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XXVII CICLO

SPERM DNA INTEGRITY IN BUFFALO, BULL AND STALLION

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The starry sky above me and the moral law within me.

I. Kant

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GLOSSARY OF ACRONYMS

AA	abnormal acrosome
ABP	androgen binding protein
AH	abnormal head
AI	artificial insemination
ALH	lateral head amplitude (μm)
AM	abnormal midpiece
ANASB	National Breeders Association of Buffalo Species
AO	acridine orange
APA	Provincial Breeders Association
ART	assisted reproductive technologies
ATP	adenosine triphosphate
AV	artificial vagina
BC	bull center
BCF	beat cross frequency (Hz)
BCS	body condition score
BFS	buffered formol saline solution
BM	bent midpiece
BSE	breeding soundness examination
BT	bent tail
CASMA	computer-assisted sperm motion analysis system
COMP-α_t	percent of cells outside main population
CT	coil tail
CTC	Central technical committee
CV	coefficient of variation
CVs	coefficients of variation

DD	distal droplet
DFI	DNA fragmentation index (percentage)
DH	detached head
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOP	Protected Designation of Origin
DPBS	Dulbecco's phosphate buffered saline
dsDNA	double stranded DNA
DSO	daily sperm output
DTT	dithiothreitol
EC	epithelial cells
EDTA	ethylenediaminetetraacetic acid
EED	early embryonic death
EN	Eosin/Nigrosin
EN-VIA	Eosin/Nigrosin-Viability
FITC	Fluorescein Isothiocyanate
FSH	follicle-stimulating hormone
GLM	General Linear Model Procedure
GnRH	Gonadotropin-releasing hormone
ICSI	intracytoplasmic sperm injection
IM	intramuscular
IMB	Italian Mediterranean Buffalo
IVF	in vitro fertilization
LH	Luteinizing hormone
LIN	percentage of sperm that are linear
LMPA	Low Melting Point Agarose
M-α_t	Mean-α_t

MAR	matrix attachment region
Mean int	Mean fluorescent intensity (Comet, Head and Tail)
Mo-α_t	Mode-α_t
NC-VIA	NucleoCounter-viability
NNR	non-return rate
NVAD	NonViable Acrosome Damaged
NVAI	NonViable Acrosome Intact
OBSM	Out of Breeding Season Mating
OTM	Olive tail moment
PC	pregnancy rate per cycle
PD	proximal droplet
PGC	premature germ cells
PGF2α	Prostaglandin F2α
PI	propidium iodide
PMOT	percentage of progressively motile sperm
PMSG	Pregnant Mare Serum Gonadotrophin
PRID	Progesterone Release Intravaginal Device
PSA	Pisum Sativum
PUFA	unsaturated fatty acids
RAP	percentage of sperm with rapid velocity
ROC	Receiver Operating Characteristic Curve
ROS	reactive oxygen species
SBH	Sperm Bos Halomax
SC	scrotal circumference
SCDt	Sperm Chromatin Dispersion Test
SCSA	Sperm Chromatin Structure Assay
SD-α_t	Standard Deviation-α_t

SMI	sperm membrane integrity
ssDNA	single stranded DNA
STR	percentage of sperm that are straight
TM	tail moment
TMOT	percentage of total motile sperm
UO	unilateral orchiectomy
V	Volt
VAD	Viable Acrosome Damaged
VAI	Viable Acrosome Intact
VAP	average path velocity (μm/s)
VCL	mean curvilinear velocity (μm/s)
VSL	straight-line velocity (μm/s)
%H- DNA	%DNA in the Comet head
%T- DNA	%DNA in the Comet tail

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CHAPTER I

1. INTRODUCTION

Male infertility is a common problem in animal husbandry, often overlooked in comparison to the female counterpart. A male may be used to breed up to 50 females by natural service and potentially thousands via artificial insemination (AI). If its breeding potential has not been estimated, it may represent an important economic loss, mainly expressed in bulls as delayed conception and prolonged breeding season (Kastelic and Thundathil, 2008).

In addition, in dairy species, a limited number of males is commonly raised for natural breeding or for being used in AI program, whereas most of them are raised for meat production, as occur in bovine (Bos Taurus), or partially eliminated after birth, as occur in water buffalo (Bubalus bubalis) in Italy, where the buffalo meat market demand is low. Only the males with high genetic value are raised for reproductive purposes. Livestock species are raised primarily for food production (i.e., milk, meat, etc.), however, those characteristics, genetically, may be independent of fertility.

Perhaps, the male factor represents 50% of the pregnancy potential and the spermatozoon delivers the genetic information to the egg resulting in fertilization. The influence of sperm on reproductive outcome has been widely investigated in the last two decades and it has been proved that paternal contribution is not only related to fertilization, but also fundamental to guarantee a normal

embryonic development in humans (Yamauchi et al., 2011; Sakkas and Alvarez, 2010; Ward WS, 2010). In animal husbandry, during the genetic era, the assisted reproductive technologies (ART) have become a necessary tool to ensure and increase income from the resulting offspring. Semen collection, AI, in-vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are routinely performed in livestock species (Ehmcke and Schlatt, 2008) and semen is commonly frozen and shipped, increasing the number of offspring from genetically valuable males. The most important reason for in vitro sperm quality evaluation is to increase the number of breeding doses per ejaculate and to make possible the global use of semen from a sire with high genetic value. Also, it allows the identification of males with reduced fertility as well as those with higher fertility potential (Amann and Hammerstedt, 1993a). Sperm quality is typically evaluated using different measures of sperm features, such as sperm motility, sperm membrane integrity (SMI) or viability, total sperm number and sperm morphology.

DNA quality can also be evaluated by different techniques, among those are the Sperm Chromatin Structure Assay (SCSA), the Comet assay and the Sperm Bos Halomax (SBH). The first step for a correct understanding of the in vitro semen evaluation is the comprehension of the reproductive physiology of the male.

1. 1. Spermatogenesis

In mammals normal spermatogenesis is a temperature dependent process, ensured by maintaining the testes below body temperature (Setchell BP, 1977). The combined action of tunica dartos, scrotal sweat glands (i.e., stallion) and pampiniform plexus is responsible for testicular thermoregulation. The duration of spermatogenesis is approximately 60 days in most domestic animals and epididymal transit takes a further 8–14 days (Noakes et al., 2001; Johnson L. 2011). Sperm from different vertebrates are represented in Figure 1.1.

Sperm are formed in the seminiferous tubules of the testis, which are suspended within the scrotum. The testicular parenchyma is composed of seminiferous tubules (Sertoli cells and layers of spermatogenetic cells) and of interstitial tissue (Leydig cells, blood and lymph vessels and connective tissue). The diploid germ cells, spermatogonia, remain quiescent until puberty, when they are activated and form primary spermatocytes, through several mitotic divisions. The spermatozoa development occurs during spermatogenesis, in which a spermatogonial stem cell through a diploid and haploid phase in the testis becomes a mature, elongated motile cell specialized to deliver the haploid male genome to the oocyte. The spermatocytes form through a meiotic division the haploid cells called spermatids. The corresponding haploid phase of the spermatogenesis is the spermiogenesis. Spermatogonia, primary spermatocytes and spherical spermatids develop in the space

between two or more Sertoli cells and are in contact with them. The spermatids are originally round cells, developed in the basal compartment and then moved by the Sertoli cells into the adluminal compartment as elongated spermatids, which have a compact nucleus and are released into the lumen of the seminiferous tubules.

The mature stage is the spermatozoon that consists of a head and a tail. The head contains a nucleus, which contains nucleoproteins and deoxyribonucleic acid (DNA) forming the tightest compacted chromatin among all the somatic cells, synthetically and transcriptionally inactive. The mammalian sperm nucleus is surrounded by a perinuclear theca except at the base (Oko R, 1995) and the acrosomal regions. The acrosome is a cap-like vesicle lying between the plasma membrane and the perinuclear theca, filled with hydrolytic enzymes that allow the sperm to penetrate the cumulus oophorus and the zona pellucida of the oocyte (Schatten and Constantinescu, 2008).

The post-acrosomal sheath of perinuclear theca is located between the plasma membrane and the nuclear membrane and this portion is responsible for activating the oocyte to initiate the embryonic cell cycle (Perry et al., 1999).

The sperm tail extends behind the head and it is responsible for the movement. It can be divided into 4 different regions: the neck, the midpiece, the principal piece and the end-piece, all surrounded by a common plasma membrane. Sperm motility derives from movement of microtubules doublets displaced in a 9+2 arrangement

within axoneme complex powered by adenosine triphosphate (ATP) synthesis in the mitochondria. The mitochondria form a helical sheath in the midpiece that surrounds the proximal portion of the axonemal complex (Youngquist and Threlfall, 2006). The entire spermatozoon is covered by the plasma membrane, which plays a role in capacitation, acrosome reaction as well as fusion with the oocyte plasma membrane.

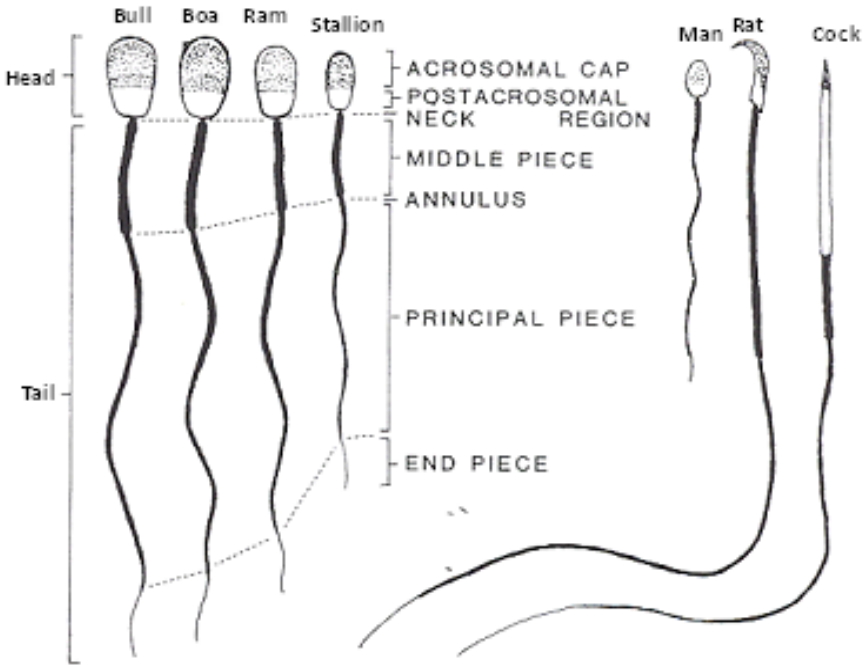
The Sertoli cells provide nutritional, physical and hormonal requirements (Barth and Oko, 1989). The endocrine control of testicular development and spermatogenesis is regulated by the pituitary-gonadal axis through the follicle-stimulating hormone (FSH), the luteinizing hormone (LH) and the androgens (testosterone). The FSH is responsible for the pubertal development of the testis, acting on the Sertoli cells within the testis to produce estradiol. The estradiol is produced by converting the testosterone, secreted by the Leydig cells stimulated by the LH that is responsible for the maintenance of the spermatogenesis in adulthood. The Sertoli cells are also stimulated by FSH to produce inhibin and androgen binding protein (ABP). Inhibin acts with a negative feedback on the pituitary gland, blocking the release of FSH but not LH. ABP forms a complex with androgens and is transported with the sperm to the epididymis. Also it transports testosterone within the Sertoli cells. Before the release of the mature spermatozoa into the lumen of the seminiferous tubules, the excess of cytoplasm of the elongated spermatids is phagocytized by the Sertoli cells.

In conclusion, each spermatocyte forms four haploid spermatids that become elongated and mature spermatozoa (sperm), which are released into the lumen of seminiferous tubules and then transported to the efferent ducts and epididymis.

Once the sperm reach the epididymis they undergo to a maturation process, consisting in the DNA-protein-complex changes, in the acquisition of progressive motility and in the migration of the proximal droplets into the distal portion of the midpiece. Sperm maturation occurs in the caput and corpus of the epididymis, while maintenance and storage of sperm is the primary role of the cauda epididymis (Amann et al., 1993b). Sperm are then transported from the cauda epididymis to the ductus deferens and its terminus, the ampullae, which contributes fructose and citric acid to the seminal plasma and serves as a minor storage area. The accessory glands include the ampulla, seminal vesicles, prostate and bulbourethral glands in both stallion and bull. The secretions of these accessory glands are regulated by androgens and are composed of numerous compounds, including citric acid, fructose and other free sugars, glycoproteins and proteins (albumin, sialomucoprotein, and globulins), glycerylphosphorylcholine, and prostaglandins (Barth and Oko, 1989).

At ejaculation, the semen is transported via the penile urethra and deposited in the female reproductive tract.

Figure 1.1.: Comparisons of the spermatozoa of vertebrates (In: Hafez B and Hafez ESE, 2000).



1. 2. Breeding Soundness Examination (BSE)

The BSE is an overall assessment of the sire's reproductive potential, performed to estimate the male's ability to get a number of females pregnant. In livestock species, as for bulls and rams, it is also important to evaluate the serving capacity that describes the number of services completed in a given time period (Blockey MDB, 1981; Ott and Memon, 1980).

The BSE evaluates the overall physical health of the sire as well as different reproductive characteristics, including the breeding history, the physical examination of the reproductive tract (i.e., scrotal circumference-SC), the libido and the assessment of the semen quality. At the end of the examination the veterinarian must categorize the breeder in one of the 3 categories: satisfactory potential breeder, unsatisfactory potential breeder or classification deferred (Chenoweth et al., 1992).

Among sperm features, motility and morphology have been associated with fertility in man (Lewis SEM, 2007), bull (Blom E, 1977; Barth AD, 2007) and stallion (Jasko et al., 1990; Love et al., 2011; Heckenbichler et al., 2011). Therefore, these measures are used for sperm quality assessment.

According to the Society for Theriogenology, a bull can be categorized as satisfactory potential breeder, when it has ≥70% morphologically normal sperm and ≥30% of progressive motile sperm (Chenoweth et al., 1992). Any bull not having minimum

requirements can be classified as unsatisfactory potential breeder or, whenever temporary conditions occur such as injury or lameness, lameness, sickness or puberty, the classification can be deferred and the bull re-evaluated at a later date.

Testicular size is an important part of the BSE because size is associated with sperm production. In bulls testes size is estimated using a scrotal tape that measures scrotal circumference (SC). Stallion testicles can be measured (length, width, and height) using either calipers or by ultrasonography. SC is highly correlated with testis weight, daily sperm output (DSO) and sperm quality (Coulter et al., 1987; Barth AD, 2007). SC of yearling bulls should exceed 30 cm, while mature bulls should be over 36-38 cm.

1. 3. Semen collection and evaluation

Sperm quality assessment has an important role in breeding activities as well as in assisted reproductive techniques in livestock species. Semen is usually collected using an artificial vagina (AV), which consists of a rubber cylinder containing a latex liner ending with a cone attached to a collection bottle. The space between the rubber and the latex liner is filled with warm water (45-50° C). Once the AV is filled, a small amount of non-spermicidal lubricant is applied and semen is collected. In bulls, particularly not used to being handled, electroejaculation can be an alternative method to collect semen and it is commonly used in USA. In this species, semen can also be collected by per rectum massage of the internal genitalia. This technique involves the location of the vesicular glands per rectum and massaging them against the pubis, which causes accessory fluid to drip from the prepuce. The ampullae are then located and 'milked' between the finger and thumb. Success is indicated by the dripping of semen from the prepuce (Noakes et al., 2001). Electroejaculation and massage are useful for the evaluation of sperm quality; however, they do not represent a physiologic ejaculate which contains higher sperm numbers.

Once semen is collected sperm quality is evaluated, typically using combined measures of different sperm features, such as sperm motility, sperm membrane integrity (SMI) or viability, total sperm number and sperm morphology. After semen collection a general

evaluation of the color and appearance of raw semen is performed. Determining gel-free volume and concentration is fundamental to calculate the total sperm number. Gel-free volume is measured with a graduated cylinder or by weight. Sperm concentration can be evaluated using a hemocytometer, spectrophotometric based counting instruments or an automated cell counter (i.e., NucleoCounterSP-100™ Chemometec, A/S, Allerød, Denmark).

The sperm concentration (as million/ml) is multiplied by the gel-free volume (mL) to give the total sperm number (as billion) in the ejaculate. Semen characteristics are reported in Table 1.1.

Table 1.1.: Semen characteristics of bull (Bos Taurus), buffalo (Bubalus bubalis), stallion and boar.

Species	Volume (mL)	Concentration (10^6/mL)	Fractionated
Bull	4 (2-10)	1250 (600-2800)	N
Buffalo	4 (1-6)	950 (600-3000)	N
Stallion	60 (30-250)	120 (30-600)	Y
Boar	250 (125-500)	100 (25-1000)	Y

Sperm motility can be assessed visually using a light or a phase-contrast microscope (at 200x or 400x) or with a computer assisted sperm motion analysis system (CASMA).

Sperm morphology is typically examined by bright-field microscopy (1000x oil immersion) after staining with eosin-nigrosin, India ink, Trypan Blue/Giemsa or by differential-interference contrast microscopy on a wet mount of fixed cells (i.e., diluted with buffered formol saline solution).

