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"Evaluation of sperm subpopulation structure in relation to *in vitro* sperm-oocyte interaction and field fertility of frozen-thawed semen from Holstein bulls"

Tesis de Master

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CERTIFIQUEN:

Que la memòria titulada "**Evaluation of sperm subpopulation structure in relation to *in vitro* sperm-oocyte interaction and field fertility of frozen-thawed semen from Holstein bulls**", presentada per Marcia de Almeida Monteiro Melo Ferraz, ha estat realitzada sota la direcció de la Dra. Maria Teresa Mogas Amorós i la Dra. Roser Morató Molet i, considerant-la acabada, autoritzen la seva presentació per a què sigui jutjada per la comissió corresponent.

I per tal que consti els efectes que corresponen, signen la present a Bellaterra, el 15 de Mayo de 2013,

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Abstract

The present study examined the relationship between sperm subpopulations, sperm-oocyte interaction and *in vivo* fertility in ejaculates from Holstein bulls. Post-thaw sperm motility was analysed by using a CASA system and evaluated to identify sperm subpopulations. Adhesion and penetration of zona pellucida (ZP) and pronucleus formation using post-thawed samples with different percentages of sperm included in the subpopulation with the fastest and most progressive subpopulation (subpopulation four) was tested. Pregnancy rates after first insemination, using sperm samples having different rates of spermatozoa belonging to subpopulation four, from six ejaculates, were determined. The correlation between the subpopulation four and the number of spermatozoa bound to the ZP (ZBA), the penetration rate (PENR) and the rate of pronucleus formation (PNR) were determined. A significant ($P < 0.05$) and positive correlation was found between the ZBA, the PENR and the PNR with the SP4 ($r=0.79$, $r=0.66$ and $r=0.63$, respectively). We also found a significant correlation between this sperm subpopulation and *in vivo* fertility ($r=0.89$, $P < 0.05$). Our results suggest that this specific high motile and progressive subpopulation is positive and significant correlated with the ability of a thawed bull semen sample in binding and penetrating the ZP and with *in vivo* fertility. Showing that this subpopulation may has a great influence on fertility. These findings emphasize the relevance of semen subpopulation structure in bull sperm fertilizing ability and also show that the analysis of such subpopulations may improve sperm quality analyses in Holstein bulls.

Keywords: bull sperm**,** sperm motility and fertilizing ability, zona pellucida binding assay; penetration and pronucleus rates; *in vivo* fertility trials

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Introduction

1. Introduction

Fertility variation is the most important factor limiting efficient production of cattle. Despite 60 plus years of commercial experience in artificial insemination (AI) in the bovine, and years of studies on new technologies and manners to improve cattle fertility, our ability to use *in vitro* assessments of semen quality to predict the fertility potential of a semen sample seldom explains more than 50 to 60% of the variation among males (Dejarnette, 2005). The effects of semen quality on reproductive efficiency in cattle are well documented but only modestly understood. Sire fertility is an important component of the AI programs. It is necessary time and money to evaluate the fertility of a young bull, since most of the evaluations are done after AI of a certain number of females, using *in vitro* approved frozen-thawed semen. Although it is the best method, it is expensive, been of utmost importance the development of laboratory methods for accurately predicting the potential fertility of AI semen.

Most of the artificial inseminations in dairy cattle are done with frozen-thawed semen. It is already known that the cryopreservation process cause damages to the sperm cell, inducing loss of sperm viability and altering the functionality of the surviving leads to lower fertilizing capacity of the frozen-thawed spermatozoa (Watson, 2000). Individual variation in semen freeze-ability is recognized to exist in most domestic species, including the bovine (Parkinson and Whitfield, 1987), and such variation has been related to the incidence of motile (Davis et al., 1995) and morphologically (Thurston et al., 2001) distinct sperm subpopulations present in the ejaculates. Studies done in several species have identified the existence of different sperm subpopulations, defined by specific movement characteristics using the CASA system.

In order to determine the sperm quality, *in vivo* and *in vitro* analysis were developed, such as the assessment of sperm motility and morphology by visual microscopy, assessment of motility by using the CASA system, fluorescence and metabolic methods, and the analysis of the interaction between the spermatozoa and the oocyte (zona binding assay and embryo production). These methods are going to be discussed in the present study.

1.1 Importance of cryopreserved semen analysis

Semen cryopreservation has been successfully used in dairy cattle for years, but the fertility rates are not as good as those seen with fresh semen (Holt and Van Look, 2004). Reduced fertility of frozen semen is largely attributed to altered membrane structure and function during cryopreservation that may cause damage to spermatozoa and modify its capability of egg fecundation (Holt and Van Look, 2004). During the cryopreservation protocols, bull spermatozoa may suffer different kinds of damaging stresses: such as cold shock, osmotic and toxic stress, caused by the addition of molar concentrations of cryoprotectants, and the formation and dissolution of ice crystals in the extracellular environment. Many observations suggest that spermatozoa surviving freezing and thawing have altered membrane properties that may render them functionally similar to capacitated or acrosome-reacted cells (Watson, 1995). In addition, cytoskeletal elements are temperature-sensitive and the cooling of the cell may result in a premature depolymerisation of actin filaments (Watson, 2000).

One of the most important impacts of the cryopreservation in the sperm cell is the decline in motility. While a minority seems to exhibit vigorous forward progression, the majority show a variable degree of impairment. Furthermore, this method increases

the formation of reactive oxygen species, which are detrimental to subsequent performance (Watson, 2000). Finally, the ability of the sperm to sustain the early embryonic development may also be modified in frozen-thawed semen, because of potential damages to the sperm nuclear structures that this procedure may cause (Ellington et al., 1998). Since the cryopreservation process may cause different alterations in the sperm cells, it is of utmost importance to evaluate the frozen-thawed sperm quality, being able to increase the fertility rates in cattle.

1.2 Sperm subpopulations

Many studies have demonstrated that any mammalian ejaculate constitutes a heterogeneous population of spermatozoa in which we can find different subpopulations (Abaigar et al., 1999; Muiño et al., 2008b). These sperm subpopulations may be analyzed by different computerized and laboratory methods for sperm quality assessment. New techniques are been developed by many laboratories, in an attempt to distinguish the subpopulation of potentially competent spermatozoa among the whole sperm population (Rubio-Guillén et al., 2007). This sperm heterogeneity makes it possible for the female reproductive tract to exert multiple selective processes which will finally reduce a population of several millions to a few competent spermatozoa (Holt and Van Look, 2004). The sperm motility is important for its transport across the female reproductive tract, allowing the sperm cell to access the egg and penetrate it. Distinct sperm populations with variable motility may have different probability of crossing the female tract and enter the oviduct, having variable fertility rates.

