inbred bulls (F = 25% vs F = 0%) and did not find any differences.

In contrast, Maximini *et al.* (2011) demonstrated a correlation between F and four sperm traits (sperm volume, total sperm number, viability and subjective motility). Interestingly, both studies were performed using subjective methods (direct observation and sample classification over a motility scale of 1–5). This methodology has been reported to be less accurate and highly affected by technicians, with differences among raters of up to 30% for the same sample (Amann 1989; Verstegen *et al.* 2005). In the present study, such methodological differences were ruled out because kinetic assessments were performed by using an automated computerised system (CASA).

Several studies have reported that inbreeding depression increases the number of spermatozoa with abnormal morphology in both wild and domestic animals (Gomendio *et al.* 2000; Gage *et al.* 2006; Pukazhenthi *et al.* 2006; van Eldik *et al.* 2006; Asa *et al.* 2007). However, knowledge of the effects of inbreeding depression on cattle spermatozoa is limited (Losdat *et al.* 2014). In the present study, no significant differences were found between HI and NI animals in terms of sperm morphology. The same results were observed for sperm viability and acrosomal status. These results are in agreement with those reported by Flade and Zeller (1992) and Ducrocq and Humblot (1995), because they show a slight but non-significant increase in the percentage of ASM associated with higher inbreeding values. More recently, Godfrey and Dodson (2005) confirmed the absence of such a correlation. In contrast, increased DNA instability or an increased percentage of immature spermatozoa were largely related to increased ASM (Persson and Soderquist 2005; Enciso *et al.* 2011*a*). In the present study, neither DFI nor HDS differed significantly between the HI and NI groups, which is in line with the lack of differences in sperm morphology between groups.

Studies assessing the effects of inbreeding on sperm osmotic resistance in mammals are scarce till now. To our knowledge, this is the first study on this topic performed in cattle. Our findings indicate that there is no effect of inbreeding on sperm osmotic tolerance in bulls. Previous studies reported decreased tolerance to osmotic stress in inbred wild mice reared in captivity (Malo *et al.* 2010), but these findings were associated with sperm morphological abnormalities. Conversely, Garde *et al.* (2003) found a relationship between F and sperm osmotic resistance in some species of gazelle. However, they were highly affected by the species studied and so they cannot be extrapolated across different genera. In the same way, Walters *et al.* (2005) demonstrated that spermatozoa of inbred strains of mice (C57BL/6 and DBA/2N) were highly sensitive to hypo-osmotic conditions, mostly because of alterations in mitochondrial morphology and function, leading to decreased resistance to cryopreservation. Despite the fact that some of these morphologically altered spermatozoa could regain motility, their ability to fertilise an oocyte may still be compromised (Nishizono *et al.* 2004). In the present study, there were no differences in the proportion of HOS+ spermatozoa or ASM and VS between the HI and LE groups. Furthermore, although some motility parameters, mostly associated with sperm hyperactivation, were increased in inbred spermatozoa, field fertility of the HI bulls was decreased, suggesting that sperm structure and osmotic resistance are not affected by inbreeding in cattle. Thus, the decreased fertility observed in inbred bulls could be more likely related to kinetic than other sperm traits.

The genetic effect on DFI in cattle spermatozoa was recently studied by Karoui *et al.* (2012), who reported that only a minimum percentage of the variability (<2.5%) was explained by genetic causes. Similar findings were made in the present study: DFI was not significantly affected by inbreeding, despite the F values were higher in the present study than those reported by Karoui *et al.* (2011) (16% vs 5.3%, respectively). However, Ruiz-Lopez *et al.* (2010) and Petrovic *et al.* (2013) demonstrated a clear correlation between DFI and inbreeding in wild ungulates and rams. This could be explained by differential resistance to oxidative stress and DNA fragmentation of spermatozoa from different species, which was also established by Enciso *et al.* (2011*b*). Interestingly, the results of the present study showed a larger than expected variability within groups despite the high number of spermatozoa analysed for each animal (at least 80 000), suggesting that DFI is more affected by bull factors (genetic background plus environmental factors) rather than inbreeding alone. Conversely, the lack of differences in percentage HDS, a marker related to sperm maturation by Rybar *et al.* (2004), in association with a normal percentage of ASM suggests that inbreeding depression does not affect or impair spermatogenesis.