Sperm morphology in bulls can be classified as normal, primary and secondary abnormalities (Chenoweth et al., 1992), according to Blom (1950); Barth and Oko (1989). Primary abnormalities arise during spermatogenesis (i.e., nuclear vacuoles), while secondary defects are formed within the epididymis (i.e. proximal droplets), and tertiary defects, which arise after ejaculation and are usually considered iatrogenic (e.g. from inadequate temperature, pH or

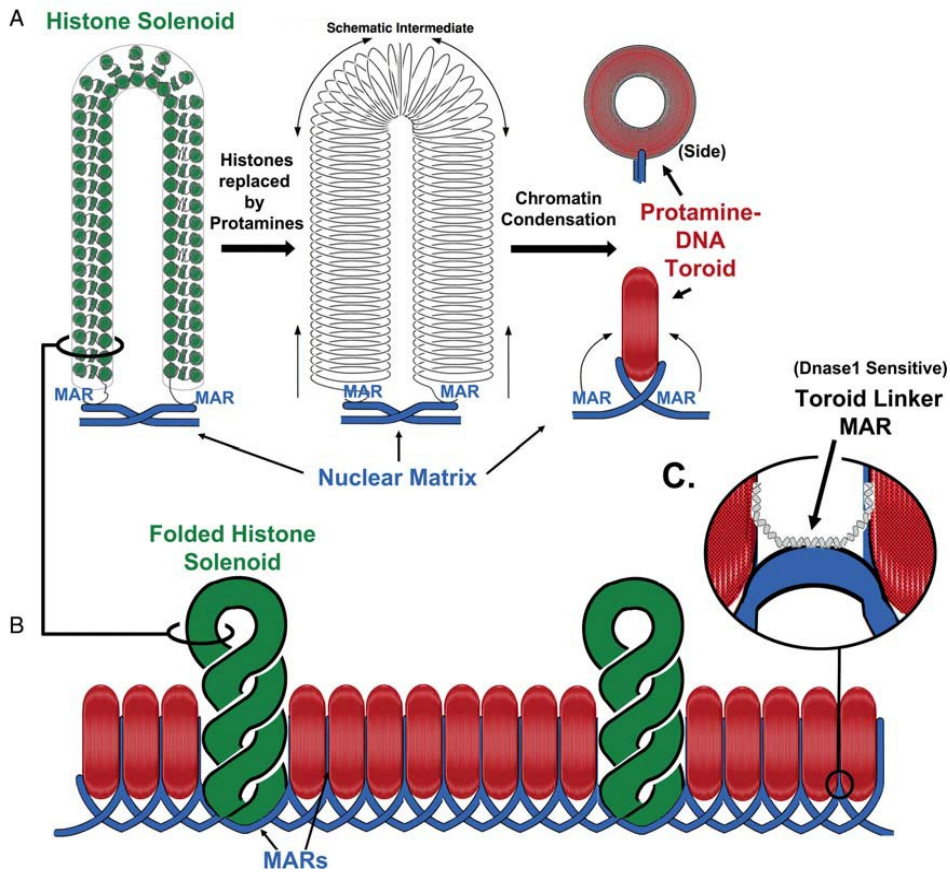
osmotic control during handling of the semen). Another system classifies sperm defects in Major and Minor (Blom E, 1977). Major defects are those that can negatively affect fertility, such as abnormal heads and midpieces. Minor defects have no impact on fertility, as distal droplets. Another system (i.e., stallion classification system) identifies the individual abnormalities and more commonly used in stallions (Kenney et al., 1983). In this system, all abnormalities identified on a sperm are recorded and a total of 100-200 sperm are counted. This system determines the percent of normal sperm, but also the percent of each abnormality. The following sperm morphologic features are identified: normal, abnormal heads (mis-shaped heads, micro- and macrocephalic sperm, and large nuclear vacuoles), abnormal acrosomes, detached heads, proximal and distal cytoplasmic droplets, swollen or irregular midpieces, bent or coiled midpieces/tails, and premature germ cells (Kenney et al., 1983).

CHAPTER 2

2. Sperm Chromatin Structure

Sperm chromatin (DNA + associated nucleoproteins) is organized in an extremely compact structure, forming a Donut-Loop model (Ward and Coffey, 1991; Figure 2.2.), which is different from the less compact structure of somatic cells. The nucleoproteins (i.e., histones and protamines) envelop and fold the DNA, which remains in a transcriptionally inert form until the paternal genome is delivered into the oocyte. During spermiogenesis the sperm DNA undergoes condensation in which histones are replaced by protamines. This causes the major part of the DNA to be coiled into stable structures called toroids, linked side by side and connected to a matrix attachment region (MAR) by toroid linker regions (Ward WS, 2010). Some of the histones, however, are not replaced by protamines and remain bound to the chromatin, representing, together with toroid linker regions, portions of the sperm chromatin more susceptible to be “damaged” (Shaman and Ward, 2006). This condensation process serves to protect the sperm DNA and maintain it in a transcriptionally inert form during epididymal maturation and transport through the female tract (Perreault SD, 1992).

Figure 2.2.: Sperm chromatin organization (Ward WS, 2010).



2. 1. Origin of DNA damage

A variety of factors have been associated with sperm DNA damage in humans, such as age, lifestyle (i.e., cigarette smoking, diabetes), drugs (i.e., antibiotics), chemotherapy and radiotherapy (O'Brien and Zini, 2005; Aitken et al., 2013). Further, many toxicological studies have demonstrated that several chemicals, such

as pesticides, phthalates, polychlorinated biphenyls, heavy metals (e.g., cadmium) and high levels of air pollution can decrease DNA quality (Barratt et al., 2010). Increased testicular temperature following scrotal insulation, or elevated ambient temperature, may alter spermatogenesis, resulting in a decrease of sperm quality in humans (Mieussett et al., 1987), bulls (Brito et al., 2003), stallions (Freidman et al., 1991) and boars (Malmgren and Larsson, 1984). In addition, post-testicular inflammation (e.g., prostatitis), male genital tract infections associated with leukocytospermia (resulting in increased numbers of immature germ cells in semen). Clinical conditions, such as spermatic cord torsion and varicocele of the testicular vein have all been associated with an increase in sperm DNA "damage" (O'Brien and Zini, 2005; Aitken and De Iuliis, 2007; Aitken et al., 2013; Barratt et al., 2010). In addition, defects in chromatin compaction have been reported in humans (Aitken et al., 2013); a lack of chromatin protamination in bulls (Rahman et al., 2011), as well as a reduction in disulphide bonds and increased susceptibility to DNA denaturation in stallions (Love and Kenney, 1999). Sperm are particularly susceptible to lipid peroxidation because of their high content of unsaturated fatty acids (PUFA). All these factors act on sperm inducing the activation of lipid peroxidation cascade that produces aldehydes, which bind to the proteins in the mitochondrial electron transport chain, stimulating the generation of reactive oxygen species (ROS). Once ROS are produced, a cascade cycle is activated, which induces oxidative

damage to the sperm mitochondria, programmed cell death (apoptosis) and single strand breaks in the DNA (Aitken et al., 2014). High levels of sperm DNA damage have been shown to interfere with embryonic development (Sakkas et al., 1998; Evenson et al., 1999; Spanò et al., 2000, Zini et al., 2001, Saleh et al., 2003; Bungum et al., 2004; Virro et al., 2004; Zini 2005), inducing miscarriage (Morris et al., 2002; Ribas-Maynou et al., 2012b) and diseases in the offspring (Cooke et al., 2003; Lewis and Aitken, 2005; Aitken et al., 2009).

However, the oocyte and the early embryo are able to repair low levels of DNA damage, but if this damage is extensive embryonic development may be compromised (Ahmadi and Ng, 1999).

Many techniques have been developed to examine sperm DNA integrity, such as the Sperm Chromatin Structure Assay (SCSA), the single cell gel electrophoresis method (Comet assay) and the Sperm Bos Halomax (SBH) assay. These tests reportedly evaluate different aspects of the sperm DNA structure. The SCSA identifies the ratio of ssDNA (abnormal) to dsDNA (native) in the exposed toroid linker regions, but not in the more compact toroids (Shaman and Ward, 2006). This assay has been widely used to evaluate sperm DNA quality in men (Evenson et al., 1980), bulls (Ballachey et al., 1987; Ballachey et al., 1988; Januskauskas et al., 2001; Januskauskas et al., 2003; Waterhouse et al., 2006; Fortes et al., 2012; D'Occhio et al., 2013), buffalo bulls (Kadirvel et al., 2009), stallions (Love and Kenney, 1998) and boars (Evenson et al., 1994). In contrast, the

Comet assays (i.e., neutral and alkaline) evaluate both the toroid and toroid linker regions (Shaman et al., 2007) for identification of ssDNA and dsDNA breaks. In the Comet assays, sperm DNA breaks migrate away from the head region to form "comets" following electrophoresis, whereas intact DNA remains in the original head position (Shaman and Ward, 2006). The SBH assay has been developed for assessment of sperm DNA integrity in bulls, based on the Sperm Chromatin Dispersion Test (SCDt) for humans (Fernandez et al., 2003). The SBH assay is similar to the Comet assays with the exception that treated sperm are not exposed to an electrophoretic field. Higher DNA fragmentation produces larger halos, whereas lower DNA fragmentation yields smaller halos (García-Macías et al., 2007).

2. 2. Aims of the thesis

In animal husbandry, males are selected based on their genetic or athletic performances and not on their reproductive capabilities. Advances in sperm quality evaluation allow for a more critical identification of subfertile and infertile individuals, which is beneficial for farmers, practitioners and producers, resulting in economic savings by reducing the number of non-pregnant females in the herd, due to poor sperm quality. In addition, animal models of reduced fertility are essential to provide to understand the basic mechanisms that could advance the knowledge of human reproduction subfertility and infertility. The evaluation of sperm quality is based on different methodologies (light or fluorescent microscopy, automated systems for sperm motility, flow cytometry) which analyze different sperm compartments. The main aim of the sperm quality evaluation is the estimation of fertility. Recently, there has been increased interest in the evaluation of sperm DNA integrity and its relationship to subfertility and infertility. This interest has resulted in the development of several sperm DNA assays. Some of these assays, such as the Sperm Chromatin Structure Assay (SCSA), have been associated with fertility in many species, while it is unclear how newer assays, such as the SBH and Comet assays, relate to fertility. The overall objective of the thesis was to compare several sperm DNA assays in buffaloes, bulls and stallions. Comparisons were performed using different environmental and laboratory conditions to simulate different levels of DNA damage

and thus, hopefully, determine the capabilities of each assay to measure DNA damage. The SCSA was used as reference assay in this study, to which other sperm DNA assays were compared. The main aim of this study was to validate the Comet assay in buffalo, bulls and stallions. In addition, this study compared the different sperm DNA assays (SCSA, Comet and SBH) and evaluated a relationship of those assays with traditional sperm features of sperm motility and morphology in buffaloes, bulls and stallions.

The experiment 1, 2, 3 and 4 cover the above mentioned aims as follows:

Experiment 1

In the experiment 1 sperm DNA integrity was assessed in frozen-thawed semen of buffaloes under progeny test, used in AI program. Fertility data were recorded to evaluate the relationship between sperm features and in vivo fertility.

The aims of this study were to:

1 evaluate traditional sperm features (motility, morphology, acrosomal and membrane integrity)

2 evaluate sperm DNA integrity by neutral Comet assay, the SCSA and the SBH;

3 identify the relationship among traditional sperm features and sperm DNA integrity;

4 describe fertility results of Italian Mediterranean Buffalo

(IMB) bulls under progeny test, expressed as pregnancy rate per cycle and calving rate;

5 identify the relationship among sperm features in vitro and fertility in vivo.

Experiment 2

In the experiment 2 sperm DNA integrity was assessed in two groups of bulls (high and low sperm quality), selected based on their sperm motility and morphology for further analysis to compare SCSA, neutral and alkaline Comet and SBH.

The aims of the study were to:

1. Objectively measure and describe comet images using a software program and determine assay repeatability (intra- and inter-assay);
2. Evaluate and compare sperm DNA integrity using the SCSA, the neutral and the alkaline Comet assays, and the SBH;
3. Compare the sperm DNA integrity tests to traditional measures of sperm quality such as sperm motility and morphology.

Experiment 3

In the experiment 3 stallion sperm DNA integrity was assessed following the induction of a mild stress to the scrotum (i.e.,

unilateral orchiectomy). DNA quality was evaluated using two assays (Comet assay and SCSA).

CHAPTER 3

EXPERIMENT 1

Progeny Test report in Italian Mediterranean Buffalo bulls: evaluation of sperm quality and its relationship to fertility in vivo

Abstract

The relationship between sperm DNA integrity and fertility in vivo has not been reported in Italian Mediterranean Buffalo (IMB) bulls under progeny test. Straws of frozen-thawed semen were evaluated for traditional sperm features (i.e., motility, viability, acrosome integrity and morphology) and for sperm DNA integrity, assessed by the neutral Comet assay, Sperm Bos Halomax (SBH) and Sperm Chromatin Structure Assay (SCSA). Sperm DNA assays were not correlated to each other ($P>0.05$). The neutral Comet assay was correlated to sperm motility and viability and to coiled tails and to the presence of epithelial cells, among morphologic abnormalities. The SCSA measures were correlated to sperm viability and to bent midpieces and distal droplets. SBH was only correlated to Non-Viable Acrosome Damaged-NVAD ($r= 0.60$; $P<0.05$) and to Viable Acrosome Damaged ($r= -0.63$; $P<0.05$). The overall pregnancy rate per cycle (at 30 and 45 days) and the calving rate on 528 buffalo cows inseminated with 3 IMB bulls were 57%, 55% and 45%, respectively. Among sperm features analyzed Receiver Operating Characteristic (ROC) Curve evidenced significant values ($P<0.05$) for sperm motility, distal droplets, Non-Viable Acrosome Damaged,

Standard Deviation-α_t (SD-α_t) and neutral comet measures of Olive Tail moment and tail moment, %DNA in head (and tail) and tail area.

1. Introduction

The Italian Mediterranean Buffalo (IMB) is an indigenous species in South Italy, dating to the XII and XIII centuries. It is a national breed, derived from the river type of water buffalo (Bubalus bubalis) and its milk is used to make Mozzarella cheese. Mozza (original name of the dough to make Mozzarella cheese) is documented to be offered to the Saints in 1294. During that period, the South Italy was a flat, swampy area with poor vegetation and the buffalo farming was the only form of livestock activity possible. The water buffalo was well adapted to this environment, compared to the cow (Bos bovis), (Zicarelli L, 2004). The IMB population has increased significantly, from roughly 12,500 head immediately after the II world war to 377,392 head at now (National Data Bank-BDN, 2014). The reason for this remarkable increase is related to the main source of income of this species, represented by the milk to produce the "Mozzarella di Bufala Campana", a fresh cheese with spun dough, which became DOP (Protected Designation of Origin) in 1996, meaning that three Italian Regions (Campania, Lazio and Puglia) produce a certified product. Currently, the IMB is

recognized as national breed, with a Studbook since 1979, held by the National Breeders Association of the Buffalo (ANASB).

IMB are short-day polyestrous breeders, with the reproductive activity influenced by the melatonin's secretion (Parmeggiani et al., 1992; Parmeggiani et al., 1994; Di Palo et al., 1997). Calving occurs from July to December. Peak milk production occurs in the fall and winter (Barile et al., 2005); however, the demand for Mozzarella cheese is during the spring and the summer. Therefore out-of-breeding-season-mating (OBSM) technique has been developed to accommodate the peak demand for Mozzarella cheese (Zicarelli L, 1997). To meet the market requirements, buffalo bulls are exposed to the cows from March to the end of September and are removed during the fall and winter. This results in the calving season moved from the end of January to the end of August, thus satisfying the demand for Mozzarella cheese.

The calving period, following the OBSM technique, is in contrast to the natural breeding season, thus, the reproductive efficiency in IMB is low (Presicce et al., 2007). For this reason, the OBSM technique is gradually applied into the herd to reduce economic loss due to the decrease in calving rate. However, the implementation of the OBSM technique over the last decades has selected animals less sensitive to seasonality, as the opened cows, more sensitive to seasonality, were culled. The National Breeders Association of Buffalo Species (ANASB) implemented the genetic selection of the IMB, based on the milk production, since 2000. The

aims of the ANASB are to: implement the Studbook and guarantee the genetic improvement of the breed.

The genetic selection of the breed is based on the increase of the milk production and of its content in protein and fat. The main goal is to maximize the Mozzarella cheese production by the PKM index, which expresses the amount in Kg of mozzarella cheese obtained from milk produced during a standard lactation of 270 days. The index is based on an algorithm found by Altiero et al. (1986) and takes into account the percentage of milk protein and fat, experimentally estimated as follows:

PKM (kg)=(milk, kg)*[3.5*(protein, %) + 1.23*(fat, %)- 0.88] /100

The use of this index allows obtaining information on both milk quantity and quality and increasing milk production without modifying its protein and fat contents, on average 4.67 and 8.16% respectively (ANASB data, 2013).

The national average milk yield is 2222 kg/lactation with some farms producing more than 3000 kg/lactation and high yielding buffaloes producing about 5030 kg/lactation (ANASB data, 2014). These productive levels have been reached through management criteria and through a genetic selection, essential to enhance specific features needed for the development of the breed. The number of IMB registered to the Studbook is currently 94,769 head, which represent the 25% of the whole population. Of those, 55,000 heads are lactating buffaloes, monitored monthly by Provincial Breeders Associations (APA) for their milk quantity and quality (ANASB

data, 2014).

In Italy, buffalo registered in the Studbook undergo a linear type trait evaluation, performed by experts of the breed. The final score is obtained as weighted average of four partial scores: 1. structure, with an influence of 15% on the final score; 2. limbs and foot, with an influence of 25%; 3. mammary apparatus, with an influence of 40%; 4. productive potential, with an influence of 30%. The first three scores are similar to those calculated for the Holstein-Friesian breed, whereas the last score is obtained by merging lactiferous traits and body's capability (Caso et al., 2010).

All the bulls used at the farms for natural breeding must pass the linear type trait evaluation with a score of at least "Good" and in addition, from 2001, their ascendancy have to be confirmed through DNA testing. Further, the ANASB, since 2001, to encourage the practice of the artificial insemination, organized progeny test cycles by choosing the bulls among the best male offspring of the "Mothers of Bulls". According to the Regulations of the Association the title of "Mother of Bull" is attributed to those cows that have the following requirements:

1) morphology of at least 80 points in the linear type trait evaluation score;

2) at least one lactation officially controlled, with a length of at least 270 days and a minimum production of 31 quintals of milk, at least a content of 7.7% in fat and 4.5% in protein;

3) pedigree of a generation of maternal and paternal ascendants registered in the Studbook and at least grandmothers;
4) to be at least in the RANK 90 with at least 0.70 accuracy of PKM index.

Cows are ranked on their PKM index and the RANK 90 represents the "best" 10% of the population. Accuracy is the proximity of measurement results to the true value, influenced by pedigree and parity. These initiatives were successful and allowed the association to organize progeny tests every year. In fact, in November the ANASB sends to all the Provincial Breeders Associations (APA) and Bull Centers (BC) lists of the "Mothers of the bulls" to permit the identification of the best bulls to be proposed for the progeny test.

Within the first week of March of the following year the applications of individuals identified must be received from the ANASB. Such nominations may come from APA, BC or directly by the farmers who want to propose their own bulls.
At the end of March, the Central Technical Committee (CTC) identifies those bulls selected as well as reserves for the progeny test, which will begin in December. So far, 84 bulls have been included in the progeny tests, of those 27 are still under evaluation, and 25 have offspring with improved milk production and quality.

The buffalo bulls chosen for the progeny test are sent to the BC for sperm collection, processing and freezing. The number of IMB bulls chosen every year for the progeny test is limited (5-6), as most

of the farmers still rely on natural breeding. This number may be reduced if a “genetically selected” bull reveals inadequate libido, poor fresh or frozen-thawed semen quality. Criteria for selecting AI sires are scrotal circumference, sperm motility, viability and morphology (Pant et al., 2003; Sansone et al., 2000; Brakat et al., 2009; Koonjaenak et al., 2007). However, there are no standardized minimum requirements of sperm features in IMB bulls. At the BC, bulls must be negative for infective diseases and have a normal karyotype, before being used for sperm production.