In different species the process of cryopreservation may affect the motility parameters of each subpopulation as the distribution of spermatozoa within them,

compared to the parameters observed in the fresh ejaculate (Flores et al., 2008; Muiño et al., 2008a). Muiño and collaborators (2008b) have described the presence of four motile sperm subpopulations within a single ejaculate in fresh and frozen-thawed semen from Holstein bulls.

1.3 Methods of semen analysis

The evaluation of the ejaculate is of utmost importance to determine its degree of normality before being processed for AI or *in vitro* fertilization (IVF). Usually, this evaluation includes recordings of volume, appearance, sperm concentration and motility. In recent years, more detailed screenings of sperm function have been incorporated other than the determination of sperm motility, which, by far, is the parameter most widely used for processed semen in farm animals (Dejarnette, 2005). Fertility can be tested *in vitro* (e.g. by IVF) or *in vivo* by using AI of a certain number of females.

In vivo testing of fertility requires a certain minimum number of females to lower the variation of the outcomes to acceptable ones, e.g. below 10%, which would require at least 100 cows to be bred per ejaculate (Amann and Hammerstedt, 1993). Such testing of *in vivo* fertility is obviously linked with time-consuming, costly procedures, thus calling for alternative methods to predict semen and male fertility *in vitro*. Nevertheless, *in vitro* methods must be related to the fertility *in vivo*, thus calling for proper fertility records issued on an acceptable number of females, a matter that has not always been contemplated elsewhere despite it being a major prerequisite (Graham and Mocé, 2005; Graham, 2001; Rodríguez-Martínez, 2003). Most current *in vitro* semen evaluation methods measure general characteristics of the spermatozoa

(morphology, motility patterns, organelle integrity etc.), all essential to fertility if maintained until the spermatozoa are confronted with the oocyte.

1.3.1 *In vitro* methods

Techniques routinely used to evaluate bull fertility, such as concentration, morphology and motility, cannot reliably predict the fertility of the bull, they only give an estimate if it is a good or a bad ejaculate. *In vitro* tests, such as sperm parameters analysis and *in vitro* fertilization (IVF) tests might afford an adequate means of assessing fertility, specially the IVF tests, which allows the evaluation of sperm–oocyte interactions that occur during fertilization, and permits different endpoints in the early stages of embryo development to be monitored.

1.3.1.1 Conventional methods

Visual microscopic assessment of sperm motility is the classic measure of semen quality. This procedure is simple, rapid, and inexpensive; however, it is also highly subjective. Visual estimates of motility can be biased and either over or under estimated as a function of sperm concentration within the sample. Repeatability of visual motility estimates is often marginal both within and across technicians evaluating the same samples. Similarly, procedural, environmental, and technician variances across laboratories make it difficult to precisely define minimum standards and it is often necessary for each laboratory to establish independent “scales” for assessment of sperm motility (Dejarnette, 2005).

Assessment of normal sperm morphology is another essential component of the spermiogram. Determination of head morphology, acrosomal membrane integrity, tail abnormalities and sperm viability are important to determine the sperm quality. However, the sperm defect believed to be more directly associated with sperm fertility is the DNA integrity, which may not be assessed by the conventional methods.

1.3.1.2 Computer-assisted semen analyzer (CASA)

The CASA is a more objective method for sperm motility assessment. In addition to percentage of sperm motility, these systems can provide information related to patterns of sperm motion including linearity, velocity, frequency and amplitude of head displacement. Besides the purchasing and operating costs, application of CASA in the domestic animal andrology laboratory is further limited by the inability to distinguish between non-motile sperm and particulate matter in the extender, which biases estimates of both concentration and motility (Verstegen et al., 2002).

Studies carried out by several researchers on fresh and frozen-thawed semen from species as diverse as marmosets (Holt et al., 1996), gazelles (Abaigar et al., 1999), boars (Abaigar et al., 1999; Cremades et al., 2005; Quintero-Moreno et al., 2004; Rivera et al., 2006), stallions (Quintero-Moreno et al., 2003), dogs (Núñez-Martínez et al., 2006) or rabbits (Quintero-Moreno et al., 2007) have demonstrated that, using CASA systems, it is possible to identify and quantify different sperm subpopulations with specific patterns of movement. This can be achieved by using different procedures of multivariate clustering analysis applied to the CASA-derived kinematic parameters obtained for each individual spermatozoon in a semen sample (Muiño et al 2008b).

The kinematic parameters generally used to determine the sperm quality by CASA are: curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s), percentage of linearity (LIN, %), percentage of straightness (STR %), wobble coefficient (WOB %), mean amplitude of lateral head displacement (ALH, μm) and beat cross frequency (BCF, Hz), described in table 1.

Muino and collaborators (2008b) described 4 different subpopulations in ejaculates from Holstein bulls:

Subpopulation 1 represented those spermatozoa with a relatively low velocity (medium VCL and VAP) but with a high progressiveness (high LIN, STR,WOB and low ALH).

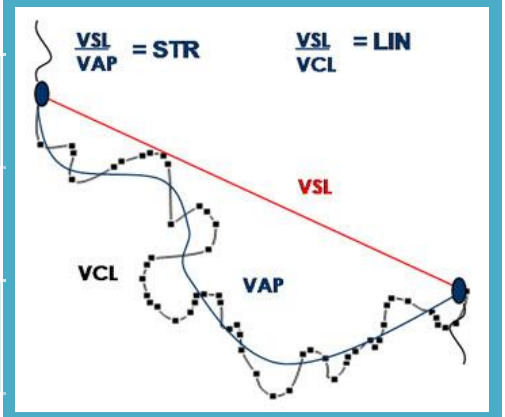
Subpopulation 2 contained highly active but non-progressive spermatozoa, as indicated by the high values of VCL and ALH together with low values of LIN and STR, and moderate BCF. They could be considered as having "hyperactivated-like" movement.