The negative effect of inbreeding on sperm motility has been well documented in several species, including horses (van Eldik *et al.* 2006), mice (Songsasen and Leibo 1997) and wild herbivores (Gomendio *et al.* 2000). In contrast, results for cattle show a neutral (Flade and Zeller 1992; Karoui *et al.* 2011) to very low (Ducrocq and Humblot 1995) effect. The present study, by assessing an extended set of CASA-derived motility parameters, allowed us to draw more accurate conclusions. The increase in kinetic parameters (VCL, VSL, VAP and ALH) in HI bulls was associated with increased velocity, erratic tracks and unexpected direction changes, typical behaviour of hyperactivated (HA) spermatozoa (Cancel 2000). Motility results were corroborated by subpopulation clustering, with the proportion of sP1 spermatozoa (highly active but non-progressive), a pattern also associated with hyperactivation (Muiño *et al.* 2008), being considerably increased in the HI group. Although this movement is important for egg penetration, premature sperm hyperactivation could impair sperm transport along the lower female reproductive tract (Olds-Clarke and Wivell 1992). This effect has been reported in mice, in which epididymal motility was increased in inbred strains but fertility was reduced (Carey and Olds-Clarke 1980), and in donkeys, where individuals with a higher percentage of HA spermatozoa were less fertile (Dorado *et al.* 2013). Therefore, we hypothesise that the early sperm hyperactivation observed in HI bulls could hinder access of the spermatozoa to the fertilisation site because of the premature exhaustion of energy reserves, subsequently reducing their fertilisation capacity. This hypothesis is in agreement with the results of the field fertility analysis, which showed significantly increased CI for calves derived from HI bulls. Interestingly, when we selected the animals for the experimental design, we noticed that bulls with such extreme F percentages were used commercially as sires. In our case (Retinta bulls), ANCRE by-laws do not prohibit their use and some breeders, against the

genetic counselling provided by the association, only take into account bull morphology and 'pedigree' to select future sires.

It is well known that the genetic response to selection is less efficient for reproductive traits, and even less for fertility, than for several productive traits because reproduction is strongly affected by non-genetic components (reproductive diseases, nutritional status, herd management, year) and has already been affected by natural selection over several generations. However, the results of the present study showed that, in the absence of external factors, HI bulls had an impaired reproductive performance and an extended CI (0.6278 extra months on average) under field conditions compared with NI bulls. These findings agree with those of Charlesworth and Willis (2009), who demonstrated that the increase in homozygosity and the over-expression of deleterious recessive alleles (partial dominance) affect the reproductive performance of animals and suggested that F values must be included in any selection scheme in cattle.

A broader genetic analysis of the ANCRE database (159 bulls) was performed using a restricted maximum likelihood (REML) animal model. This methodology takes into account several genetic and non-genetic effects very efficiently, including all the relationship information among animals at any level. In the present study, with this analysis, we demonstrated that CI was highly influenced by the sire. However, most of this effect was explained by non-genetic factors, such as herd–year–season (HYS) of first calving or PEE. These results agree with those of Mackinnon *et al.* (1990), who found that the male component of fertility under extensive breeding systems with a prevalence of natural mating is larger than in populations with heavy use of AI. In the present study these results were unexpected because Retinta bulls are screened to avoid the use of individuals with poor sperm quality. Therefore, we hypothesise that such differences could be explained by sperm parameters not included in the basic semen analysis performed, such as the detection of premature hyperactivated motility. Conversely, the effect of inbreeding on CI was better explained by the F value of the cow than the mating bull (4.98% vs 0.84%, respectively). This has also been reported by González-Recio *et al.* (2007) and McParland *et al.* (2009), who found lower pregnancy rates associated with highly inbred cows. However, the joint analysis of both genetic studies (higher CIs on HI bulls in narrow model and no differences in extended model) may suggest that the effect of inbreeding on sperm is only significant when certain F value threshold is reached (13.5% in our case). These results agree with previous studies assessing the productive traits of dairy cows, where an inbreeding 'threshold' was also proposed (Hansen 2000; Sewalem *et al.* 2006).

Conclusions

In the present study, using objective and automated methodology, we demonstrated for the first time that inbreeding affects bull sperm motility. Inbred bulls showed a premature hyperactive-like motility pattern, associated with increased sperm velocity (VCL, VSL and VAP) and ALH. In addition, cows mated with inbred bulls had an increased CI under field conditions. However, we cannot determine whether the reproductive impairment observed was caused by effects associated with the spermatozoa (premature hyperactivation) or inbreeding (partial dominance, overdominance or epistasis). Further complex experiments including genotyping (single nucleotide polymorphism array genotyping or full sequencing) of embryos produced from highly and non-inbred animals are needed to clarify this issue.

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