Semen is usually collected with a Danish model AV (Andrabi SMH, 2014) and each collection consists of two ejaculates obtained at 30 min intervals. Assessment of semen quality is based on the evaluation of its color, volume, sperm concentration, motility, acrosomal and membrane integrity (viability), and morphology (Sansone et al., 2000; Vale WG, 1994). Raw semen with more than 30% “dead” sperm may not be suitable for storage and freezing (Sansone et al., 2000).

AI programs are restricted by other species-specific female factors, such as lack of marked estrous signs, variable estrous length and low prediction of the ovulation time (Neglia et al., 2003). Synchronization of the estrous and the induction of ovulation ensure a pregnancy rate of around 40% (Baruselli et al., 2003; Neglia et al., 2003). Currently, natural breeding is most common form of producing calves, with AI applied to the 6% of the IMB population

registered to the Studbook (ANASB, 2014). However, AI programs are also performed by farms not registered to the Studbook.

The IMB is also affected by a high incidence of late embryonic mortality (45%), greater during the season with an increase in daylight (Campanile et al., 2005; Campanile at al., 2013). Late embryonic mortality has been attributed to a low progesterone level in the IMB cows during the seasonal increase in day length (Vecchio et al., 2012), which corresponds to a decline in the reproductive function (Campanile et al., 2005). However, the male may represent another cause of the embryonic mortality. In fact, sperm DNA transfers half of the genetic information to the offspring and whether it is "damaged" the embryonic and fetal development may be highly compromised, as reported in humans (Sakkas et al., 1998; Evenson et al., 1999; Spanò et al., 2000, Zini et al., 2001, Saleh et al., 2003; Bungum et al., 2004; Virro et al., 2004; Morris et al., 2002; Ribas-Maynou et al., 2012b; Cooke et al., 2003; Lewis and Aitken, 2005; Aitken et al., 2009).

Reliable estimation of fertility is still vague and the best approach include multiple tests to evaluate several sperm characters, such as sperm motility, morphology, viability, mitochondrial function, acrosome and DNA integrity. Different methods are available to estimate those different sperm compartments, based on light or fluorescent microscopy, automated systems (i.e., computer-assisted sperm motion analysis-CASMA) or flow cytometry. The use of flow cytometry in sperm evaluation warrants assessment of

different sperm compartments simultaneously, counting thousands of cells in few minutes. Studies on sperm DNA integrity on buffalo sperm are still limited. Sperm DNA integrity can be determined by flow cytometry (i.e., Sperm Chromatin Structure Assay- SCSA) or by fluorescent microscopy (i.e., Comet assay and Sperm Bos Halomax). In addition, its relationship with traditional sperm features (i.e., sperm motility, acrosomal status, membrane integrity and morphology) has not been investigated in the buffalo species.

The SCSA has been widely applied to humans (Evenson et al., 1980), bulls (Ballachey et al., 1987; Ballachey et al., 1988; Januskauskas et al., 2001; Januskauskas et al., 2003; Waterhouse et al., 2006; Fortes et al., 2012; D'Occhio et al., 2013), stallions (Love and Kenney, 1998) and boars (Evenson et al., 1994). In buffaloes, sperm DNA integrity decreased following storage at 4°C, as measured by the SCSA, whereas there was no effect of freezing-thawing, as compared to the fresh (Kadirvel et al., 2009). The Comet assay is able to identify strand breaks in the whole sperm chromatin structure in comparison to the SCSA, which measures the DNA "damage" only in the toroid linker region (Shaman and Ward, 2006). Higher DNA damage has been reported in cryopreserved buffalo sperm compared to fresh, as detected by the Comet assay (Kumar et al., 2011). Further, the SBH has been recently developed to assess sperm DNA integrity in bulls and applied to the buffalo (Pawar and Kaul, 2011). It is based on the Sperm Chromatin Dispersion Test (SCDt) for humans (Fernandez et al., 2003), with larger halos

expression of fragmented DNA and smaller halos of lower DNA fragmentation (García-Macías et al., 2007).

The evaluation of sperm DNA integrity by different methods in frozen-thawed IMB semen, used in AI program, has not been reported. The aims of this study were to:

1) evaluate traditional sperm features (motility, morphology, acrosomal and membrane integrity or viability);

2) evaluate sperm DNA integrity by neutral Comet assay, the SCSA and the SBH;

3) identify the relationship among traditional sperm features and sperm DNA integrity;

4) describe fertility results of IMB bulls under progeny test, expressed as pregnancy rate per cycle and calving rate;

5) identify the relationship among sperm features in vitro and fertility in vivo.

2. Materials and Methods

2. 1. a Sampling

This study used straws of frozen semen of three IMB bulls (aged 2-3 years) under progeny test provided by the Centro Tori Chiacchierini (Perugia, Italy), where semen was regularly collected, processed and analyzed. According to the standard quality procedure of the BC, one straw (0.5 mL) from three semen batches for each bull was thawed in a water bath at 37° C for 30' to assess sperm concentration and membrane integrity by an automated cell counter (NucleoCounterSP-100™ Chemometec, A/S, Allerød, Denmark). Total sperm motility (TMOT) was evaluated by a light microscope, equipped with a warm stage set at 37°C. The mean of sperm quality features assessed is reported in Table 1. 3.

Table 1.3.: The mean ± SD, range () of sperm concentration, motility and viability assessed at the bull center

Bull	Concentration (x 10^6)	TMOT (%)	NC-VIA (%)
1	53 ± 10 (44-64)	40 ± 10 (30-50)	33 ± 12 (19-41)
2	95 ± 22 (80-136)	40 ± 4 (35-45)	56 ± 13 (45-68)
3	78 ± 18 (58-89)	47 ± 6 (40-50)	68 ± 4 (63-71)
Total	75 ± 21 (53-95)	42 ± 4 (40-47)	52 ± 18 (33-68)

Measures are abbreviated as follows: TMOT-total motility (visually assessed), NC-VIA-viability (NucleoCounter).

Straws of the same three batches for each IMB bull were transferred and stocked at the Laboratory of Biotechnology applied to Animal Production (Department of Veterinary Medicine and Animal Production, Naples, Italy) to be used for further analyses, which were performed on two straws of each of the three batches of each bull.

Analyses included TMOT, sperm viability by Eosin/Nigrosin (EN-VIA, light microscopy) and by PSA/PI (flow cytometry), sperm morphology, neutral Comet assay, SBH, SCSA. The SCSA and PSA/PI were performed at the Istituto Nazionale di Fecondazione Artificiale, Cadriano (Bo, Italy).

2. 1. b Laboratory processing

Semen was thawed in a water bath at 37° C for 30' and TMOT was visually evaluated by a phase-contrast microscope equipped with a warm stage set at 37°C.

The microscopic evaluation evidenced particulate matter, related to the egg yolk in which the semen was diluted for freezing. To avoid interference of those particles with methods based on microscopic evaluation (i.e., Comet assay), semen was centrifuged with 2 mL of Dulbecco's phosphate buffered saline (DPBS) at 400 g for 10 min. Following centrifugation, the supernatant was discarded and the sperm pellet concentration was determined by a hemocytometer. A drop of the sperm pellet was stained with eosin/nigrosin (EN) to

evaluate sperm membrane integrity (viability) by light microscopy (magnification 1000x). Two slides were prepared for each sample and 100 sperm for slides were examined.
An aliquot was diluted to a concentration of 1 million/mL in DPBS for further analysis with the Comet Assay.

2. 1. c Sperm morphology

Semen prior and post sperm centrifugation was diluted with buffered formol saline solution in a1.5 mL Eppendorf tube and evaluated by differential-interference contrast microscopy (Nikon Diaphot 300, Nikon Inc., Melville, NY, USA; 1000x magnification). A total of 100 sperm per sample were evaluated and all abnormalities identified on a sperm were recorded, as reported by Kenney et al. (1983). The following sperm morphologic features were identified: normal, abnormal heads (mis-shaped heads, micro- and macrocephalic sperm, and large nuclear vacuoles), abnormal acrosomes, detached heads, proximal cytoplasmic droplets, distal cytoplasmic droplets, swollen or irregular midpieces, bent or coiled midpieces/tails, and premature germ cells.

2. 1. d Sperm acrosome intactness and viability (Pisum sativum PSA/propidium iodide-PI)

Samples were diluted to a final concentration of 0.05 mg/mL fluorescein isothiocyanate (FITC)-conjugated agglutinin derived

from *Pisum sativum* (FITC-PSA) staining (Sigma-Aldrich, Milan, Italy) and 12 μM PI in DPBS, incubated for 10 min at room temperature and immediately evaluated by flow cytometry (BD FACSCalibur Flow Cytometry System, BD biosciences, CA, USA), as described by Love et al. (2012). A total of 5000 cells were analyzed and sperm were classified as non-viable acrosome intact (NVAI), non-viable acrosome damaged (NVAD), viable acrosome intact (VAI) and viable acrosome damaged (VAD).

2. 2. Neutral Comet assay

The protocol for the neutral Comet assay was modified from a previous study (Tice et al., 2000). Low Melting Point Agarose (LMPA; Trevigen Inc. Gaithersburg, MD) was melted in a microwave for 10 s and then transferred to a 15 mL plastic tube and floated in a water bath at 37°C to prevent gel solidification. After 5 min the temperature of the gel was checked to be sure that it did not exceed 37°C. From the aliquots containing 1 million/mL sperm in DPBS 25 μL were dispensed into 250 μL of LMPA in an 1.5 mL Eppendorf tube, vortexed for 5 s then floated in the water bath. Seventy-five microliters of sperm/LMPA mixture was quickly pipetted onto a horizontal Comet microscope slide (CometSlide™ 2 well/slide; Trevigen Inc. Gaithersburg, MD). For each semen sample 2 slides (2 wells/slide) were prepared and 50 sperm/slide were counted (25 sperm/well). In addition, a control bull semen sample

was included in each trial to monitor day-to-day repeatability. The slides were incubated at 4°C for a minimum of 5 min, before being placed horizontally in a rack (CometSlide™ Rack System Trevigen Inc. Gaithersburg, MD) and immersed in a cold (5-8°C) lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris HCl, 1% Triton X-100, 20 mM dithiothreitol, pH 10) for 30 min in the refrigerator, followed by immersion and incubation for 1h and 15 min at 37°C in the same lysis solution with the addition of 0.1 mg/mL Proteinase K (Proteinase K from Tritirachium album, Promega, Madison, WI; USA). All solutions were made fresh and stored either in the refrigerator or incubator before adding to the comet slides. Following incubation in the second lysis solution, slides were washed 3 times with distilled water at 5 min intervals and then placed in an electrophoretic horizontal unit (Single Cell Gel Electrophoresis system, Scie-Plas Ltd, UK).

Slides were kept for 30 min in a chilled electrophoresis solution (500 mM NaCl, 0.1 M Tris-Base, 1 mM EDTA, 0.2% DMSO, q.s to 800 mL deionized water, pH 9). Electrophoresis was performed at 0.7 V/cm for 30 min at room temperature in the dark. Slides were rinsed drop-wise 3 times at 5 min intervals with a neutralization buffer (0.4 M Tris Base, pH 7.5), then dehydrated with cold 70% ethanol and left overnight, in the dark, at room temperature.

Samples were evaluated by fluorescent microscopy (Leica microscope model DMRA2, equipped with an objective HC Plan APO, magnification 20 x/ 0, 70, ∞/ 0.17/c). Slides were stained with

40 µl of diluted propidium iodide (PI, final concentration 0.38 µM) from a stock concentration of 9.6 µM PI in 1 ml Tris-EDTA buffer (10 mM Tris HCl and 1mM EDTA) and incubated for 10 min in the dark. Images acquisition was performed using a software program (Leica QFluoro, Wetzlar, Germany). The exposure time (1 s) and image size (1360 x 1024 pixels) were preset. Microscope images were acquired and stored in bitmap format prior to evaluation with software (CometScore Version 1.5 TriTek Corp, VA). The software was pre-calibrated in microns by a picture of a slide glass micrometer (acquired with the same settings described for the Comet images above), regulated with the bar provided by the software in the calibration settings. Comet measurements included the following parameters, grouped as dimension measures (i.e., comet length, tail length, comet height, comet area, head diameter, head area, tail area), intensity measures (comet intensity, comet mean intensity, head intensity, head mean intensity, tail intensity, tail mean intensity), %DNA in head-%H-DNA and tail-%T-DNA, tail moment-TM and Olive tail moment-OTM (see Appendix A).

2. 3. Sperm Bos Halomax (SBH)

The SBH (Sperm Bos Halomax, Bos Taurus, Halotech DNA, SL, Madrid, Spain) was performed as previously described (Halotech DNA, SL, Madrid, Spain). Briefly, sperm samples were thawed in a water bath at 37°C and diluted to 20 mil/mL in DPBS

and 25 μL of diluted semen was transferred to an empty Eppendorf tube in a water bath at 37°C and 50 μL of liquefied agarose was added into the tubes and mixed. A drop of 2 μL of the mixture was spread onto the well of the SBH slide and every 4 wells were covered with a 22 x 22 mm glass coverslip. Each sperm sample was run in duplicate on different slides. The slide was maintained horizontally at 4°C in the refrigerator for 5 min, the coverslip was removed and the lysis solution was applied for 5 min. The slide was then washed for 5 min in distilled water, dehydrated in sequential ethanol solutions 2 min each (70% and 100%) and left to dry overnight at room temperature. The slide was then stained with propidium iodide (final concentration 0.03 μM) from a stock solution that was diluted (1:40x; stock solution 1.2 μM) in deionized water and then 1:1 mixed with antifade mounting medium (Vectashield, Vector Laboratories, Burlingame, CA, USA). A final volume of 2 μL was used to stain each well and 300 sperm per sample were counted with an Olympus microscope model BX60, equipped with an objective U Plan FL N, magnification 40 x/ 0, 0.75 Ph2, ∞/ 0.17. Sperm were categorized as those with presence or absence of a halo and expressed as percentage on 300 sperm (DFI).

2. 4. Sperm Chromatin structure assay (SCSA)

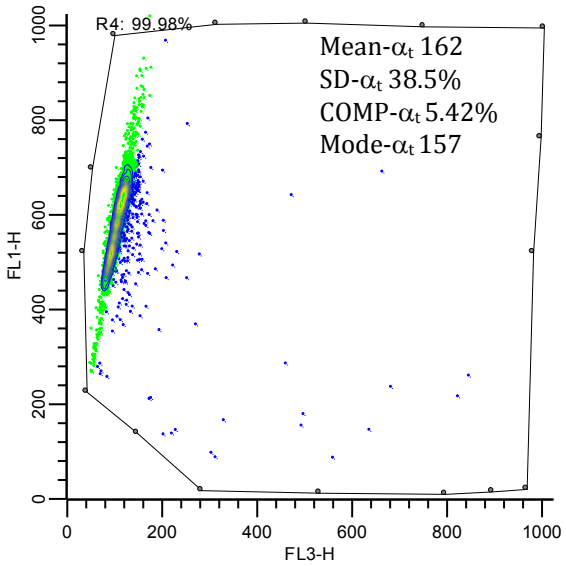
The SCSA was performed as previously described by Love and Kenney (1998). Briefly, frozen semen samples were thawed in a

37°C water bath and 2-7 μL aliquot of semen was diluted to 200 μL in a buffer solution (0.186 g disodium EDTA, 0.790 g Tris-HCl, 4.380 g NaCl in 500 mL deionized water, pH 7.4). This was mixed with 400 μL of acid-detergent solution (2.19 g NaCl, 1.0 mL of 2N HCl solution, 0.25 mL Triton-X, qs. 250 mL deionized water). After 30 s, 1.2 mL of the acridine orange (AO) solution was added (3.8869 g citric acid monohydrate, 8.9429 g Na_2HPO_4, 4.3850 g NaCI, 0.1700 g disodium EDTA, 4 μg/mL acridine orange stock solution-1 mg/mL), qs. 500 mL deionized water, pH 6.0. The sample was then allowed to equilibrate for 30 s on the flow cytometer. The cell flow rate was 100-200 cells/s. Following acquisition of 5000 cells the sip tube was thoroughly cleaned with a dry KimWipe to remove residual sample. Sperm from a control bull (i.e., good sperm quality based on a low percent COMP-α_t) was used to standardize instrument settings prior to analysis of study samples. The flow cytometer was adjusted such that the mean green fluorescence was set at 500 channels (FI-1 ~ 500) and mean red fluorescence at 150 channels (FI-3 @ 150). This results in scatter plots displaying cells with fragmented DNA, non-fragmented DNA, and any present debris. The *main population* represents the spermatozoa that emit more green than red fluorescence due to the predominantly normal double-stranded configuration of their DNA. Sperm cells located to the right and down of this main population represent those cells that have an increase in the amount of red fluorescence and a decrease of green fluorescence when compared with spermatozoa in the main

population. Data was stored in List-Mode and subsequently analyzed using WinList software (Verity Software House, Topsham, ME, USA). Measures included Mean-α_t, Standard Deviation-α_t (SD-α_t), Cell Outside Main Population (COMP-α_t) and Mode-α_t.

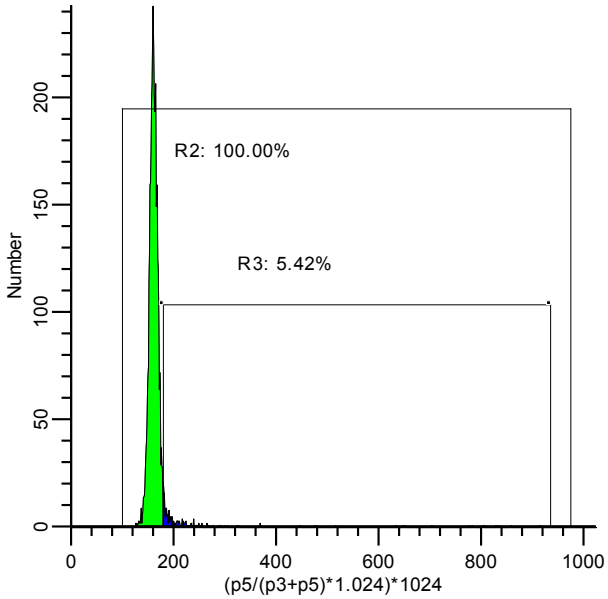
SCSA Scattergram from a water buffalo bull is presented in Figure 1.3.