Subpopulation 3 was poorly motile and non-progressive. Those spermatozoa showed the lowest values of VCL, ALH and BCF together with low LIN and STR.

Subpopulation 4 represented those spermatozoa which moved most rapidly and progressively, as indicated by the highest values of VCL, VAP, LIN, STR and also the highest BCF.

Table 1. Description of the most important CASA parameters, utilized to define sperm subpopulations.

Characteristic	Description
VCL (µm/s)	Point to point velocity (total distance traveled) per second.
VSL (µm/s)	The average path velocity of the sperm head along a straight line from its first to its last position.
VAP (µm/s)	The average velocity of the sperm head along its average trajectory.
LIN (%)	The ratio VSL/VCL, describes path curvate.
STR (%)	The ratio between VSL and VAP.
WOB (%)	The ratio between VAP and VCL.
BCF (Hz)	The frequency with which the actual sperm trajectory crosses the average path trajectory.
ALH (µm)	The average value of the extreme side-to-side movement of the sperm head in each beat cycle.



1.3.1.3 Fluorescence and metabolic methods

Generally the fluorescence methods are used to determine cell viability, mitochondrial potential, acrosomal membrane integrity, plasmatic membrane integrity,

DNA integrity and others parameters. Acrosomal membrane integrity is another common measure of sperm viability known to be associated with fertility. Numerous live/dead staining procedures are available to facilitate estimations of the "viable" populations of spermatozoa in a semen sample under field conditions with relatively inexpensive brightfield-microscopy (Celeghini et al., 2007).

Abnormalities in the level of sperm nucleus with implications on reproduction, include DNA strand breaks, numerical and structural chromosomal abnormalities. A variety of tests have been performed to evaluate the DNA damage, such as TUNEL, comet, DNA breakage detection-fluorescence in situ hybridization and sperm chromatin structure assay (SCSA), all based on fluorescence methods.

Flow cytometry in different technical applications offers many advantages for the analysis of sperm quality. Flow cytometers can acquire data on several subpopulations within a sample in a few minutes, making it ideal for assessment of heterogeneous populations in semen sample. Using such a separation approach, cellular patterns can be identified by assessing, in individual cells within a population, protein expression using fluorescently labelled antibodies and other fluorescent probes (Baumgarth and Roederer, 2000; Herzenberg et al., 2006).

Similar to CASA analysis of sperm motility, flow cytometric analysis of sperm characteristics presents an opportunity to increase the precision and repeatability of sperm analysis. However, like the CASA, this increased precision comes at considerable increase in the costs, which limits its application in commercial andrology laboratories serving domestic livestock species. However, in addition to equipment expense, these procedures appear to be quite tedious and time/environment sensitive, which limits implementation for use in domestic animals (Dejarnette, 2005).

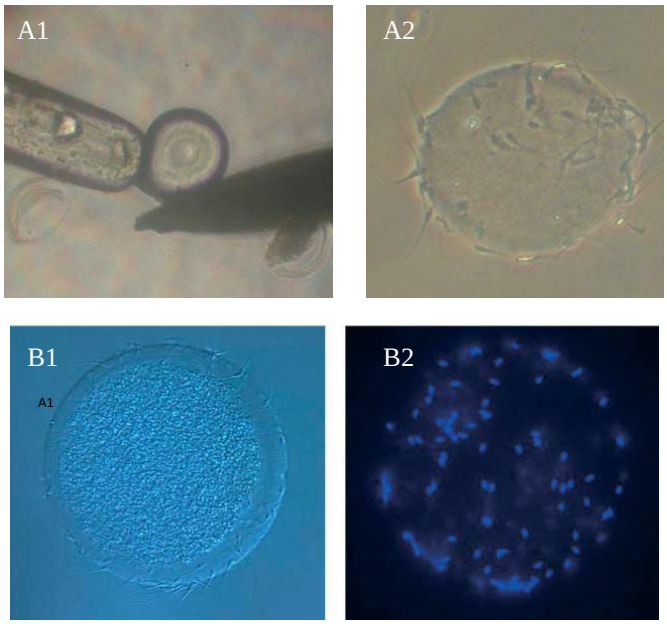
As metabolic methods the Hypo-osmotic swelling (HOS) test is a wide used analysis, especially in cases when there are very few or no motile sperm in the ejaculate. This test is based in the ability of live spermatozoon to withstand moderate hypo-osmotic stress (Franken and Oehninger, 2012). Another test is the determination of Reactive oxygen species (ROS). Since the sperm cell needs to convert oxygen into energy and that this conversion is not perfectly efficient, 1-5% of the consumed oxygen is converted into free radicals, we can say that the spermatozoa are potent producers of ROS (Franken and Oehninger, 2012). Excessively ROS-producing by the sperm cell seem to damage its own DNA, having a particularly clinical importance (Henkel et al., 2003).

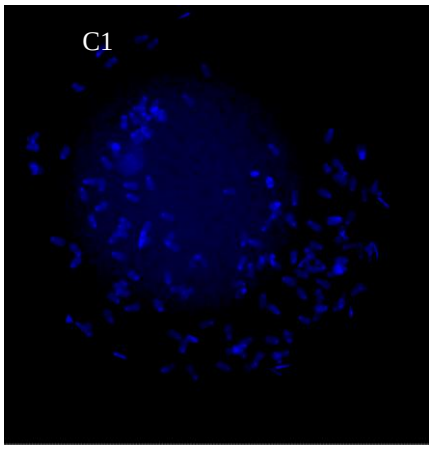
1.3.1.4 Zona Pellucida binding assay

Mammalian oocytes Zona Pelucida (ZP) is critical for sperm-oocyte interaction. This interaction between the sperm cell and the ZP may reflect distinct functions of the spermatozoa, like morphology, motility, viability, acrosomal status and the ability to penetrate the oocyte; thus the analysis of this interaction may be a good tool to sperm evaluation. Assays based in the number of sperm cells bound to the ZP have been studied as diagnostic test to predict bull fertility in the field (Zhang et al., 1998). ZP binding assays (ZBA) have been developed as diagnostic test for several species, including bovine, equine, donkey and buffalo (Clulow et al., 2010; Park et al., 2012; Selvaraju et al., 2008; Taberner et al., 2010; Zhang et al., 1998). Both heterologous and homologous, hemi-zona or intact zona binding assay were tested (Clulow et al., 2010; Park et al., 2012; Thundathil et al., 2001; Zhang et al., 1998) (Figure 1). Zhang and collaborators (1998) in a ZBA utilizing frozen-thawed semen from bulls, found a

significant correlation between the ZBA and the non-return rate (NRR), the ZBA was also correlated with linear motility post-thawing. Positive results were also found in buffalos, where the number of sperm bound to the ZP had significant correlations with the mitochondrial membrane potential and plasmalemma integrity (Selvaraju et al., 2008). According to Thundathil et al (2001) sperm with proximal droplets fail during the sperm-oocyte binding due to impaired motility or structural and/or functional inadequacy, showing that the ZBA may be used as a diagnostic tool for sperm quality.