Figure 1.3.: SCSA Scattergram from a IMB bull



The X-axis (FL3-H) represents the red fluorescence and the Y-axis (FL1-H) the green fluorescence. The COMP-α_t, Mean-α_t, SD-α_t, Mode-α_t are reported on the right.

Figure 2.3.: One-dimensional histogram of the distribution of alpha-t values represented in Fig. 1.3.



The X-axis represents the alpha-t that is the ratio between red fluorescence/ red + green fluorescence for each sperm in 1024 channels. The Y-axis represents sperm number. The bar represents the COMP-a_t.

2. 5. Estrous synchronization and AI

Buffalo cows (n=528), raised in 11 farms located in South Italy, were synchronized from January to May by Ovsinch protocol with timed-artificial insemination (AI) as previously reported (Neglia et al., 2003). Briefly, 0.2 mg of buserelin acetate (GnRH agonist, Receptal, Intervet) was administered to each buffalo cow IM (day 0); followed by 0.52 mg of synthetic prostaglandin on day 7^{th} ($PGF_{2\alpha}$, Clorprostenol, Estrumate, Merk Animal Health, Intervet) and 0.2 mg of buserelin acetate on day 9^{th}. Buffalo cows were palpated per rectum to verify estrous and AI with frozen/thawed semen was performed within 16-20 hours after the second injection of GnRH agonist. Heifers were implanted with an intravaginal device (PRID, Gellini, Italy) for 10 days containing 1.5 g of progesterone and a 10 mg capsule of estradiol benzoate, similarly to a previous study (Barile et al., 2001). On the 7^{th} day after PRID insertion, a dose of 1000 I.U. of pregnant mare serum gonadotrophin (PMSG; Ciclogonina, Fort Dodge, Italy) was administered IM. On day 10^{th}, PRID was removed, followed by an administration of 0.52 mg of synthetic prostaglandin ($PGF_{2\alpha}$, Clorprostenol, Estrumate, Merk Animal Health, Intervet). Heifers were inseminated with frozen–thawed semen at 16-20 hours after the PRID removal.
All animals were inseminated with frozen/thawed semen for one cycle.

Different batches of IMB bulls under progeny test were used

for AI. Insemination records on cows included: parity, farm, days in milk, bull and batch of the bull. Fertility was expressed as pregnancy rate per cycle (%), defined as the number of buffalo cows pregnant at 30 days following AI divided by the total number of cows inseminated per cycle. In addition, cows were transrectally examined at 45 days following AI to confirm or not the pregnancy. Further, calving dates were recorded to evaluate the incidence of fetal losses.

2. 5. Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics 20.0 (2012). The coefficient of variation (CV) was used to measure inter and intra-assay variability. Kolmogorv-Smirnov test was used to determine data normality distribution. Spearman rank correlations were used to test the relationship among traditional sperm features of sperm morphology, motility, viability and acrosome integrity with DNA integrity, as the data were not normally distributed.

In order to identify the relationships among fertility rate and the sperm features assessed with the different analyses, Receiver Operating Characteristic (ROC) curve analysis was used. ROC curve is a plot of the true positive rate against the false positive rate at different cut-off levels. The area under the curve measures the accuracy of the test, expressed as the ability of the test to correctly classify the cases according to the positive value of the state variable (Akobeng AK, 2007). In our data set, pregnancy rate at 45 d was considered the discriminant factor and the different in vitro sperm features were considered test variables. The cut-off value of the variable for each test was chosen as the value for which the percentage of pregnant buffaloes detected pregnant by the test (test sensitivity) ranged from 70% to 90%.

3. Results

3. 1. Sperm features post sperm centrifugation

Mean sperm concentration (x 10^6), measured by hematocytometer, was 27 ± 10 (range 10-38) lower than that assessed at the BC and mean EN-VIA was 74 ± 6 (range 62-83), similar to that assessed at the BC.

Results of sperm morphology are showed in Table 2.3.: no differences were found in uncentrifuged and centrifuged samples (Table 2.3.).

Table 2.3.: The mean ± SD, range () of sperm features prior and post centrifugation

Sperm morphology (%)	Prior-centrifugation	Post-centrifugation
Normal	64 ± 8 (45-76)	69 ± 10 (55-79)
AH	10 ± 3 (5-14)	10 ± 3 (7-18)
AA		
DH	1 ±1 (0-4)	2 ± 2 (0-4)
PD	1 ± 1 (0-1)	1 ± 1 (0-3)
DD	1 ± 1 (0-2)	1 ± 1 (0-2)
AM	1 ± 1 (0-2)	1 ± 0.5 (0-1)
BM	22 ± 8 (11-38)	16 ± 7 (6-29)
BT	2 ± 2 (0-6)	2 ± 1.5 (0-6)
CT	1 ± 1 (0-2)	1 ± 1 (0-3)
PGC		
EC	0.5 ± 0.5 (0-1)	0.5 ± 0.5 (0-1)

The morphologic categories are abbreviated as: AH-abnormal head, AA-abnormal acrosome, DH-detached head, PD-proximal droplet, DD-distal droplet, AM-abnormal midpiece, BM-bent midpiece, BT-bent tail, CT-coil tail, PCG- premature germ cells and EC-epithelial cells.

3. 2. Sperm motility and viability by flow cytometry

Mean sperm motility and viability are presented in Table 3.3.

Table 3.3.: The mean ± SD, range () of sperm features in vitro

Measure (%)	Mean ± SD (min-max)
TMOT	52 ± 26 (10-80)
VAI	51 ± 15 (28-74)
NVAI	22 ± 11 (7-43)
NVAD	24 ± 6 (14-37)
VAD	2 ± 1 (0-4)

Sperm features are abbreviated as follows: TMOT-total motility (visually estimated), VAI-viable acrosome intact, NVAI-nonviable acrosome intact, NVAD-nonviable acrosome damaged, and VAD-viable acrosome damaged (flow cytometry).

3. 3. Relationship between sperm morphology, motility and viability

As regard centrifuged samples, TMOT (%) was positively related to EN-VIA ($r= 0.81$; $P<0.05$) and inversely to the presence of epithelial cells ($r= -0.60$; $P<0.05$). EN-VIA was inversely correlated to coiled tails ($r= -0.66$; $P<0.05$). VAI and NVAI were correlated to coiled tails ($r= -0.62$ and 0.59 respectively; $P<0.05$). VAI was also inversely correlated to abnormal midpieces ($r= -0.63$). VAD were positively correlated to bent tails and inversely to proximal droplets

(r= 0.68 and -0.58 respectively; P<0.05).

3. 4. Comet assay

3. 4. a. Repeatability among days within the control

The mean neutral comet measures and their relative coefficients of variation among days are displayed in Table 4.3. In the neutral Comet assay the lowest CVs within the dimension measures were related to comet height and length (3%; respectively), comet area (5%), head diameter (4%), head area (5%), whereas the CVs for the tail length and area were 46% and 54%; respectively (Table 4.3). The other variable with a low CV was %H-DNA (6%), whereas for the %T-DNA was 38%. Among the intensity measures the measures with a low CV were the head mean intensity (8%) and comet mean intensity (7%), whereas tail mean intensity and tail intensity had a higher CV (16% and 44; respectively). The CV for the Olive tail moment was 87%, whereas for the tail moment was 57%.

Table 4.3.: The mean± SD, range () and the coefficient of variation (CV) for the control measures of the neutral Comet assay.

Measure	Mean ± SD	Range (min-max)	CV (%)
Comet Length (μm)	24 ± 1	11 - 40	3
Tail Length (μm)	3 ± 1	0 - 26	46
Comet Height (μm)	23 ± 1	10 - 36	3
Comet Area (μm²)	344 ± 16	74 - 874	5
Head Diameter (μm)	20 ± 1	10 - 29	4
Head Area (μm²)	289 ± 14	70 - 539	5
Tail Area (μm²)	55 ± 30	1 - 499	54
Comet Intensity	356953 ± 32571	108554 - 834726	9
Comet Mean Intensity	110 ± 8	65 - 162	7
Head Intensity	304525 ± 30413	67446 - 654573	10
Head Mean Intensity	110 ± 8	69 - 163	8
Tail Intensity	52428 ± 23228	1 - 532793	44
Tail Mean Intensity	117 ± 18	1 - 1143	16
%H-DNA	87 ± 5	25 - 100	6
%T-DNA	13 ± 5	0 - 75	38
Tail Moment	3 ± 3	0 - 59	87
Olive Tail Moment	4 ± 2	0 - 37	57

Intensity (Comet, Head and Tail) corresponds to the sum of pixels intensity. Mean intensity (Comet, Head and Tail) corresponds to the mean intensity of pixels.

3. 4. b. Repeatability between replicates (i.e., between slides)

Comet assay variables had lower variability between replicates (i.e., between slides; Table 5.3.) compared to day-to day variability. The CVs for the dimension measures ranged from 0.2 (head diameter) to 6% (tail area). The CVs for the intensity measures ranged from 0.03% (tail intensity) to 6% (head intensity). The CV for %H-DNA was 1%; whereas the %T-DNA was 3.5%. The CVs for tail and Olive moments were 9 and 6%, respectively.

Table 5.3.: The mean±SD, range () and the coefficient of variation (CV) for neutral Comet measures between replicates (n=2).

Measure	Mean ± SD	Range (min-max)	CV (%)
Comet Length (μm)	24 ± 0.1	18-29	0.5
Tail Length (μm)	4 ± 0.1	2-7	2.0
Comet Height (μm)	23 ± 0.2	17-28	1.0
Comet Area (μm²)	359 ± 1.3	215-511	0.4
Head Diameter (μm)	20 ± 0.05	15-24	0.2
Head Area (μm²)	294 ± 3	162-405	1.0
Tail Area (μm²)	65 ± 4	30-136	6.0
Comet Intensity	366632 ± 18604	186833-659952	5.0
Comet Mean Intensity	106 ± 4	59-136	4.0
Head Intensity	308407 ± 18623	143761-532060	6.0
Head Mean Intensity	107 ± 4	60-139	4.0
Tail Intensity	58225 ± 19	25759-127891	0.03
Tail Mean Intensity	105 ± 0.4	70-200	0.4
%H-DNA	85 ± 0.5	77-91	1.0
%T-DNA	15 ± 0.5	9-22	3.5
Tail Moment	4 ± 0.3	1-10	9.0
Olive Tail Moment	4 ± 0.2	2-7	6.0

Intensity (Comet, Head and Tail) corresponds to the sum of pixels intensity. Mean intensity (Comet, Head and Tail) corresponds to the mean intensity of pixels. * r-values are significant at $P<0.05$, ** r-values are significant at $P<0.01$.

3. 5. Relationship among neutral Comet measures, sperm morphology, motility and viability

Correlations among morphologic categories of centrifuged samples and neutral Comet measures are showed in Table 6.3. Coiled tails were inversely correlated to %H-DNA, and positively to tail length, tail and Olive tail moment (r= -0.80; 0.71; 0.70 and 0.65; respectively, $P<0.05$; Table 6.3.). Epithelial cells were inversely correlated to %H-DNA (r= -0.67; $P<0.05$), and positively to tail length, area and intensity (r= 0.82; $P<0.05$), tail and Olive tail moment (r= 0.82 and 0.77; respectively, $P<0.05$).

TMOT (%) and EN-VIA were correlated to several Comet head and tail measures (Table 6.3.), whereas VAD was inversely correlated only to tail mean intensity (r= -0.61; $P<0.05$, not in table).

Table 6.3.: Relationship among neutral comet measures, sperm morphology, motility and viability

	Neutral								
	Head				Tail			Moment	
Measure	diameter	area	intensity	%DNA	length	area	intensity	tail	Olive
CT				-0.80	0.71			0.70	0.65
EC				-0.67	0.82	0.82	0.82	0.82	0.77
TMOT	0.72	0.60	0.61	0.74	-0.58	-0.66		-0.69	-0.76
EN-VIA	0.60			0.81	-0.71	-0.66		-0.73	-0.75

Measures are abbreviated as follows: PC-post-centrifugation, CT-coiled tail, and EC-epithelial cells, TMOT-total motility, EN-VIA Eosin/Nigrosin-Viability. Intensity (Head and Tail) corresponds to the sum of pixels intensity. Mean intensity (Head and Tail) corresponds to the mean intensity of pixels. r-values are significant at least at $P<0.05$.

3. 6. SBH, SCSA and their relationship to neutral comet measures

Mean values of sperm DNA integrity measures by the SBH and the SCSA are presented in Table 7.3. SBH, SCSA and neutral comet measures were not correlated to each other (P>0.05).

Table 7.3.: The mean ± SD, range () of sperm DNA measures by SBH and SCSA

Measure	**Mean ± SD (min-max)**
DFI (%)	8 ± 3 (6-15)
Mean-α_t	219 ± 38 (158-254)
SD-α_t (%)	36 ± 4 (28-43)
COMP-α_t (%)	6 ± 2 (3-10)
MODE-α_t	215 ± 38 (156-251)

Sperm features are presented as DFI-defragmentation index (% SBH), Mean-α_t, SD-α_t, COMP-α_t and Mode-α_t (SCSA).

3. 7. Relationship among SBH, SCSA measures, sperm morphology, motility and viability

SBH was not correlated to sperm morphology and motility, whereas it was positively correlated to NVAD and inversely to VAD ($r= 0.60$ and $–0.63$; respectively, $P<0.05$). Mean-α_t and Mode-α_t were correlated to bent midpieces ($r= 0.63$ and 0.61; respectively, $P<0.05$). COMP-α_t was inversely correlated to distal droplets ($r= -0.61$; $P<0.05$). SCSA measures were not correlated to sperm motility and viability, except COMP-α_t with VAI ($r= -0.60$; $P<0.05$). No relationship was found among SBH, SCSA measures and EN-VIA ($P>0.05$).

3. 8. Fertility in vivo

The overall pregnancy rate/cycle (PC) at 30 days was 57% and 55% at 45 days, with a decrease of 3.8% at 45 days. The overall calving rate was 45% (Table 8.3.), with a decrease of 21% and 18% as compared to PC at 30 and 45 days, respectively. There was no difference among farms in pregnancy and calving rates (P>0.05).

Table 8.3.: Overall pregnancy rate per cycle and calving rate within bull

IMB bull	PC 30 d (%)	PC 45 d (%)	Calving rate (%)
1	53	48	39
2	57	55	45
3	63	61	50
Total	57	55	45

Measures are abbreviated as follows: IMB-Italian Mediterranean Buffalo, PC-pregnancy rate per cycle (at 30 and 45 days).

Among the analyzed batches, the overall pregnancy rate/cycle (PC) at 30 days was 63% and 62% at 45 days, with a decrease of 2% at 45 days. The overall calving rate was 50% (Table 9.3.), with a decrease of 21% and 19% as compared to PC at 30 and 45 days, respectively. No differences were found in pregnancy and calving rates among farms (P>0.05).

Table 9.3.: Pregnancy rate per cycle and calving rate of the batches analyzed

IMB bulls	PC 30 d (%)	PC 45 d (%)	Calving rate (%)
1	63	59	48
2	63	63	49
3	65	65	51
Total	63	62	50

Measures are abbreviated as follows: IMB-Italian Mediterranean Buffalo, PC-pregnancy rate per cycle (at 30 and 45 days).

3. 9. Receiver Operating Characteristic (ROC) curve

ROC curve analysis showed significant values when sperm motility, NVAD, SD-α_t, distal droplets, Olive and tail moments, %H-DNA (and its relative %T-DNA) and tail area were used as variables ($P<0.05$; Table 10.3). The areas under the curve were 0.59 for sperm motility, 0.57 for the Olive tail moment and 0.56 for all the other significant sperm features (respectively $P<0.05$). Cut-off values and respective levels of sensitivity and specificity are presented in Table 10.3.

Table 10.3.: ROC curve analysis of sperm features in IMB bulls

Measure	Area*	Cut-off value (%)	Sensitivity (%)	Specificity (%)
TMOT (%)	0.59	≥47.5	86	25
NVAD (%)	0.56	≤26	82	17
DD (%)	0.56	≤1.25	73	33
SD-α_t (%)	0.56	≤40	83	15
OTM (μm)	0.57	≤3.5	80	26
TM (μm)	0.56	≤3	80	26
H-DNA (%)	0.56	≥86	81	26
T-DNA (%)	0.56	≤14	81	26
TA (μm²)	0.56	≤58	80	26

*Area under the ROC curve. Measures are abbreviated as follows: TMOT-total motility, NVAD-not viable acrosome damaged, DD-distal droplets, SD-α_t-Standard Deviation-α_t, OTM-Olive tail moment, TM-tail moment, H-DNA-head DNA, T-DNA-tail DNA, TA-tail area.

4. Discussion

This study reported in vitro sperm quality in IMB bulls under progeny test using traditional and newer methods, such as sperm DNA assays.

Traditional sperm features of motility, viability, acrosome integrity and morphology were evaluated. Semen was centrifuged to avoid interference of granular particles, evidenced under microscopy, and contained in the egg yolk used to freeze the straws. The centrifugation was adopted to eliminate those particles and to avoid a background, which could have interfered with the Comet images. The purpose for centrifugation was not to select the "best" sperm population and in fact sperm morphology was not different between uncentrifuged and centrifuged samples. However, sperm concentration was different between the automated cell counter provided from the bull center and the hematocytometer. This may be related to the centrifugation, which may have partially changed the final number of sperm, and to a possible interference of the automated cell counter with those granular particles, as previously reported for other species (Parks et al., 1985).

The % morphologically normal sperm was high in this group of bulls and the main abnormalities observed were bent midpieces, which may be iatrogenic (i.e., cold or osmotic shock) or may be due to the bull. These abnormalities may be motile, but swim backwards. This backwards swimming motion gives the impression of adequate

motility when evaluated at a low magnification, but it is unlikely that these sperm have the capability to fertilize.

Sperm viability assessed by the automated cell counter from the bull center and by the flow cytometer was similar to what reported in Swamp buffalo in a previous study (Koonjaenak et al., 2007); and higher than that reported in IMB bulls by Minervini et at. (2013). In our study, sperm viability measured by EN exhibited higher values compared to the fluorescent methods (PI), in agreement with previous studies in boars, bulls and stallions (Tamuli and Watson, 1994; Gadea and Matas, 2000; Pintado et al., 2000; Brito et al., 2003; Foster et al., 2011). Stain properties may affect its ability of the stain to penetrate the sperm membrane, such that a damaged sperm membrane allows entrance of PI more than EN (Foster et al., 2011).

This study described for the first time in IMB bulls the comet measures through a software program and the day-to-day variation of the Comet assay. In general, among the dimension measures, comet height, length and area, head diameter and area were less variable among days, as for the *intensity* measures head and comet intensity and their mean intensity. The composite measures (tail and Olive tail moments) had high variability among days, as they combine *dimension* and *intensity* measures into their value.