Figure 1. Hemizona, heterologous and homologous zona pellucida binding assay.





A. Hemizona binding assay, A1: bisection of the oocyte in two equals halves; A2: matching hemizonaes with boar spermatozoa.

(Tsakmakidis et al., 2007)

B. Heterologous zona pellucida binding assay. Light microscopy (B1) and fluorescence microscopy (B2) of bovine oocytes incubated with equine spermatozoa.

(Coutinho da Silva et al., 2012)

C. Homologous zona pellucida binding assay. Fluorescence microscopy (C1).

1.3.1.5 In vitro fertilization (IVF)

IVF has been developed and used for producing offspring in many domestic animal species (Brackett et al., 1982; Palmer et al., 1991). This technique implies *in vitro* maturation of oocytes (IVM), *in vitro* fertilization (IVF) of oocytes and *in vitro* culture (IVC) of fertilized oocytes up to the blastocyst stage (Larsson and Rodríguez-Martínez, 2000). Over the years, many studies, performed mainly in cattle, have shown that the donor of the semen largely influences the outcome of both IVF and IVC (Eyestone and First, 1989). This difference in *in vitro* fertility between semen donors led to studies designed to investigate whether there was a correlation between *in vivo* and *in vitro* fertility (Zhang et al., 1997). Although a correlation between IVF and IVC results and *in vivo* fertility has not been found in all investigations (Schneider et al., 1999), the findings of several studies support the concept of using IVF to assess the fertilizing ability of bull (Marquant-Le Guienne et al., 1990; Zhang et al., 1997), where the authors found a significant positive correlation between cleavage rates *in vitro* and *in vivo* fertility, expressed as NRRs after AI.

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Aim of the study

2. Aim of the study

The present study aim to investigate the effect of sperm subpopulation in the ability of sperm cell to bind to the zona pellucida, penetrate the oocyte, forming the pronucleous and *in vivo* bull fertility. For this purpose, in a first experiment, the ability of the sperm from the higuer and progressive subpopulation to bind, penetrate the ZP and form the pronucleus of *in vitro*-matured bovine oocytes was evaluated. In the second experiment, field fertility and offsprings of frozen-thawed bull ejaculates were determined and correlated with the rates of the fastest and most progressive sperm subpopulation within such ejaculates

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Evaluation of sperm subpopulation structure in relation to *in vitro* sperm-oocyte interaction and field fertility of frozen-thawed semen from Holstein bulls

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Abstract

The present study examined the relationship between sperm subpopulations, sperm-oocyte interaction and *in vivo* fertility in ejaculates from Holstein bulls. Post-thaw sperm motility was analysed by using a CASA system and evaluated to identify sperm subpopulations. Adhesion and penetration of zona pellucida (ZP) and pronucleus formation using post-thawed samples with different percentages of sperm included in the subpopulation with the fastest and most progressive subpopulation (subpopulation four) was tested. Pregnancy rates after first insemination, using sperm samples having different rates of spermatozoa belonging to subpopulation four, from six ejaculates, were determined. The correlation between the subpopulation four and the number of spermatozoa bound to the ZP (ZBA), the penetration rate (PENR) and the rate of pronucleus formation (PNR) were determined. A significant ($P < 0.05$) and positive correlation was found between the ZBA, the PENR and the PNR with the SP4 ($r=0.79$, $r=0.66$ and $r=0.63$, respectively). We also found a significant correlation between this sperm subpopulation and *in vivo* fertility ($r=0.89$, $P < 0.05$). Our results suggest that this specific high motile and progressive subpopulation is positive and significant correlated with the ability of a thawed bull semen sample in binding and penetrating the ZP and with *in vivo* fertility. Showing that this subpopulation may has a great influence on fertility. These findings emphasize the relevance of semen subpopulation structure in bull sperm fertilizing ability and also show that the analysis of such subpopulations may improve sperm quality analyses in Holstein bulls.

Keywords: bull sperm**,** sperm motility and fertilizing ability, zona pellucida binding assay; penetration and pronucleus rates; *in vivo* fertility trials

3.1 Introduction

Fertility variation is the most important factor limiting efficient production of cattle. The effects of semen quality on reproductive efficiency in cattle are well documented but only modestly understood (Rodríguez-Martínez, 2003). Our ability to use *in vitro* assessments of semen quality to predict the fertilizing ability of a semen sample seldom explains more than 50 to 60% of the variation among males (Dejarnette, 2005). In order to determine the sperm quality, *in vivo* and *in vitro* analyses have been developed. The analysis of sperm motility by visual microscopy or using a Computer Assisted Sperm Analyzer (CASA) system is the parameter most commonly used. The sperm motility is an important factor for its transport across the female reproductive tract, allowing the sperm cell to access the egg and penetrate it. But this parameter is poorly correlated with fertility (Puglisi et al., 2012; Sancho et al., 2006).

Studies performed in several species have identified the existence of different sperm subpopulations using CASA systems (Abaigar et al., 1999; Martínez-Pastor et al., 2011; Quintero-Moreno et al., 2004). This sperm heterogeneity seems to be related with the ability of the female reproductive tract to perform distinct selective processes which will reduce a population of several millions to a few competent spermatozoa (Holt and Van Look, 2004), although more studies are needed to confirm this hypothesis. The presence of four motile sperm subpopulations within a single ejaculate in fresh and frozen-thawed semen have been described in sperm from Holstein bulls (Muiño et al., 2008b). Distinct sperm populations with variable motility may have different

probability of crossing the female tract, enter the oviduct and interact with the oocyte, leading to variable fertility rates.