The intra-assay variability (between slides) was lower, as compared to inter-assay variability for the control sample. This result suggests that the main contributor to the variability is sample processing

(reagent preparation) rather than slide preparation and it occurs among days rather than among slides. The %H-DNA was the less variable measure among days and between slides. The latter finding was in accordance with a previous study in human (Hughes et al., 1997), which reported only repeatability between slides and not among days. However, in our study, %H-DNA and dimension measures were more reliable among the comet measures.

Despite the Comet assay is a relatively simple procedure, it requires several steps: inclusion of the sperm in agarose, lysis, preincubation in electrophoretic solution, electrophoresis, staining, image acquisition and scoring. Differences in reagent preparation, electrophoresis (i.e., voltage, time), staining (i.e., fluorescent microscope, dye), evaluation system (i.e., visual, software) are accountable for inter- and intra-laboratory variation. Several papers have been published using the Comet assay on sperm (see Appendix B), none in our knowledge has reported day-to-day-variation. In our experience, it is beneficial to include a control in the procedure to make the results among and within laboratories comparable.

This was the first study, in our knowledge, that evaluated in a group of IMB bulls sperm DNA integrity with different sperm DNA assays. The 6% of COMP-α_t (or %DFI) was lower than that reported from Minervini et al. (2013) in IMB bulls and similar to that found by Pawar and Kaul (2011) in Murrah buffalo bulls. However, this latter study used SBH by bright field microscopy rather than fluorescent microscopy.

The high and similar sperm quality of the bulls in our study could partially explain the lack of correlation among the sperm DNA assays (i.e., Comet assay, SBH and SCSA). However, lack of relationship among the sperm DNA assays may be also related to the characteristics of the assays, which evaluate differently sperm DNA in terms of methodologies and targets. As mentioned, the SCSA measures the susceptibility of sperm DNA to denaturation only in the toroid linker region, while the Comet assay and similarly the SBH remove both protamines and histones, allowing access to the whole chromatin structure. In a group of individuals with high sperm quality it would be likely that the chromatin is highly stable and this may probably limit its accessibility to the assays.

Further studies are needed to verify the consistency of the comet measures in wide trials with different sperm quality groups in order to improve our knowledge about the comet measures. A broader range of sperm quality should be assessed to determine the utility of sperm DNA assays in practice.

The relationship between Comet measures and %COMP-α_t with sperm viability and between Comet measures and sperm motility suggests that sperm DNA integrity was related to traditional sperm features. An increase in morphologic abnormalities (i.e., coiled tails, abnormal midpieces and bent tails) and a decrease in sperm motility (presence of epithelial cells) were associated with Comet assay measures (i.e., increase in %T-DNA, tail length and tail moment and Olive tail moment). Distal droplets were the only

morphologic abnormality associated with %COMP-α_t and bent midpieces to Mean-α_t and Mode-α_t. These results are similar to a previous study, in which sperm motility and morphology decreased as DNA quality decreased (Blanchard et al., 2013).

Immature sperm forms (i.e., coiled tails and distal droplets) and epithelial cells originating from excurrent ducts, accessory glands or urinary tract, may reduce sperm motility and sperm DNA integrity.

This study also reported in vivo fertility results of IMB bulls. The 3 bulls revealed high pregnancy rate per cycle (at 30 and 45 d) and calving rate, higher than that reported in buffalo inseminated with frozen semen in previous studies (Barile et al., 1999; Senatore et al., 2004; Neglia et al., 2015) and similar to what reported for Nili-Ravi buffalo bulls (Andrabi et al., 2006). However, Barile et al. (1999) performed pregnancy diagnosis at 40 and 60 days post AI, Neglia et al. (2015) at 45 and 70 days, while Andrabi et al. (2006) at 75 days. This study, in addition to PC, monitored long-term reproductive outcomes represented by the calving rate. None of those previous studies reported calving rate. This information was considered to give an estimation on the potential number of calves born, which could be taken into account to design progeny tests. However, differences in how fertility is estimated limit comparisons among studies in this species. Variation in semen quality within individuals and among individuals is another component that may change fertility and this variability is mainly related to season, age, nutrition, frequency of ejaculation and testicular size, as reported for

bulls (Brito et al., 2002; Chacon et al., 2002) and Nili-Ravi, Murrah and IMB bulls (Saeed et al., 1990; Kumar et al., 1993; Galli et al., 1993; Presicce et al., 2003). Considering the relatively high sperm quality of the bulls evaluated, the 19% decrease in calving rate compared to PC at 45 days may be mainly related to the female counterpart, for which age, seasonality, parity and management may determine pregnancy loss or abortion (Zicarelli L, 2010).

The results of ROC curve analysis pointed out the relationship between sperm motility, NVAD and SD-α_t with fertility, in accordance with previous studies (Ballachey et al., 1987; Correa et al., 1997; Love and Kenney, 1998; Januskauskas et al., 2001; Alm et al., 2001). Interestingly, in addition to those sperm features, Olive tail moment and tail moment, %H-DNA, %T-DNA and comet tail area were also sensitive in estimating fertility. A previous study (Ribas-Maynou et al., 2013) in men reported a positive relationship between fertility and alkaline and neutral Comet assays, visually estimated. In the latter study the alkaline Comet assay had a stronger relationship with fertility as compared to the neutral. However, differences between methods, number of individuals evaluated and species make the results difficult to compare. The low levels of specificity found in our study may be related to the female component, which plays an important role in determining fertility outcomes. Our study was the first in our knowledge to combine a wide set of in vitro sperm quality tests with fertility in vivo in IMB species. Our preliminary results allowed the identification of some

sperm features and their predictive levels of fertility in vivo, which may be used to select water buffalo bulls for sperm production. Further studies should be conducted to verify the relationship of the sperm DNA assays to fertility in vivo in trials with a larger number of IMB bulls.

CHAPTER 4

EXPERIMENT 2

Sperm DNA integrity measured by the Sperm Chromatin Structure Assay, the alkaline and neutral Comet assay, the Sperm Bos Halomax and their relationship to sperm motility and sperm morphology in bulls (Bos taurus).

Abstract

The relationship among sperm DNA assays in bulls with different sperm motility and morphology measures has not been reported. The objectives of this study were to 1) describe Comet assay measures and examine their repeatability (inter- and intra-assay); 2) compare sperm DNA quality assays (i.e., Sperm Chromatin Structure Assay-SCSA; alkaline and neutral Comet assays and Sperm Bos Halomax assay-SBH) in a group of bulls selected on sperm motility and morphology (higher vs. lower); 3) determine the relationship among DNA assays and sperm motility and morphology values. Inter-assay repeatability was higher for the neutral Comet assay as compared to the alkaline Comet assay. Intra-assay repeatability was higher than inter-assay repeatability for both Comet assays. Comet assay dimension measures and % tail DNA were the most repeatable for both Comet assays. Among sperm DNA quality assays, only SCSA measures and neutral Comet assay Ghosts (% Ghosts), head diameter and area, and comet area were different between high and low sperm quality groups ($P < 0.05$). The SCSA measures were inversely correlated with neutral Comet head

measures (diameter, area, and intensity) and % Ghosts ($P < 0.05$). The % Ghosts and COMP-α_t were correlated with some measures of sperm morphology and sperm motility. The neutral Comet assay was more appropriate for sperm evaluation than the alkaline Comet assay for distinguishing between groups with different sperm quality.

1. Introduction

Traditional semen evaluation includes assessment of sperm motility and morphology, however, DNA integrity has been shown to be an independent feature of a sperm, necessary to sustain embryo development (Zini et al., 2005; Yamauchi et al., 2011). Sperm has the most condensed chromatin among eukaryotic cells, organized in toroids, extremely stable and compact structures, attached to the nuclear matrix by the toroid linker regions, which represent the most accessible and susceptible parts of the sperm chromatin (Ward and Coffey, 1991; Sotolongo et al., 2003). Sperm DNA can be damaged, producing single (ssDNA) or double strand breaks (dsDNA) as result of oxidative and enzymatic damage, respectively (Aitken et al., 2013). Independent of the cause, human ssDNA may have a better prognosis since it is easier to repair than dsDNA (Sakkas et al., 2010). In addition, ssDNA may impair fertilization (Ribas-Maynou et al., 2012b; Simon et al., 2011b) while dsDNA may interfere with embryonic development and implantation (Lewis and Aitken, 2005), as well as cause recurrent miscarriages (Lewis and

Simon, 2010). Many techniques have been developed to examine sperm DNA integrity, including the Sperm Chromatin Structure Assay (SCSA), the Single cell gel electrophoresis (Comet) assay and the Sperm Bos Halomax (SBH). These tests evaluate different aspects of the sperm DNA structure. The SCSA identifies the presence of ssDNA in the exposed *toroid linker region*, but cannot identify changes in the more compact toroid region because the assay conditions (i.e., low pH) are not sufficient to decondense toroid DNA and allow dye penetration. In contrast, the Comet assays (i.e., neutral and alkaline) allow stain access to both the toroid and toroid linker regions (Shaman et al., 2007) and thus ssDNA and dsDNA *breaks* can be identified in both of these regions. Both the SBH and the Comet assays, use disulfide bond reducing agents (i.e., β-mercaptoetanol and dithiothreitol-DTT, respectively) in the lysis solution to completely expose DNA strands and allow strand break detection in the toroid linker and toroid regions. The Comet assay embeds sperm in agarose into slides, then treats the sperm with detergent and high salt solution to remove cellular and nuclear membranes and proteins, resulting in a decondensed DNA structure referred to as a nucleoid (Cook et al., 1976). These sperm are then exposed to an electrophoretic field under either a neutral (pH-9.0) or alkaline (pH-13.0) solution. The DNA from sperm with breaks migrates away from the "intact" head region to form a "comet" towards the anode following electrophoresis resulting in a comet-like appearance whereas intact DNA remains in its original position

(Shaman and Ward, 2006). The extent of DNA migration is determined by the size of the DNA fragments, such that small fragments migrate farther than large. The SCSA, a flow cytometric assay, uses a metachromatic dye, acridine orange, to evaluate ssDNA (abnormal) and dsDNA (native) after acid treatment and has been used to evaluate DNA quality in men (Evenson et al., 1980), bulls (Ballachey et al., 1987; Ballachey et al., 1988; Januskauskas et al., 2001; Januskauskas et al., 2003; Whaterhouse et al., 2006; Fortes et al., 2012; D'Occhio et al., 2013), stallions (Love and Kenney, 1998) and boars (Evenson et al., 1994).

It is unclear what type of DNA breaks are detected by the neutral and alkaline Comet assays. Shaman and Ward (2006) suggested that the alkaline Comet identifies ssDNA and dsDNA breaks while the neutral Comet assay (pH 8-9) detects mainly dsDNA breaks; whereas in contrast, Baumgartner et al. (2009) proposed the neutral Comet assay identifies dsDNA and *closely associated* ssDNA and the alkaline Comet identifies only ssDNA breaks. Several studies in human sperm (Singh et al., 1989; Hughes et al., 1996, Hughes et al., 1997; McKelvey-Martin et al., 1997) identify high levels of ssDNA breaks when the alkaline Comet is used. These ssDNA "breaks" however, may be artifacts rather than DNA pathology since sperm DNA is known to have a high level of *alkaline labile sites* (i.e., sites on the DNA that normally separate when exposed to alkaline conditions), (Haines et al., 1998; Singh et al., 1989). Due to the "milder" electrophoretic conditions (i.e., pH-8-

9) the neutral Comet assay may be better able to detect "pathologic" ssDNA and dsDNA breaks (Collins, 2004; Fraser et al., 2011) since it does not affect alkaline labile sites.

Several techniques have been used to evaluate the comet image. Initially, visual evaluation was performed and sperm were determined to either have a comet tail or not, or were classified into 4 or 5 categories, based on the length of the comet tail and on the amount of DNA in the tail; however, recently computer software programs have been developed to objectively evaluate, among many parameters, the size of the comet head and tail as well as the intensity of the fluorescence given off by the image. Due to the variation in evaluation method comparisons between studies can be difficult.

Other factors that vary between studies include the type and amount of the reducing agents; electrophoretic voltage settings; time of the slide in the electrophoresis solution; and evaluation criteria (i.e., visual scoring, micrometer or software). For example, there was no difference between fresh and frozen-thawed human sperm when a micrometer was used (Duty et al., 2002); however, low levels of DNA damage were reported when fresh and frozen-thawed bull sperm were evaluated using a software program (Słowińska et al., 2008), or more severe damage was reported with *visual* evaluation (i.e., having a comet tail or not), (Mukhopadhyay et al., 2011). Further, in boars, DNA fragmentation in frozen-thawed sperm, measured *visually* by having a comet tail or not, was higher

than fresh (Fraser and Strzeżek, 2007a). However, these results may have been confounded by the methods used to evaluate the comet since the human sperm comets were measured using a micrometer, the bull were scored either by software or visually and boar sperm were only evaluated visually.

Solution composition may also differ between studies. For example, Słowińska et al. (2008) included 40 mM of dithiothreitol (DTT) in the lysis solution after incubating the slides in an alkaline solution and then performing the electrophoresis for 10 min in a neutral solution (pH 8.4). In contrast, Mukhopadhyay et al. (2011) *did not use DTT*, and the electrophoresis was performed for *30 min* in a neutral solution (pH 9) *without* an alkaline pretreatment. Electrophoresis time could make a difference in the tail length, with a longer time resulting in longer tail. Also, the use of DTT (a strong disulphide bond reducing agent) would allow toroid DNA to ”relax” more, which in turn could also result in a longer comet tail. It is also likely that there is species-species variability with respect to Comet assay conditions; therefore similar assay conditions may elicit a different degree of DNA decondensation, and thus a different comet response depending on the species studied.

It is unclear how neutral and alkaline Comet assays differ with respect to evaluation of DNA quality since there have been few direct comparisons in humans and none in bulls. In men, fertile sperm donors had low levels of both ssDNA and dsDNA breaks compared to different groups of subfertile patients, such as

asthenoteratozoospermic individuals either with or without varicocele or oligoasthenoteratozoospermic patients with structural chromosome rearrangements (Ribas-Maynou et al., 2012a). In bulls, the alkaline Comet assay has not been evaluated, while the neutral Comet assay detected a higher tail moment (i.e., more DNA breaks) in conventional processed than sex-sorted semen (Boe-Hansen et al., 2005). In the same study, there was no correlation between neutral Comet and SCS assay values.

Recently the Sperm Bos Halomax (SBH) has been developed as a quick test for assessment of sperm DNA integrity in bulls, based on Sperm Chromatin Dispersion Test (SCDt), (Fernandez et al., 2003). Similar to the Comet assays sperm are immersed in agarose on a microscope slide and are treated with a lysis buffer to remove sperm membranes and nuclear proteins, however, the sperm DNA is allowed to passively disperse without exposure to an electrophoretic field, which results in round *nucleoids* with a central core and a symmetrical peripheral halo of passively dispersed DNA loops. DNA fragmentation produces *large* halos whereas those sperm with low levels of fragmentation show *small* halos (García-Macías et al., 2007). Currently, there has been no comparison of the four assays (alkaline and neutral Comet assays, Sperm Chromatin Structure Assay, and the Sperm Bos Halomax) available to evaluate bull sperm DNA. In addition, there has been no study comparing these DNA tests with conventional sperm quality measures such as sperm motility and morphology. The aims of this study were to:

1. Objectively measure and describe comet images using a software program and determine assay repeatability (intra- and inter-assay);
2. Evaluate and compare sperm DNA quality using the SCSA, the neutral and the alkaline Comet assays, and the SBH;
3. Compare the sperm DNA quality tests to traditional measures of sperm quality such as sperm motility and morphology.

2. Materials and Methods

Thirty-three (33) beef-type (Angus - Brangus) bulls, between 3-11 years old, underwent a routine breeding soundness examination (BSE) in a farm located in South Texas, USA. The BSE was performed in accordance with guidelines set forth by the Society for Theriogenology (Chenoweth et al., 1992). As part of the BSE, the body condition score (BCS) was measured using a 9 point scale (1-very emaciated, 9-very fat), (Richards et al., 1986) and scrotal circumference (SC) was recorded with a tape measure (Scrotal tape, Lane Manufacturing, INC., Denver, Colorado, USA). Semen was collected by electronic ejaculator (ElectroJac 6, IDEAL Instruments, Lexington, KY, USA). Twenty (20) of 33 bulls were selected based on sperm motility and morphology for further analysis to compare SCSA, neutral and alkaline Comet and SBH. Ten of the 20 bulls were those with the highest motility and morphology values, while the other 10 were those with the lowest motility and morphology values.

Immediately following semen collection 10 µl of raw semen was diluted (1:100) in 990 µl of Dulbecco's phosphate buffered saline (DPBS Corning cellgro, Mediatech, INC., VA, USA) in each of 4 Eppendorf tubes (1.5 ml), and one was diluted with a milk-based semen extender (INRA 96; IMV, Maple Grove, MN, USA). The 4 tubes with DPBS were mixed with semen inverted five times and immediately flash frozen in dry ice for DNA integrity evaluation

(i.e., neutral and alkaline Comet, SCSA and SBH). The remaining aliquot with semen extender was kept in an Equitainer (Hamilton, Thorne Biosciences, Beverly, MA, USA) at 5-8°C to be analyzed for sperm concentration and sperm motility on the following day in a laboratory.

2. 1. a Sperm concentration

Sperm concentration was assessed after 24 hours of cooled storage using an automated cell counter (NucleoCounterSP-100™ Chemometec, A/S, Allerød, Denmark).

2. 1. b Sperm motility

Total sperm motility (TMOT) was evaluated in raw semen immediately following semen collection by a light microscope, (MI-5 Microscope, Binoc S/N, Jorgensen Labs, Loveland, CO, USA) equipped with a warm stage set at 37°C. *Mass sperm motility* was evaluated by placing one 10 µl drop of semen on a clean microscope slide and evaluating it using light microscopy at 400x magnification. *Individual sperm motility* was evaluated by diluting (1:1 or 1:2 semen:solution based on the visual estimation of the raw concentration) raw semen in a warm (37°C) sterile saline solution and observed at 400x. The raw semen was also diluted in a warm (37°C) milk-based extender (INRA 96; IMV, Maple Grove, MN, USA) to a final sperm concentration of approximately $30x10^6$ sperm/ml, cool-stored in an Equitainer for approximately 24 hours and then evaluated for sperm motility with a computer-assisted sperm motion analysis system (CASMA), (IVOS Version 12.2.l, Hamilton, Thorne Biosciences, Beverly, MA, USA). For analysis, samples were allowed to warm for 10 minutes on a slide warmer.