For production animals and humans, conventional methods of semen quality evaluation, such as sperm motility, morphology, viability and concentration, are routinely used to rapidly estimate male reproductive potential, although their correlations with actual fertility can be sometimes unsatisfactory (Amann and Hammerstedt, 2002; Bonet et al., 2012; Kuisma et al., 2006; Love, 2011; Rodríguez-Martínez, 2003).

Potential male fertility can be also estimated by the capacity of spermatozoa to penetrate homologous and heterologous oocytes *in vitro* (García-Alvarez et al., 2009; Macedo et al., 2010; Taberner et al., 2010), as the mammalian zona pellucida (ZP) is critical for sperm-oocyte interaction (Zhang et al., 1998). In fact, assessing sperm-ZP interaction may lead to determine both how the sperm function are and their ability to penetrate the oocyte and form the pronucleus (Burkman et al., 1988). In this context, it is worth noting that ZP binding and penetrating assays have been developed as diagnostic tools for several species (bull: Zhang et al., 1998; Park et al., 2012; stallion:Clulow et al., 2010; donkey: Taberner et al., 2010; and buffalo: Selvaraju et al., 2008) and both heterologous and homologous, hemi-zona or intact zona assays have been tested (Clulow et al., 2010; Park et al., 2012; Selvaraju et al., 2008; Thundathil et al., 2001; Zhang et al., 1999, 1998).

A correlation between the number of sperm cells bound to the ZP and field fertility (determined by the non-return rate at 56 days) has been also demonstrated in cattle (Fazeli et al., 1997; Zhang et al., 1998), and the number of sperm bound to the ZP has also been found significantly correlated with the mitochondrial membrane potential

and plasmalemma integrity in buffalos. However, and to best of our knowledge, there are no reports evaluating whether sperm subpopulation structure in bull ejaculates are correlated with *in vitro* and field fertility in cattle. Against this background, and given that the importance of sperm subpopulation structure in bull ejaculates has not been well documented yet, the present work sought to determine how different rates of the fastest and most progressive subpopulation (Muiño et al., 2008b) affect *in vitro* and *in vivo* fertility. For this purpose, in a first experiment, the ability of the sperm to bind, penetrate the ZP and form the pronucleus of *in vitro*-matured bovine oocytes was evaluated. In the second experiment, field fertility and offsprings of frozen-thawed bull ejaculates were determined and correlated with the rates of the fastest and most progressive sperm subpopulation within such ejaculates.

3.2 Materials and Methods

3.2.1 Reagents and laboratory supplies

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated. Plastic dishes, four-well plates and tubes were obtained from Nunc (Roskilde, Denmark).

3.2.2 Semen sources and determination of sperm motility

A total of 21 ejaculates collected from 7 Holstein bulls (three ejaculates per bull) were used for this study. Sperm collection, cryopreservation and analyses were conducted as previously described by Muiño et al., (2008b). The overall sperm motility of frozen-thawed sperm as well as the kinematic parameters of individual spermatozoa

were determined using a computer assisted sperm analysis system (CASA system) (Sperm Class Analyzer, Microptic SL; Barcelona, Spain) to investigate the existence of separate motile subpopulations (Muiño et al., 2008b).

The CASA system used was based on the analysis of 16 consecutive, digitalized photographic images which were taken in a time lapse of 0.64 s, which implied a velocity of image-capturing of 1 photograph every 40 ms. Images were taken from 5 µl semen aliquots, which were placed on slides and covered with 20 × 20mm coverslips. Three microscopic fields were analyzed in each sample using a phase-contrast microscope supplied with a prewarmed stage at 37^{o}C and at 200× magnification. The number of spermatozoa analyzed per sample ranged between 100 and 200, including the immotile sperm. Objects incorrectly identified as spermatozoa were minimized on the monitor by using the playback function. Total motility was defined as the percentage of spermatozoa with mean velocity (VAP) above 10 µm/s. The kinematic parameters recorded for each spermatozoa were: curvilinear velocity (VCL, µm/s); straight-line velocity (VSL, µm/s); average path velocity (VAP, µm/s); percentage of linearity (LIN, %); percentage of straightness (STR, %); wobble coefficient (WOB, %); mean amplitude of lateral head displacement (ALH, µm); and beat cross frequency (BCF, Hz) (Mortimer, 2000, 1997). Based on these parameters, four different sperm subpopulations were determined (Muiño et al., 2008b) (Table 1).

Table 1. Description of the four sperm subpopulations determined by CASA analysis and used in the study.

Subpopulation	Characteristics
1	Low velocity (medium VCL and VAP) and high progressiveness (high LIN, STR,WOB and low ALH).
2	Highly active but non-progressive spermatozoa, as indicated by the high values of VCL and ALH together with low values of LIN and STR, and moderate BCF.
3	Poorly motile and non-progressive. Those spermatozoa showed the lowest values of VCL, ALH and BCF together with low LIN and STR.
4	Spermatozoa which moved most rapidly and progressively, as indicated by the highest values of VCL, VAP, LIN, STR and also the highest BCF.

VCL: curvilinear velocity (µm/s); VSL: straight-line velocity (µm/s); VAP: average path velocity (µm/s); LIN: percentage of linearity (%); STR: percentage of straightness (%); WOB: wobble coefficient (%); ALH: mean amplitude of lateral head displacement (µm); and BCF: beat cross frequency (Hz).

3.2.3 Bovine oocyte collection and *in vitro* maturation

Bovine ovaries were obtained from a local slaughterhouse and transported to the laboratory in phosphate buffered saline (PBS) at 36–38^{o}C. . Cumulus oocyte complexes (COCs) were obtained by aspirating 2–10 mm follicles. Only COCs with three or more layers of cumulus cells and showing homogeneous cytoplasm were selected for maturing *in vitro*. Groups of up to 50 COCs were placed in 500 µL of maturation medium in four-well dishes and cultured for 24 h at 38.5^{o}C in a 5% CO_2 humidified air atmosphere. The maturation medium was comprised of TCM-199 supplemented with 10% (v/v) fetal calf serum, 10 ng/mL epidermal growth factor and 50 µg/mL gentamicin.

3.2.4 Sperm preparation and in vitro fertilization

After maturation, the COCs were washed twice in PBS and then the cumulus cells were removed by gentle pipetting. The oocytes were washed again in PBS and randomly transferred in groups of 10 to a 45 µL drops of fertilization medium (Tyrode's medium supplemented with 25 mM sodium bicarbonate, 22 mM Na-lactate, 1 mM Na-pyruvate, 6 mg/mL fatty acid-free BSA and 10 mg/mL heparin sodium salt [Calbiochem, Darmstadt, Germany]).

Semen straws were thawed in a water bath at 38.5^{o}C for 40 s and immediately diluted in fertilization medium without heparin. Spermatozoa were then centrifuged in the BoviPure® solution (Nidacon International AB, Göthenborg, Sweden) for 10 min at 100 x g at room temperature. The supernatant was removed, the pellet resuspended in 3 mL of BoviPure® solution and centrifuged again at 100 x g for 5 min. Spermatozoa were counted in a Neubauer chamber and diluted in an appropriate volume of fertilization medium to give a final concentration of 10 x 10^6 spermatozoa/mL. A 5 µL aliquot of each sperm suspension was then added to each fertilization drop to obtain a final rate of 5000 spermatozoa/oocyte. Plates were incubated for 20 h at 38.5^{o}C in a 5% CO_2 humidified air atmosphere.

3.2.5 Evaluation of sperm ZP binding and penetration assay

At 20 h post-insemination, the presumptive zygotes were pippeted ten times with a pippete of 0.45-mm inner diameter to remove those spermatozoa that were not strongly attached to ZP, washed three times in PBS, fixed for 30 min in 4% (v/v) paraformaldehyde in PBS, stained with DAPI (4',6-diamidino-2-phenylindole; Vysis Inc., Downer's Grove, IL, USA) (125 ng/mL) and mounted on glass slides. The number of sperm bound to the ZP, the number of spermatozoa that penetrated the ZP and the

number of pronucleus were assessed at 400× under an epifluorescence microscope. A total of three replicates for each ejaculate were performed.

3.2.6 Cows and Reproductive management

This experiment was conducted in collaboration with a dairy farm from Pascual company located in Zuzone (Burgos, Spain). Cows were housed in cross-ventilated and freestall barns. Cows were submitted to a Presynch-Ovsynch protocol for first service AI. Briefly, cows received 2 intramuscular injections of PGF2α (25 mg i.m. of Dinoprost tromethamine, Lutalyse; Pfizer Animal Health, New York, NY) 14 d apart at 39 ± 3 and 53 ± 3 days in milk. Cows detected in estrus based on tail chalk removal conducted daily after the second PGF2α injection of the presynchronization protocol were inseminated by veterinarians. The pregnancy rate after the first insemination was determined at day 45 post-artificial insemination (AI) by ultrasonography.

3.2.7 Experimental design

3.2.7.1 Experiment 1: Evaluation of sperm ability to bind and penetrate the ZP

In vitro-matured cow oocytes were randomly fertilized using fifteen frozen-thawed ejaculates with distinct rates of spermatozoa from subpopulaion 4. Criteria for determining sperm subpopulation 4 were established according to Muiño et al. (2008b). After a 20 h co-incubation period, the presumptive zygotes were fixed and the number of spermatozoa bound to the ZP, those that penetrated the ZP and the number of pronucleus were determined.

3.2.7.2 Experiment 2: Sperm subpopulation in relation to field fertility of frozen-thawed semen from Holstein bulls

Six frozen-thawed semen ejaculates from two Holstein bulls were used to perform the AI trials. The frozen-thawed semen samples have been previously divided according to the percentages of spermatozoa belonging to high motile and progressive subpopulation (subpopulation 4). A total of 207 cows were used in this experiment. The pregnancy rate was determined at day 45 post first AI by ultrasonagraphy. Pregnancies after first AI as well as deliveries of calves were determined.

3.2.8 Statistical analysis

All statistical analyses were conducted with the IBM® SPSS® 20 for Windows (IBM corp.; Chicago, Illinois). Data are presented as percentages and mean ± standard error of the mean (SEM). Each ejaculate was considered as an independent observation and the minimal level of significance was set at $P < 0.05$ in all the statistical analyses.

First, penetration and pronucleus rates were transformed using logistic (logit) transformation as follows: logit=ln(PENR/(1 – PENR), and logit=ln(PNR/(1-PNR)), where PENR was the penetration rate and PNR the pronucleus rate. The resulting log-odds were used for further calculations. Next, these two logit-transformed variables, together with the numbers of spermatozoa attached to zona pellucida (ZBA), of pronucleus, of penetrated oocytes and of *in vivo* fertility, as well as data from sperm quality analyses (%SP4, VCL, VSL, …) were tested for normality and homocedasticity using Kolmogorov-Smirnov and Levene tests. When needed, data on percentages were

recalculated using the arcsine square root (x) transformation to match the parametric assumptions. However, numbers of sperm attached to ZP (ZBA) in the different ejaculates within each bull were compared through Kruskal-Wallis and Mann-Whitney tests, as linear transformations did not solve heterocedasticity and non-parametric alternatives to parametric ANOVA were required.

On the other hand, the ability of sperm motility analysis to correlate with and predict sperm fertilising ability was investigated through linear regression analyses (Pearson correlation and multiple regression) where ZBA, PENR, PNR and *in vivo* fertility were the dependent variables, and the percentage of SP4 and the other sperm parameters were the independent variables. The procedure used (the forward stepwise model) was the same described by Yeste et al., (2010) and consisted of optimizing the regression equation ($y=a+b_1x_1+b_2x_2+ \ldots + b_kx_k$) to increase the determination coefficient (R^2). The significance level for introducing each parameter in the multiple regression model was 0.10 and the significance level (α) for the model was 0.05.

3.3 Results

3.3.1 Experiment 1

The mean number of spermatozoa bound to the zona pellucida ranged between 2.1 to 14.7. For all five bulls a significant difference ($P < 0.05$) was found between the different ejaculates, when considering the ZBA, the PENR and the PNR; except for bull number 4, in which no difference was found. The post-thawing motility, the rate of sperm from subpopulation 4, the number of spermatozoa attached to the ZP, the rate of penetration and pronucleus formation are described in Table 2.