Six μl of each sample was placed on a microscope slide (leja Standard Count 2 Chamber slides; Leja Products, B.V., Nieuw-Vennep, The Netherlands). Slides were inserted into the CASMA. The following sperm motility parameters were evaluated: percentage of total motile sperm (TMOT); percentage of progressively motile sperm (PMOT); percentage of rapid (RAP), average path velocity (VAP; μm/s); mean curvilinear velocity (VCL; μm/s); straight line velocity (VSL; μm/s); lateral head amplitude (ALH; μm), beat cross frequency (BCF; Hz), percentage linearity (LIN) and percentage straightness ([VAP/VCL] x 100; STR). Preset values for the IVOS system consisted of the following: frames acquired -45/s; frame rate -60 Hz; minimum contrast -60; minimum cell size -4 pixels; straightness (STR) threshold for progressive motility -50%; average path velocity (VAP) threshold for progressive motility -30 μm/s; VAP threshold for static cells -15 μm/s; cell intensity- 106 pixels.

2. 1. c Sperm morphology

Sperm morphology was evaluated immediately in the field using an eosin-nigrosin stain (Society for Theriogenology, Montgomery, AL, USA), and 100 sperm were evaluated under oil immersion at magnification of 1000x. Sperm were classified by a Theriogenologist, according to the Society for Theriogenology guidelines (Chenoweth et al., 1992) as normal, primary and secondary abnormalities (bull classification system), according to

Blom (1950); Barth and Oko (1989). A second morphology sample was prepared, in which the raw semen was diluted with buffered formol saline solution in a1.5 ml Eppendorf tube and evaluated by differential-interference contrast microscopy (Olympus BX60, Olympus America, Inc., Melville, NY, USA; X 1562 magnification). A total of 100 sperm per sample were evaluated and all abnormalities identified on a sperm (stallion classification system) were recorded such that the total sperm counted was 100, but the sum of the percent normal sperm and the abnormalities was greater than 100. This technique determines the percent incidence of a sperm abnormality in the population. The following sperm morphologic features were identified: normal, abnormal heads (mis-shaped heads, micro- and macrocephalic sperm, and large nuclear vacuoles), abnormal acrosomes, detached heads, proximal cytoplasmic droplets, distal cytoplasmic droplets, swollen or irregular midpieces, bent or coiled midpieces/tails, and premature germ cells (Kenney et al., 1983).

2. 2. Neutral and alkaline Comet assay (single cell gel electrophoresis)

The initial protocol for both the neutral and alkaline Comet assays was modified from a previous study (Tice et al., 2000). Low Melting Point Agarose (LMPA; Trevigen Inc. Gaithersburg, MD) was melted in a microwave for 10 s and then transferred to a 15 ml plastic tube and floated in a water bath at 37°C to prevent gel solidification. After 5 minutes the temperature of the gel was checked to be sure that it did not exceed 37°C. Frozen semen samples were thawed in a water bath at 37°C and diluted to a concentration of 1 million/ml in DPBS and 25µl were dispensed into 250 µl of LMPA in an 1.5 ml Eppendorf tube, vortexed for 5 s then floated in the water bath. Seventy-five microliters of sperm/LMPA mixture was quickly pipetted onto a horizontal Comet microscope slide (CometSlide™ 2 well/slide; Trevigen Inc. Gaithersburg, MD; Figure 3.4). Two Comet slides were prepared for each Comet (i.e., neutral, alkaline) assay from each semen sample. In addition, a control bull semen sample was included in each trial to monitor day-to-day repeatability. The slides were incubated at 4°C for a minimum of 5 minutes, before being placed horizontally in a rack (CometSlide™ Rack System Trevigen Inc. Gaithersburg, MD) and immersed in a cold (5-8°C) lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris HCl, 1% Triton X-100, 20 mM dithiothreitol, pH 10) for 30 min in the refrigerator, followed by immersion and incubation for 1h and 15 minutes at 37°C in the same lysis solution

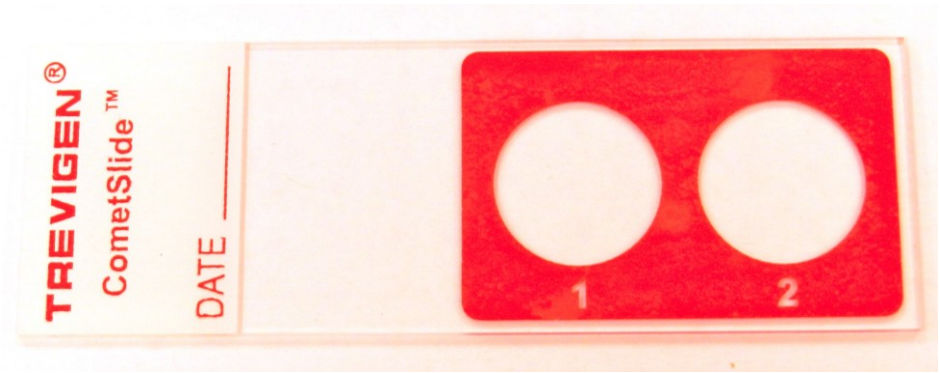
with the addition of 0.1 mg/ml Proteinase K (Proteinase K from Tritirachium album, MP Biomedicals, LLC, CA; USA). All solutions were made fresh and stored either in the refrigerator or incubator before adding to the comet slides. Following incubation in the second lysis solution, slides were washed 3 times with distilled water at 5 minute intervals and then placed in an electrophoretic horizontal unit (Fisher Scientific Electrophoresis Systems FB-SBR-2025).

Neutral Comet Assay- neutral Comet assay slides were chilled for 30 min in a chilled electrophoresis solution (500 mM NaCl, 0.1 M Tris-Base, 1 mM EDTA, 0.2% DMSO, q.s to 1.6 L deionized water, pH 9, osmolarity (mean±SD-999.5±1.34 osmol/L; range-998-1001 osmol/L). Electrophoresis was performed at 32 V (0.7 V/cm), 0.44-0.52 A, for 30 min at room temperature in the dark. Slides were rinsed drop-wise 3 times at 5 minute intervals with a neutralization buffer (0.4 M Tris Base, pH 7.5), then dehydrated with cold 70% ethanol and left overnight, in the dark, at room temperature.

Alkaline Comet Assay- This assay was performed immediately after the neutral, then the electrophoresis unit was rinsed with tap and de-ionized water. Slides were kept for 5 min in chilled electrophoresis solution (500 mM NaCl, 0.1 M Tris-Base, 1 mM EDTA, 0.2% DMSO, q.s.to 1.6 L distilled water, pH 13, osmolarity (mean±SD-1476±175.05 osmol/L; range-1270-1682 osmol/L) Electrophoresis was performed at 32 V (0.7 V/cm), 0.73-0.97 A, for

15 min at room temperature in the dark. The neutralization and the dehydration steps were similar as for the neutral Comet.

Figure 3.4.: Comet microscope slide with two wells (CometSlide™ 2 well/slide; Trevigen Inc. Gaithersburg, MD).



2. 2. b Slides staining, acquisition and scoring methods.

Slides were stained with 40 µl of diluted propidium iodide (PI, final concentration 0.38 µM) from a stock concentration of 9.6 µM PI in 1 ml Tris-EDTA buffer (10 mM Tris HCl and 1mM EDTA) and incubated for 10 min in the dark. For each semen sample 2 slides (2 wells/slide) were prepared, 50 sperm/slide were counted (25 sperm/well). Samples were evaluated by fluorescent microscopy (Olympus microscope model BX60 equipped with an objective U Plan FL N, magnification 20 x/ 0, 50 U/S2, ∞/ 0.17/ FN 26.5). Images acquisition was performed using a software program (DP Manager, Version 3.1.1.208, Olympus). The exposure time (1 s) and image size (1360x1024 pixels) were preset. The whole well was evaluated, starting from the top to the bottom, but to avoid artifactual images, the edge of the slide, air bubbles and overlapping comets were avoided.

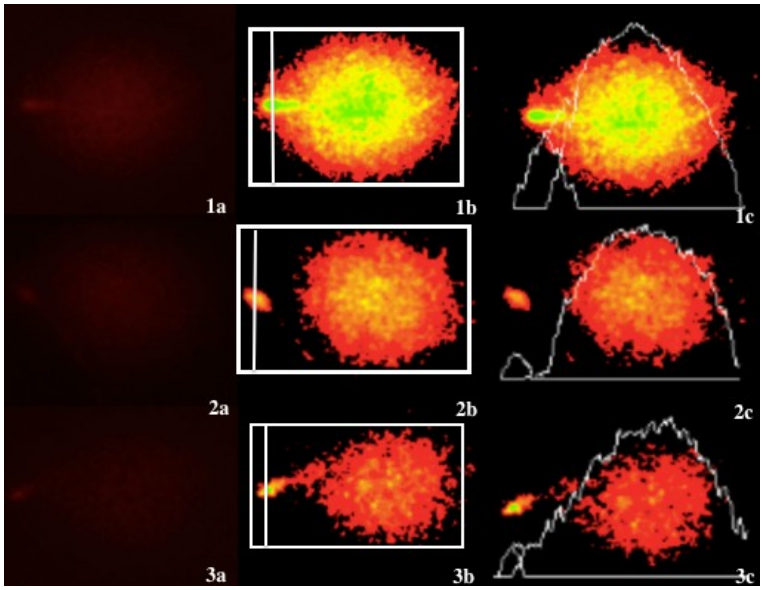
Stain (0.38 µM propidium iodide), exposure time (1 s) and image size (1360x1024 pixels) were standardized and PI concentration was optimized to avoid excess background dye. Microscope images were acquired and stored in bitmap format prior to evaluation with software (CometScore Version 1.5 TriTek Corp, VA). *Full spectrum* color settings, which increases the color variance sensitivity by a factor of 6 were chosen, and the *cutoff slider bar* was adjusted so that the background appeared black as suggested by the software guide. Software comet images were

evaluated by outlining the whole comet with a rectangle and placing a vertical line through the head (nucleoid), (Figures 4.4.b and 5.4.b). The software comet image is composed of individual pixels, which are enumerated by the software and the total pixel count corresponds to DNA content. Once released the cursor, the software shows a diagram and the comet measurements, saved as .txt (image c). In order to facilitate dimension measurements of comet (i.e., length, height, area), the software was pre-calibrated in microns by a picture of a slide glass micrometer (acquired with the same settings described for the Comet images above), calibrated with the bar provided by the software in the calibration settings. Comet measurements included the following tail parameters, grouped as dimension measures, intensity measures, %DNA in head and tail and combined measures (tail moment and Olive tail moment):

comet length, tail length, comet height, comet area, head diameter, head area, tail area, comet intensity, comet mean intensity, head intensity, head mean intensity, tail intensity, tail mean intensity, %DNA in head (%H-DNA), %DNA in tail (%T-DNA), tail moment (TM), Olive tail moment (OTM), (see Appendix A). The software measures the comet dividing it into a head (on the left) and a tail (on the right). A high % DNA of DNA in the tail is indicative of high level of “loose” defragmented DNA. The tail length has been measured starting from the edge of the head by Singh et al. (1988) and from the center of the head by Olive et al. (1990) and it reflects the size of the “loose” DNA fragments, such that small DNA

fragments migrate farther than large DNA fragments. The amount of dye binding to the DNA is proportional to the amount of DNA fragments, so that the tail intensity may vary, since long tails can contain small pieces of DNA, while a short tail can be also very intense (Ashby et al., 1995). The %T-DNA is calculated by the software from the tail intensity. Olive et al. (1990) introduced the OTM which is the product of the %T-DNA and the distance between the center of the head and the end of the tail. In addition, a measure based on the combination of tail length and %T-DNA has been introduced by computerized image analysis systems (Tice et al., 2000).

Figure 4.4.: Three different bull sperm nucleoids (1, 2, 3) using the alkaline Comet assay.



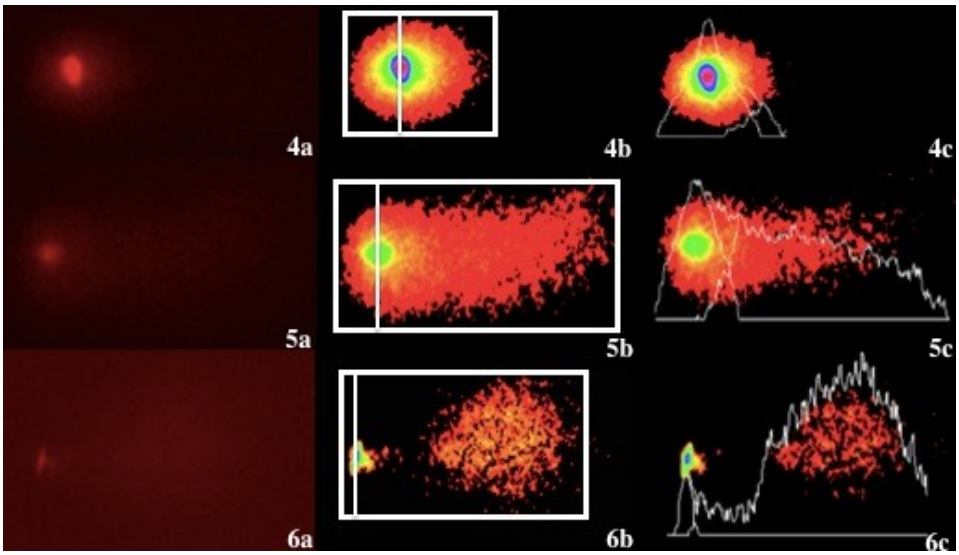
Images 1 a, 2 a, 3 a show the microscopic image; images 1 b, 2 b, 3 b show the same image enhanced and acquired by the CometScore software with the placement of the box around the comet image. The middle vertical line identifies the center of the comet head while the left and right vertical lines mark the limits of the comet image. Images 1 c, 2 c, 3 c show the software's graphic representation of the size and fluorescent intensity of the nucleoid and comet areas used for analysis. When the software measures the images b (1, 2, 3) visually, it automatically reduces the size of the comets, once the graphic representation is generated. Starting from the top to the bottom, different levels of DNA migration have been selected, in order to show the most intact (1 a, b, c) and the most defragmented DNA (images 3 a, b, c). Measures for fig. 4.4 are reported in Table 1.4.

Table 1.4.: Comet measures generated by the CometScore software from images 1, 2, 3 in Figure 4.4.

Measure	Image number		
	1	**2**	**3**
Comet Length (µm)	54	45	47
Tail Length (µm)	39	38.7	42
Comet Height (µm)	45	30	31
Comet Area (µm²)	1729	856	864
Head Diameter (µm)	15	6	5
Head Area (µm²)	521	76	16
Tail Area (µm²)	1208	780	847
Comet Intensity	251821	108661	102228
Comet Mean Intensity	15	13	12
Head Intensity	61088	8663	2174
Head Mean Intensity	12	12	14
Tail Intensity	190733	99998	100054
Tail Mean Intensity	16	13	12
%H-DNA	24	8	2.1
%T-DNA	76	92	98
Tail Moment	92	110	128
Olive Tail Moment	52	67	75

Intensity (Comet, Head and Tail) corresponds to the sum of pixels intensity. Mean intensity (Comet, Head and Tail) corresponds to the mean intensity of pixels.

Figure 5.4.: Three different bull sperm nucleoids (4, 5, 6) using the neutral Comet assay.



Images 4 a, 5 a, 6 a show the microscopic view; images 4 b, 5 b, 6 b show the same image enhanced and acquired by the CometScore software with the placement of the box around the comet image that is used for analysis. The middle vertical line identifies the center of the comet head while the left and right vertical lines mark the limits of the comet image. Images 4 c, 5 c, 6 c show the software's graphic representation of the size and fluorescent intensity of the nucleoid and comet areas used for analysis. When the software measures the images b (4, 5, 6) visually, it automatically reduces the size of the comets, once the graphic representation is generated. Starting from the top to the bottom, different levels of DNA migration have been selected, in order to show the most intact (4 a, b, c) and the most defragmented DNA (6 a, b, c). Measures for fig. 5 are reported in Table 2.4.

Table 2.4.: Comet measures generated by the CometScore software from images 4, 5, 6 in Figure 5.4.

Measure	**Image number**		
	4	**5**	**6**
Comet Length (µm)	36	79	66
Tail Length (µm)	7.4	57	59
Comet Height (µm)	31	32	42
Comet Area (µm²)	771	1672	1176
Head Diameter (µm)	28	22.5	6
Head Area (µm²)	615	515	37
Tail Area (µm²)	156	1157.5	1139
Comet Intensity	179713	283586	280857
Comet Mean Intensity	24	18	25
Head Intensity	153145	97932	10159
Head Mean Intensity	26	20	28.5
Tail Intensity	26568	185654	270698
Tail Mean Intensity	18	17	25
%H-DNA	85	34.5	4
%T-DNA	15	65.5	96
Tail Moment	3.4	115	177
Olive Tail Moment	6	64.5	113.5

Intensity (Comet, Head and Tail) corresponds to the sum of pixels intensity. Mean intensity (Comet, Head and Tail) corresponds to the mean intensity of pixels.

2. 2. c Ghost, Clouds or Hedgehogs in the neutral Comet assay

Neutral Comet assay images in which the head was completely separated from the tail are termed Ghost, Clouds or Hedgehogs (Figure 5.4.; image 6 a, b, c) because of their similarity to the spiny mammals (Collins et al., 2008) and were visually identified, recorded and scored as percentage of the total sperm counted. Previous studies suggest these comet images represent a *high level* of DNA "damage" such as apoptosis or necrosis (Kumaravel et al., 2009).

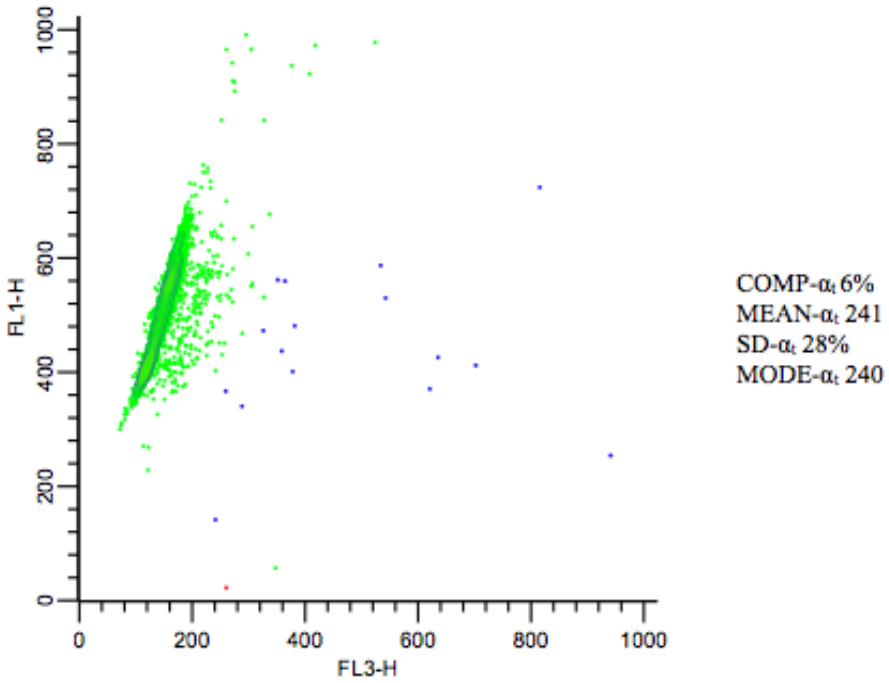
2. 3. Sperm Chromatin structure assay (SCSA)

The SCSA was performed as previously described by Love and Kenney (1998). Briefly, frozen semen samples were thawed in a 37°C water bath and 2-7 µl aliquot of semen was diluted to 200 µl in a buffer solution (0.186g disodium EDTA, 0.790 g Tris-HCl, 4.380 g NaCl in 500 ml deionized water, pH 7.4). This was mixed with 400 µl of acid-detergent solution (2.19 g NaCl, 1.0 ml of 2N HCl solution, 0.25 mL Triton-X, qs. 250 ml deionized water). After 30 s, 1.2 mL of the acridine orange (AO) solution was added (3.8869 g citric acid monohydrate, 8.9429 g Na_2HPO_4, 4.3850 g NaCI, 0.1700 g disodium EDTA, 4 µg/ml AO stock solution-1 mg/mL), qs. 500 ml water, pH 6.0. The sample was then allowed to equilibrate for 30 seconds on the flow cytometer. The cell flow rate was 100-200

cells/s. Following acquisition of 5000 cells the sip tube was thoroughly cleaned with a dry KimWipe to remove residual sample. Sperm from a control bull (i.e., good sperm quality based on a low percent of Cell outside main population-COMP-α_t) was used to standardize instrument settings prior to analysis of study samples. The flow cytometer was adjusted such that the mean green fluorescence was set at 500 channels (FI-1 ~ 500) and mean red fluorescence at 150 channels (FI-3 @ 150). This results in scatter plots displaying cells with fragmented DNA, non-fragmented DNA, and any present debris. The *main population* represents the spermatozoa that emit more green than red fluorescence due to the predominantly normal double-stranded configuration of their DNA. Sperm cells located to the right and down of this main population represent those cells that have an increase in the amount of red fluorescence and a decrease of green fluorescence when compared with spermatozoa in the main population. Data was stored in List-Mode and subsequently analyzed using WinList software (Verity Software House, Topsham, ME, USA). Measures included Mean-α_t (M-α_t), Standard Deviation-α_t (SD-α_t), COMP-α_t and Mode-α_t (Mo-α_t).

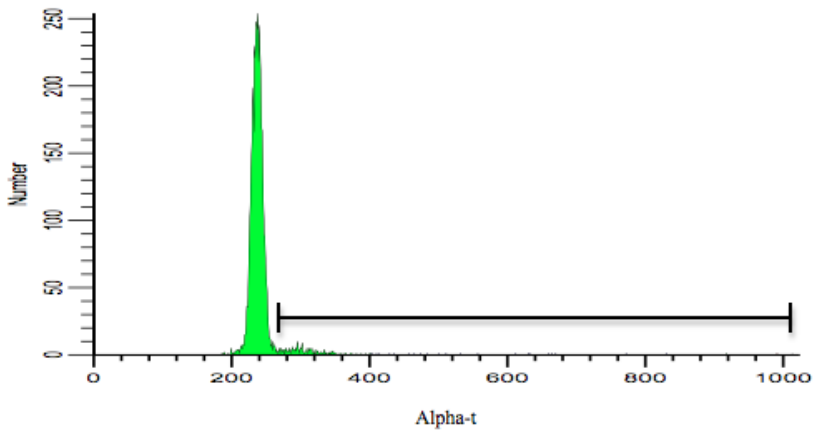
Different levels of COMP-α_t are presented in Figures 6.4.-11.4.

Figure 6.4.: SCSA Scattergram from a bull with low % COMP-a_t.



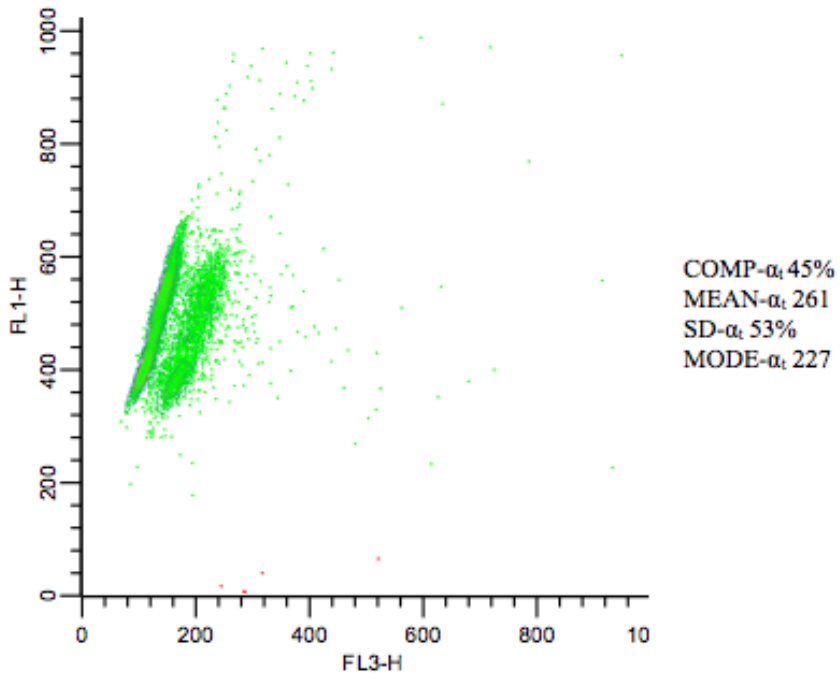
The X-axis (FL3-H) represents the red fluorescence and the Y-axis (FL1-H) the green fluorescence. The COMP-α_t, Mean-α_t, SD-α_t, Mode-α_t are reported on the right.

Figure 7.4.: One-dimensional histogram of the distribution of alpha-t values represented in Fig. 6.4.



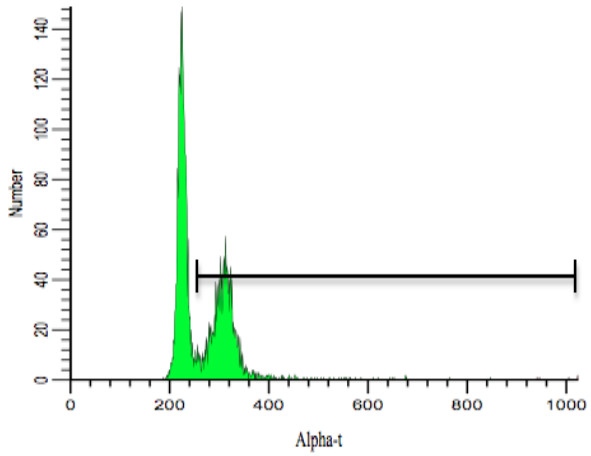
The X-axis represents the alpha-t that is the ratio between red fluorescence/ red + green fluorescence for each sperm in 1024 channels. The Y-axis represents sperm number. The bar represents the COMP-a_t.

Figure 8.4.: SCSA Scattergram from a bull with high % COMP-a_t.



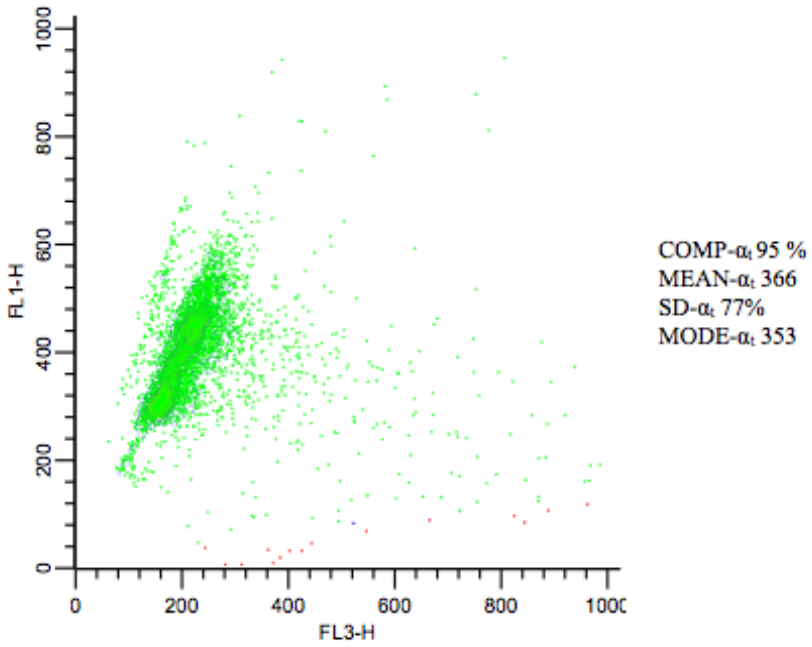
The X-axis (FL3-H) represents the red fluorescence and the Y-axis (FL1-H) the green fluorescence. The COMP-α_t, Mean-α_t, SD-α_t, Mode-α_t are reported on the right.

Figure 9.4.: One-dimensional histogram of the distribution of alpha-t values represented in Fig. 8.4.



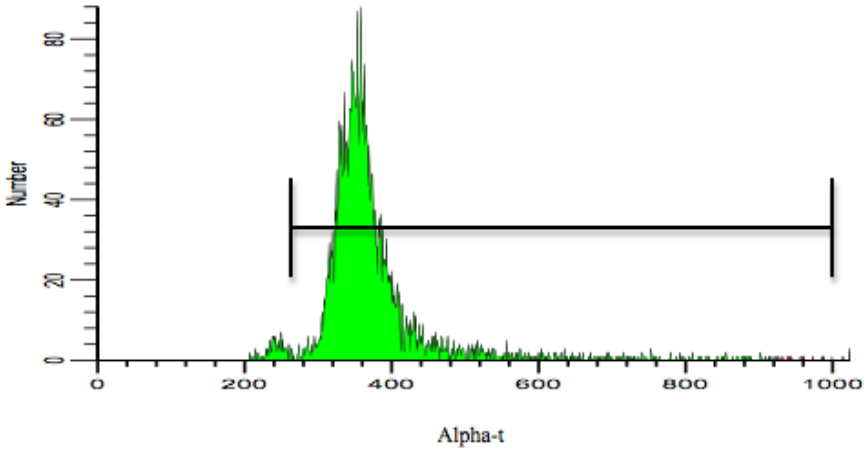
The X-axis represents the alpha-t that is the ratio between red fluorescence/ red + green fluorescence for each sperm in 1024 channels. The Y-axis represents sperm number. The bar represents the COMP-a_t.

Figure 10.4.: SCSA Scattergram from a bull with very high % COMP-a_t (95%).



The X-axis (FL3-H) represents the red fluorescence and the Y-axis (FL1-H) the green fluorescence. The COMP-α_t, Mean-α_t, SD-α_t, Mode-α_t are reported on the right.

Figure 11.4.: One-dimensional histogram of the distribution of alpha-t values represented in Fig. 10.4.



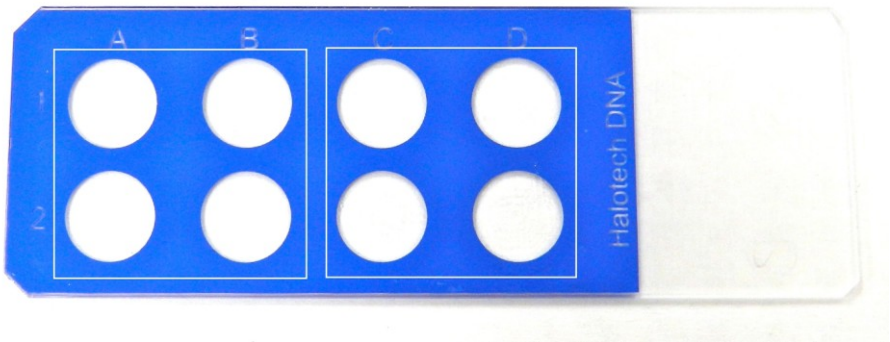
The X-axis represents the alpha-t that is the ratio between red fluorescence/ red + green fluorescence for each sperm in 1024 channels. The Y-axis represents sperm number. The bar represents the COMP-a_t.

2. 4. Sperm Bos Halomax (SBH)

The SBH (Sperm Bos Halomax, Bos Taurus, Halotech DNA, SL, Madrid, Spain) was performed as previously described (Halotech DNA, SL, Madrid, Spain). Briefly, sperm samples were thawed in a water bath at 37°C and diluted to 20 mil/ml in DPBS and 25 µl was transferred to an empty Eppendorf tube in a water bath at 37°C and 50 µl of liquefied agarose was added into the tubes and mixed. A drop of 2 µl of the mixture was spread onto the well of the SBH slide (shown in figure 12.4.) and every 4 wells were covered with a 22 x 22 mm glass coverslip. Each sperm sample was run in duplicate on different slides. The slide was maintained horizontally at 4°C in the refrigerator for 5 min, the coverslip was removed and the lysis solution was applied for 5 min. The slide was then washed for 5 min in distilled water, dehydrated in sequential ethanol solutions 2 min each (70% and 100%) and left to dry overnight at room temperature. The slide was then stained with propidium iodide (final concentration 0.03 µM) from a stock solution that was diluted (1: 40x; stock solution 1.2 µM) in deionized water and then 1:1 mixed with antifade mounting medium (Vectashield, Vector Laboratories, Burlingame, CA, USA). A final volume of 2 µl was used to stain each well and 300 sperm per sample were counted with an Olympus microscope model BX60, equipped with an objective U Plan FL N, magnification 40x/ 0, 0.75 Ph2, ∞/ 0.17. Sperm were categorized as those with presence or

absence of a halo.

Figure 12.4.: SBH slide (Sperm Bos Halomax, Halotech DNA, SL, Madrid, Spain).



Each box denotes a 22 x 22 glass coverslip that covers 4 wells.

2. 5. Statistical analysis

Statistical analysis was performed using SAS, version 9.2 (SAS Institute, Inc. Corp., Cary, NC, USA). Kolmogorv-Smirnov test was used to determine data normality distribution. The coefficient of variation (CV) was calculated to measure inter- and intra-assay variability. Spearman rank correlations were used to test the relationship between non-parametric sperm parameters. A general linear model procedure (GLM) was used to determine differences between means for sperm quality values between selected groups of bulls. P-values less than 0.05 were considered statistically significant.

3. Results

3. 1. Repeatability between days within the control

In the neutral Comet assay the lowest coefficients of variation (CV) were related to dimension measures and included comet length (11%) and height (11%), tail length (14%), head area (15%), head diameter (16%). The %T-DNA (13%) and the Olive tail moment (19%) were the only other variables with a CV less than 20%. The alkaline Comet assay measures less than 20% were comet height (11%) and comet length (18%). The coefficient of variation for the intensity measures between days within the control ranged from 21 to 34% in the neutral and from 37 to 59% in the alkaline (Table 3.4.

and 4.4.). The coefficient of variation for %H-DNA ranged from 25% in the neutral to 26% in the alkaline, whereas the %T-DNA varied from 13% in the neutral to 20% in the alkaline. The coefficient of variation for the tail moment ranged from 21% in the neutral to 47% in the alkaline, while the Olive tail moment ranged from 19% in the neutral to 35% in the alkaline.

3. 2. Comparison of neutral and alkaline Comet measures

The mean comet length was higher in the neutral (mean- 71±9 μm; range-62-78 μm) than in the alkaline (mean- 40±7 μm; range-31-45 μm). Tail length varied from 38-55 μm in the neutral assay and from 10-31 μm in the alkaline version. Also, the neutral assay had a larger head diameter (ranged 18-26 μm) than the alkaline (10-23 μm). The neutral Comet assay had a higher %T-DNA (58-76%), than the alkaline (37-65.5%). Tail moment and Olive tail moment were higher in the neutral versus the alkaline.

Table 3.4.: The mean±SD, range () and the coefficient of variation (CV) for the control measures of the neutral Comet assay.

Measure	Control neutral Comet	CV
Comet Length (μm)	71±9 (62-78)	11
Tail Length (μm)	48±7 (38-55)	14
Comet Height (μm)	45±5 (40-51)	11
Comet Area (μm²)	2322.5±526 (1725-2886)	23
Head Diameter (μm)	22±4 (18-26)	16
Head Area (μm²)	716±104 (613-886.5)	15
Tail Area (μm²)	1606±501 (998-2194)	31
Comet Intensity	372763±124808 (230032-566733)	34
Comet Mean Intensity	16.5±4 (12-21)	23
Head Intensity	116981±26520 (81406-139409)	23
Head Mean Intensity	17.5±4 (13-22)	24
Tail Intensity	255783±107031 (134055-427324)	42
Tail Mean Intensity	16±3 (12-20)	21
%H-DNA	34±9 (24-42)	25
%T-DNA	66±9 (58-76)	13
Tail Moment	105±21 (80-127)	20
Olive Tail Moment	66±12 (53-79)	19

Intensity (Comet, Head and Tail) corresponds to the sum of pixels intensity. Mean intensity (Comet, Head and Tail) corresponds to the mean intensity of pixels.

Table 4.4.: The mean±SD, range () and the coefficient of variation (CV) for the control measures of the alkaline Comet assay.

Measure	Control alkaline Comet	CV
Comet Length (μm)	40±7 (31-45)	18
Tail Length (μm)	24± 9(10-31)	37
Comet Height (μm)	36.5±6 (26.5-41)	11
Comet Area (μm²)	1130±381 (585-1443)	34
Head Diameter (μm)	15±5(10-23)	30
Head Area (μm²)	540±153 (271-643)	28
Tail Area (μm²)	590±262 (293-803)	45
Comet Intensity	162488± 95347(41985-297439)	59
Comet Mean Intensity	14±5 (7-21)	38
Head Intensity	69657±36997 (18707-120279)	53
Head Mean Intensity	13±5 (7-20)	37
Tail Intensity	92830±62273.5 (23278.5-177159)	67
Tail Mean Intensity	15±6 (8-23)	38
%H-DNA	43±11 (34.5-63)	26
%T-DNA	57±11 (37-65.5)	20
Tail Moment	46±21 (12-64.5)	47
Olive Tail Moment	29±10 (13-37)	35

Intensity (Comet, Head and Tail) corresponds to the sum of pixels intensity. Mean intensity (Comet, Head and Tail) corresponds to the mean intensity of pixels.