Table 2. Sperm-oocyte binding assay to determine the effect of containing different rates of spermatozoa belonging to the subpopulation which moved rapidly and progressively (SP4) on the ability to attach with the zona pellucida and to penetrate *in vitro* matured bovine oocytes.

Bulls	Rate of sperm from SP4 (%)	Number of oocytes	ZBA (mean±SE)	PENR (%)	PNR (%)
1	19.30	72	5.0 ± 0.5^{a}	25.0^{a}	7.0^{a}
	31.96	110	7.2 ± 0.8^{a}	20.0^{b}	11.0^{b}
	42.55	95	14.7 ± 1.9^{b}	21.7^{b}	12.0^{b}
2	5.94	90	2.1 ± 0.3^{a}	7.7^{a}	3.3^{a}
	13.09	80	8.3 ± 2.1^{b}	12.8^{b}	10.2^{b}
	26.07	66	11.6 ± 1.7^{b}	30.3^{c}	21.2^{c}
3	12.25	67	3.8 ± 0.5^{a}	11.9^{a}	6.0^{a}
	19.35	72	5.8 ± 0.8^{a}	19.4^{a}	8.3^{a}
	38.47	78	13.5 ± 2.5^{b}	27.6^{b}	18.4^{b}
4	8.36	95	2.6 ± 0.3^{a}	11.7^{a}	5.9^{a}
	11.94	93	4.1 ± 0.7^{a}	19.3^{a}	11.8^{a}
	26.12	80	3.6 ± 0.7^{a}	13.7^{a}	5.0^{a}
5	7.87	48	5.9 ± 0.8^{a}	14.6^{a}	6.2^{a}
	17.83	96	4.4 ± 0.5^{a}	24.0^{b}	13.5^{b}
	29.89	53	7.2 ± 1.4^{b}	45.3^{c}	20.7^{c}

ZBA: number of spermatozoa attached to the zona pellucida; PENR: rate of penetrated oocytes; PNR: rate of pronucleus formation. Different superscripts between rows, for each bull separately, indicate significant difference ($P < 0.05$).

Table 3 shows the correlations between the number of attached spermatozoa (ZBA), penetration rates and the sperm motility parameters. A significant positive correlation was found between the rate of sperm from subpopulation 4 and the number of spermatozoa bound to the ZP ($r = 0.79$, $P < 0.01$). When CASA parameters were analyzed, a significant correlation was obtained between VCL and the number of attached spermatozoa. The relevance of %SP4 in predicting PNR and PENR, previously logit-transformed, was confirmed by linear regression analyses, as regression equations obtained following the forward stepwise model only included the %SP4 as independent

variable. In addition, all regression equations presented a determination coefficient higher than 0.35 (R^2), and in all cases the model was found to be significant (P <0.05) (Table 4).

Table 4. Regression equation, R^2, R, F and P value for number of sperm cells bound to the ZP (ZBA), the rate of penetrated oocyte (PENR) and the rate of pronucleus formation (PNR).

	Regression equation	R^2	R	F	*P* value model
ZBA	ZBA=0.27(SP4)+1.128	0.60	0.77	21.35	0.000
PENR	Logit (PENR)=0.035(SP4)-2.189	0.39	0.62	9.93	0.008
PNR	Logit (PNR)=0.034(SP4)-2.956	0.35	0.59	8.45	0.012

3.3.2 Experiment 2

When pregnancy rates were compared between the ejaculates, significantly higher rates of pregnancies were obtained for the ejaculates with higher rates of sperm from SP4 (n= 63; 71.4%) than for medium (n= 84; 53.6%) and low (n= 60; 45.0%) rates. Furthermore, significantly higher percentages of offspring were observed in those cows inseminated with ejaculates from the higher rates of sperm from SP4 (n=63; P <0.05). However, when data was analyzed comparing bulls, one of the bulls showed no differences in pregnancy rates. A significant positive correlation was found between the rate of sperm from subpopulation 4 and the *in vivo* fertility (r = 0.89, P < 0.05).

Table 3. Correlations between sperm parameters: rate of sperm from subpopulation 4 (SP4), post-thawing sperm motility (MOT), VCL, VSL, VAP, LIN, STR, WOB, ALH and BCF, the number of sperm cells bound to the ZP (ZBA), the percentage of penetrated oocytes (PENR), the rate of pronucleus formation (PNR) the logit of the percentage of penetrated (logit PENR) and logit of the percentage of pronucleus formation (logit PNR)

	ZBA	PENR	PNR	MOT (%)	SP4 (%)	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF	Logit PENR	Logit PNR
ZBA	1.00														
PENR	0.47	1.00													
PNR	0.63*	0.86**	1.00												
MOT (%)	0.47	0.50	0.50	1.00											
SP4 (%)	0.79**	0.60*	0.60*	0.54*	1.00										
VCL	.62*	0.14	0.19	0.15	0.58*	1.00									
VSL	0.49	-0.00	0.06	-0.11	0.45	0.84**	1.00								
VAP	0.50	0.01	0.07	-0.09	0.46	0.85**	1.000**	1.00							
LIN	0.36	-0.13	-0.07	-0.21	0.36	0.64*	0.89**	0.89**	1.00						
STR	0.41	-0.11	-0.01	-0.16	0.32	0.75**	0.94**	0.93**	0.96**	1.00					
WOB	0.36	-0.12	-0.07	-0.18	0.42	0.57*	0.84**	0.84**	0.99**	0.90**	1.00				
ALH	0.49	0.07	0.12	-0.05	0.46	0.95**	0.93**	0.94**	0.75**	0.85**	0.68**	1.00			
BCF	0.38	0.08	0.01	-0.09	0.44	0.89**	0.90**	0.90**	0.80**	0.87**	0.73**	0.94**	1.00		
Logit (PENR)	0.54*	0.98**	0.86**	0.50	0.66**	0.21	0.06	0.07	-0.09	-0.07	-0.09	0.14	0.12	1.00	
Logit (PNR)	0.70**	0.84**	0.97**	0.52*	0.63*	0.24	0.11	0.12	-0.06	-0.00	-0.06	0.17	0.04	0.87**	1.00

VCL: curvilinear velocity (µm/s); VSL: straight-line velocity (µm/s); VAP: average path velocity (µm/s); LIN: percentage of linearity (%); STR: percentage of straightness (%); WOB: wobble coefficient (%); ALH: mean amplitude of lateral head displacement (µm); and BCF: beat cross frequency (Hz). (**) The correlation is significant at 0.01 level. (*) The correlation is significant at 0.05 level.

3.4 Discussion

Fertilization is a complex process requiring several steps that gametes must complete successfully. To precisely predict fertility, it is likely that a number of different tests must be performed to identify those steps that may be defective (Amann, 1989). It has been argued that data from *in vitro* tests could be used to calculate a predictive value that, when compared to real fertility rates obtained after AI, can be considered a good predictor (Rodríguez-Martínez, 2003). With the intention of better predicting the fertility ability of a semen sample, and the importance of sperm subpopulations, a combination of the results of various methods of semen evaluation *in vitro* and *in vivo*, must be performed. In addition, the influence of laboratory semen characteristics on fertility has been discussed and reviewed previously (Aitken, 2006; Bonet et al., 2012; Franken and Oehninger, 2012; Rodríguez-Martínez, 2003; Wood et al., 1986; Xu et al., 1998). However, little information is available on data correlating the sperm subpopulations and the ejaculate quality and fertilizing ability in bulls.

So far, the correlation between sperm motility and *in vivo* fertility has not been completely understood and results vary among authors (r = 0.15-0.83) (Rodríguez-Martínez, 2003). Several studies have found a positive correlation between these two parameters (Farrell et al., 1998; Jouannet et al., 1988; Selvaraju et al., 2008) but there are also studies that shows no correlation among them (Sancho et al., 2006; Yeste et al., 2010). Certainly, the influence of sperm characteristics on the success of *in vivo* fertilization needs to be well studied.

The existence of three or four well-defined sperm subpopulations has been previously described in bulls ejaculates (Muiño et al., 2009, 2008a, 2008b; Rubio-Guillén et al., 2007). In our study, four sperm subpopulations resulted to be distinguishable in the frozen-thawed semen from Holstein bulls. Sperm cells from

subpopulation 4 were the most rapid and progressive ones. Many authors suggest that this high motile and progressive subpopulation is the most suitable for being part of the fertilizing population (Muiño et al., 2008b; Rubio-Guillén et al., 2007). According to previous reports, it is likely that different distribution of subpopulations could be defined among different males (Muiño et al., 2008b). Furthermore, in our study, differences in the rate of spermatozoa from subpopulation 4 between ejaculates of the same male (ranging from 5.94 to 42.55%) were also observed.

In the absence of studies on the fertility prediction by means of the sperm subpopulations rates in the bull, the present study used the sperm-oocyte binding assay and the AI to determine the effect of containing different rates of spermatozoa which moved rapidly and progressively (SP4) on the ability to attach with the ZP and to penetrate the oocyte as well as the ability to outcome in pregnancies and offsprings.

Sperm-binding assay has been well established as an indicator of the fertilizing capacity of spermatozoa (Zhang et al., 1998; Park et al., 2012; Clulow et al., 2010; Taberner et al., 2010; Selvaraju et al., 2008). In the present work, co-incubation of bull spermatozoa from different ejaculates (with different percentages of rapid and progressive spermatozoa) and bovine oocytes resulted in the binding of higher number of sperm cells to the surface of the ZP when the ejaculate presented high rate of spermatozoa from SP4. The logistic regression model found that the rate of sperm from SP4 significantly predicted the number of spermatozoa attached to the ZP in Holstein bulls, and the number of spermatozoa attached to the ZP increased when rate of sperm from SP4 increased.

These results appear to confirm that the most motile and linear spermatozoa, believed to have a great influence on fertility, has a higher number of sperm bound to the ZP. In contrast, no correlation was found when comparing the zona binding assay

with most of the post-thawing sperm motility parameters (all except VCL) evaluated without distinguishing sperm subpopulations. These results are in accordance with Selvaraju and collaborators (2008), where no correlation between motility and *in vitro* fertility was found. Similar results were found by Sancho et al. (2006) and Yeste et al. (2010), when comparing sperm motility and *in vivo* fertility in pigs.

When the CASA parameters were analyzed separately, only the VCL had a significant and positive correlation with the number of sperm cells bound to the ZP. Relationships between motility characteristics in both *in vitro* and *in vivo* fertility studies have been reported by different authors. In donkey (*in vitro*), Taberner et al. (2010) detected a significant positive correlation between certain CASA parameters (VAP, R^2 =0.56; VCL, R^2 =0.61 and mean ALH, R^2 =0.68) and *in vitro* fertilization rates using a heterologous binding assay. Likewise, in bovines (*in vivo*), Farrell et al., (1998) reported a strong correlation between several motility characteristics (BCF, LIN, VAP, VSL and VCL) and fertility (R^2 =0.97).

In the case of field fertility, a positive correlation between the rate of sperm from subpopulation 4 and *in vivo* fertility at first insemination was detected. Thus, those ejaculates with high percentage of spermatozoa from SP4 again presented a greater number of pregnancies than ejaculates with medium and low rates of SP4 spermatozoa.

To our knowledge this is the first study correlating the sperm subpopulations and the *in vitro* and *in vivo* fertility in bulls. These results demonstrate that the sperm subpopulations have a great influence on bull fertility and the analysis of these subpopulations may be used to determine the quality of the ejaculate and to predict the sperm fertilizing ability.

In conclusion, our results suggest that the presence of a high percentage of spermatozoa that move rapidly and progressively (SP4) in a bull ejaculate is positively

and significantly correlated with the *in vitro* and *in vivo* fertility, so that evaluating the percentage of SP4 in a given bull ejaculate may improve the assessment of their quality and fertilizing ability. Despite these results, further research will help to clarify the role of this sperm subpopulation.

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Conclusions

4. Conclusions

4.1 The presence of a high percentage of spermatozoa that move rapidly and progressively (SP4) in a bull ejaculate is positively and significantly correlated with the number of sperm cells bound and penetraded to the ZP and with the rate of pronucleus formation.

4.2 The presence of a high percentage of spermatozoa that move rapidly and progressively (SP4) in a bull ejaculate is positively and significantly correlated with the *in vivo* fertility.

4.3 Evaluating the percentage of SP4 in a given bull ejaculate may improve the assessment of their quality and fertilizing ability.