3. 3. Repeatability between replicates (i.e., between slides) within bull

Comet assay variables had higher repeatability between replicates (i.e., between slides) within the same bull (Table 5.4. and 6.4.) than the among-days repeatability for the control samples. The coefficient of variation for the intensity measures between slides ranged from 1-15% in the neutral assay and from 6-29% in the alkaline. The dimension measures ranged from 3 to 14% in the neutral and from 4 to 18% in the alkaline. The coefficient of variation for %H-DNA ranged from 5% to 10% in the neutral and alkaline assays, respectively; whereas the %T-DNA was 4% in both the neutral and alkaline Comet assays. The coefficients of variation were higher in the neutral than in the alkaline for tail (7 vs. 2%) and Olive moments (7 vs. 2%). Comet measures were higher in the neutral than in the alkaline for comet length (71 vs. 40 μm), tail length (48 vs 24 μm) and head diameter (22 vs 15 μm).

Table 5.4.: The mean±SD, range () and the coefficient of variation (CV) for the measures of the neutral Comet assay between replicates (n=2) within-bull.

Measure		**CV**
Comet Length(μm)	69±3 (67-71)	5
Tail Length (μm)	47±3 (45-49)	5
Comet Height (μm)	35±2 (33-37)	7
Comet Area (μm²)	1632±190 (1498-1766)	12
Head Diameter (μm)	22±1 (21.6-22.5)	3
Head Area (μm²)	584±49 (550-619)	8
Tail Area (μm²)	1048±141 (948-1148)	14
Comet Intensity	269859±32271 (247040-292678)	12
Comet Mean Intensity	17±1 (16.8-17)	1
Head Intensity	105429±7196 (100340-110517)	7
Head Mean Intensity	18.5±1 (18.3-18.7)	2
Tail Intensity	164430±25075 (146700-182161)	15
Tail Mean Intensity	15.5±1 (15.4-15.6)	1
%H-DNA	43±2 (41.3-44.5)	5
%T-DNA	57±2 (55.5-58.7)	4
Tail Moment	98±7 (92.6-102.7)	7
Olive Tail Moment	65±4 (62-68)	7

Intensity (Comet, Head and Tail) corresponds to the sum of pixels intensity. Mean intensity (Comet, Head and Tail) corresponds to the mean intensity of pixels.

Table 6.4.: The mean±SD, range () and the coefficient of variation (CV) for the measures of the alkaline Comet assay between-replicates (n=2) within-bull.

Measure		**CV**
Comet Length (μm)	42.6±3 (41-44)	6
Tail Length (μm)	29.4±2 (28.5-30.3)	4
Comet Height (μm)	33.7±2 (32.4-35)	5
Comet Area (μm²)	1065±129 (974-1157)	12
Head Diameter (μm)	13±1 (12-14)	10
Head Area (μm²)	333±60 (291-375)	18
Tail Area (μm²)	732±70 (683-782)	10
Comet Intensity	174480±34807 (149867-199092)	20
Comet Mean Intensity	15.6±1 (14.8-16.3)	7
Head Intensity	51275±14697 (40883-61667)	29
Head Mean Intensity	15±1 (14-15.3)	7
Tail Intensity	123205±20111 (108984-137425)	16
Tail Mean Intensity	16±1 (15.5-17)	6
%H-DNA	27.6±3 (25.7-29.5)	10
%T-DNA	72.4±3 (70.5-74.3)	4
Tail Moment	68.6±1 (67.6-69.5)	2
Olive Tail Moment	42±1 (41-43)	2

Intensity (Comet, Head and Tail) corresponds to the sum of pixels intensity. Mean intensity (Comet, Head and Tail) corresponds to the mean intensity of pixels.

3.4. Differences in age, body condition score, scrotal circumference and morphology between selected groups of study

There was no difference in age, BCS and SC between the groups with high and low sperm quality (Table 7.4.). Evaluation of sperm morphologic features based on the individual categories more than based on normality, primary and secondary abnormalities (BSE system) was more able to distinguish between the 2 groups. The percent morphologically normal sperm was higher in the high than the low sperm quality group (mean-81±6 vs. 43±18) in the samples fixed in buffered formol saline (Table 7.4.). The percent abnormal heads were higher in the high than the low sperm quality group (mean-6±3 vs. 11±7; $P<0.05$), as detached heads (mean-2±1 vs. 9±19; $P<0.05$), proximal droplets (mean-0.8±1 vs. 6.3±9; $P<0.05$), bent midpieces (mean-5±8 vs. 14±12; $P<0.05$), bent tails (mean-0.2±0.4 vs. 2±3; $P<0.05$), coiled tails (mean-3.5±3 vs. 12.3±8; $P<0.05$) and premature germ cells (mean-0.6±0.7 vs. 3.4±2; $P<0.05$).

Table 7.4.: The mean (± SD) and the range (in parentheses) for age, BCS, scrotal circumference (SC) and morphologic features (BSE system and individual morphologic categories) between selected groups of study.

Measure	Group high quality (n=10)	Group low quality (n=10)
Age	5±2 (3-8)	7±3 (3-11)
BCS	5.45±0.44 (5-6)	5.5±0.44 (5-6)
SC	41±3 (36-46)	43±4 (37-47)
Morphology classification		
Bull		
Normal	85±5 (74-93)	77±12 (52-85)
Primary	7±4 (4-17)	13±14 (5-45)
Secondary	7.5±2.5 (3-12)	10±4 (3-16)
Individual		
Normal*	81±6 (63-93)	43±18 (14-69)
AH	6±3 (1-13)	11±7 (0-21)
AA	0.4±0.7 (0-2)	0.5±0.7 (0-2)
DH	2±1.13 (1-4)	9±19 (0-62)
PD	0.8±1 (0-3)	6.3±9 (0-25)
DD	1.5±4.4 (0-14)	3±4 (0-11)
AM*	0.8±1 (0-3)	2±2 (0-5)
BM*	5±8 (0-24)	14±12 (0-39)
BT	0.2±0.4 (0-1)	2±3 (0-9)
CT*	3.5±3.1 (0-9)	12.3±8 (3-28)
PGC*	0.6±0.7 (0-2)	3.4±2 (1-9)

Morphology includes BSE system (normal, primary and secondary abnormalities) and individual morphologic categories, abbreviated as follows: AH-abnormal head, AA-abnormal acrosome, DH-detached head, PD-proximal droplet, DD-distal droplet, AM-abnormal midpiece, BM-bent midpiece, BT-bent tail, CT-coil tail and PGC-premature germ cells. For each group N=10.

* Within row means are different ($P<0.05$).

3.5. Differences in visual and automated analysis of sperm motility between selected groups of study

Percent total sperm motility estimated by microscopy was not different between groups (P>0.05; Table 8.4.). Total motility by CASMA (approximately 24 hours later than visual motility) was higher in the high than the low sperm quality group (mean-90±4 vs. 23±26; P<0.05), as progressive motility (mean-65±6 vs. 12±16; P<0.05), % rapid (RAP), (mean-81±6 vs. 15±20; P<0.05), average path velocity (VAP), (mean-107±12 vs. 48±27; P<0.05), straight line velocity (VSL), (mean-77±8 vs. 35±22; P<0.05), curvilinear velocity (VCL), (mean-207±34 vs. 96±36; P<0.05), lateral head amplitude (ALH), (mean-10±2 vs. 5±4; P<0.05).

Table 8.4.: The mean±SD, and range () for motility values between groups of high and low sperm quality.

Measure		High sperm quality (n=10)	Low sperm quality (n=10)
Visual Motility	Total	75±0 (75)	74±2 (70-75)
Motility by CASMA	Total∗	90±4 (83-95)	23±26 (0-74)
	Progressive∗	65±6 (55-79)	12±16 (0-43)
	RAP∗	81±6 (71-89)	15±20 (0-54)
	VAP∗	107±12 (92-126)	48±27 (18-88)
	VSL∗	77±8 (66-89)	35±22 (9-76)
	VCL∗	207±34 (161-264)	96±36 (53-145)
	ALH∗	10±2 (7.5-12.5)	5±4 (0-9)
	BCF	29±4 (26-39)	34±6 (20-43)
	STR	68±6 (59-77)	68±15 (43-92)
	LIN	37±7 (29-49)	35±12 (20-60)

Sperm motility values are expressed as total motility (visually scored), and measured by a CASMA system as total and progressive motility, % rapid (RAP), average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL), lateral head amplitude (ALH), beat cross frequency (BCF), straightness (STR) and linearity (LIN).

∗ Within row means are different ($P<0.05$).

3.6. Differences in SCSA measures, neutral Ghosts and SBH between selected groups of study

SCSA measures were different between sperm quality groups (Table 9.4.). Mean-α_t (M-α_t) was lower in the high than the low sperm quality group (mean-231±10 vs. 268±48; $P<0.05$), as Standard Deviation-α_t (SD-α_t, mean-30±9 vs. 69±34; $P<0.05$), and Cell outside main population (COMP-α_t, mean-6±1 vs. 39±27; $P<0.05$). Neutral Ghosts (N-Ghosts) were lower in the high than low quality group (mean-3±2 vs. 17±12; $P<0.05$).

Table 9.4.: The mean ± SD and the range (in parentheses) for SCSA, SBH and neutral Ghosts measures between sperm quality groups.

Measure	High sperm quality (n=10)	Low sperm quality (n=10)
M-α_t*	231±10 (205-242)	268±48 (224-365)
SD-α_t*	30±9 (16-43)	69±34 (41-160)
COMP-α_t*	6±1 (4-8)	39±27 (13-96)
Mo-α_t	228±11 (200-240)	240±42 (216-358)
N-Ghosts*	3±2 (0-5)	17±12 (6.5-44.5)
SBH	12±4 (8-20)	20±13 (7-45)

M-α_t -Mean-α_t, SD-α_t -Standard Deviation-α_t, COMP-α_t -Cell outside main population, Mo-α_t -Mode-α_t, N-Ghosts-neutral Ghosts, SBH - Sperm Bos Halomax.
* Within row means are different ($P<0.05$).

3. 7. Differences in neutral Comet measures between selected groups of study

Among the neutral intensity measures, the mean intensities (comet, head and tail) were not different between the selected groups of study (Table 10.4.; $P>0.05$). Head intensity was higher in the high than low sperm quality group (mean-125608±40760 pixels vs. 85249±39639; $P<0.05$), as among the dimension measures, head diameter (mean-24±3 μm vs. 20±5; $P<0.05$), head area (mean-703±95 μm² vs. 466±164; $P<0.05$) and comet area (mean-1865±578 μm² vs. 1400±331; $P<0.05$).

Table 10.4.: The mean ± SD and the range (in parentheses) for the measures of the neutral Comet assay between selected groups of study.

Neutral Comet measures		**Group high quality (n=10)**	**Group low quality (n=10)**
Comet	length	72±14 (43-88)	66±11 (51-86)
	area*	1865±578 (1048-2618)	1400±331 (881-1862)
	height	36±5 (29-45)	34±4 (27-39)
	Mean int	17±4 (12-23)	17±4 (10-22)
	intensity	311977±159869 (128358-584783)	227741±86252 (94717-348933)
Head	diameter*	24±3 (19-29)	20±5 (12-25)
	area*	703±95 (535-887)	466±164 (166-675)
	Mean int	19±4 (12-24)	19±4 (12-24)
	intensity*	125608±40760 (73839-207079)	85249±39639 (37476-138559)
	% DNA	45±14 (25-73)	40±16 (12-59)
Tail	length	48±15 (16-64)	46±14 (28-72)
	area	1162±535 (343-1963)	934±330 (411-1258)
	Mean int	16±4 (12-23)	15±4 (10-22)
	intensity	186370±129133 (48214-437098)	142492±74296 (41642-262678)
	% DNA	54±14 (27-74)	60±16 (41-88)
Moment	tail	91±37 (24-145)	104±46 (49-188)
	Olive	59±23 (20-94)	72±33 (35-134)

Comet measures are grouped as Comet (length, area, height, Mean int, intensity), Head (diameter, area, Mean int, % DNA), Tail (length, area, height, Mean int, intensity, % DNA), Moment (Tail and Olive). Intensity (Comet, Head and Tail) corresponds to the sum of pixels intensity. Mean intensity-Mean int (Comet, Head and Tail) corresponds to the mean intensity of pixels.* Within row means are different ($P<0.05$).

3.8. Differences in alkaline Comet measures between selected groups of study

Alkaline measures were similar between sperm quality groups (Table 11.4.; P>0.05).

Table 11.4.: The mean ± SD and the range (in parentheses) for the neutral COMET assay measures between selected groups of study.

Alkaline Comet measures		Group high quality (n=10) ∗	Group low quality (n=10)
Comet	length	43±9 (31-63)	42±6 (36-52)
	area	1122±427 (590-1869)	1008±344 (593-1676)
	height	34±6 (29-45)	33±6 (26-44)
	Mean int	16±5 (9-24)	15±4 (10-21)
	intensity	189014±121672 (55296-435013)	159945±80863 (58599-307849)
Head	diameter	13±4 (9-21)	13±4 (8-20)
	area	340±141 (171-619)	326±147 (147-542)
	Mean int	15±5 (9-24)	14.5±3.5 (10-20)
	intensity	53639±39797 (15908-148947)	48910±30617 (14008-95899)
	% DNA	26±8 (14-40)	29±13 (15-51)
Tail	length	31±7 (19-41)	28±7 (16-35)
	area	782±319 (368-1249)	683±291 (368-1160)
	Mean int	16±5 (9-24)	16±4 (10-22)
	intensity	135375±85400 (39388-286066)	111035±66698 (44591-231766)
	% DNA	74±8 (60-86)	71±13 (49-85)
Moment	tail	71±20 (36-91)	66±22 (25-93)
	Olive	43±11 (24-55)	41±11 (20-55)

Comet measures are grouped as Comet (length, area, height, Mean int, intensity), Head (diameter, area, Mean int, % DNA), Tail (length, area, height, Mean int, intensity, % DNA), Moment (Tail and Olive). Intensity (Comet, Head and Tail) corresponds to the sum of pixels intensity. Mean intensity-Mean int (Comet, Head and Tail) corresponds to the mean intensity of pixels. ∗ Within row means are not different (P>0.05).

4. Relationship between SCSA and neutral Comet measures

There was no correlation between Mean-α_t and neutral Comet measures ($P>0.05$), except with head area ($r= -0.67$; $P<0.05$). COMP-α_t and SD-α_t were correlated to head intensity ($r= -0.58$ and-0.49; respectively $P<0.05$). There was no correlation between SCSA measures and %T DNA, tail moment and Olive moment ($P>0.05$). SD-α_t was correlated with head area, head diameter and head intensity ($r= -0.63, -0.46, -0.49$; respectively $P<0.05$). COMP-α_t was correlated with head area, head intensity and head diameter ($r= -0.73, -0.52, -0.58$; respectively $P<0.05$). Mode-α_t was not correlated with any neutral measure ($P>0.05$).

4. 1. Relationship between SCSA and alkaline Comet measures

There was no correlation between SCSA and alkaline Comet measures ($P>0.05$).

4. 2. Relationship among neutral Comet assay measures

There was no correlation between the length measures (comet length and tail length) and neutral comet intensity measures (Table

12.4.), except tail intensity (r= 0.75; 0.64; respectively P<0.05). Comet length and tail length were correlated to tail area (r= 0.91; 0.8; respectively P<0.05), comet length with comet area (r= 0.81; P<0.05) and tail length with tail area (r= 0.80; P<0.05). Length measures (comet and tail) were correlated to %T-DNA (r= 0.82; 0.92; respectively P<0.05), tail moment (r= 0.84; 0.94; respectively P<0.05), Olive moment (r= 0.78; 0.9; respectively P<0.05), whereas the correlation between comet length and tail length with %H-DNA was negative (r= -0.82; -0.92; respectively P<0.05). There was a high correlation between comet area, comet height, head diameter, head and tail area (range- r= 0.47; 0.92; P<0.05; Table 12.4.) and the intensity values. Tail area was correlated to tail intensity (r= 0.92; P<0.05), %T-DNA, tail moment, Olive moment (r= 0.73; 0.72; 0.67; respectively P<0.05). The correlations between most neutral Comet intensity measures and %H-DNA, %T-DNA, tail moment, Olive moment was not significant (P>0.05), except tail intensity that was correlated to %T-DNA, tail moment, Olive moment (r= 0.67; 0.64; 0.95; respectively P<0.05). Tail intensity was correlated to %T-DNA (r= 0.67; P<0.05). % T-DNA was correlated to tail moment, Olive moment (r= 0.97; 0.95; respectively P<0.05), whereas the %H-DNA was negatively correlated to tail moment, Olive moment (r= -0.97; -0.95; respectively P<0.05).

Table 12.4.: Correlation coefficients among neutral Comet measures.

		Neutral														
		Comet				Head				Tail					Moment	
	Neutral	length area	height	Mean int	intensity	diameter	area	Mean int	intensity	%DNA	length	area	Mean int	intensity	%DNA	tail	Olive
Comet	length	0.81	0.60		0.64					-0.82	0.95	0.91		0.75	0.82	0.84	0.78
	area		0.85	0.47	0.9		0.53	0.50	0.65	-0.47		0.94	0.56	0.87	0.47	0.47	
	height			0.83	0.96			0.83	0.7	-0.46		0.84	0.87	0.91	0.46	0.46	
	Mean int							0.97	0.63				0.95	0.69			
	intensity							0.75	0.72	-0.45	0.46	0.87	0.84	0.96	0.45		
Head	diameter					0.92		0.88	0.56						-0.56	-0.56	-0.60
	area							0.83	0.45						-0.45	-0.47	-0.53
	Mean int							0.66				0.51	0.88	0.66			
	intensity												0.54	0.49			
	% DNA										-0.92	-0.73		-0.67	-1	-0.97	-0.95
Tail	length											0.80		0.64	0.92	0.94	0.90
	area												0.61	0.92	0.73	0.72	0.67
	Mean int													0.83	0.41		
	intensity														0.67	0.64	0.60
	% DNA															0.97	0.95
Moment	tail																0.99

Comet measures are grouped as Comet (length, area, height, Mean int, intensity), Head (diameter, area, Mean int, % DNA), Tail (length, area, height, Mean int, intensity, % DNA), Moment (tail and Olive). Intensity (Comet, Head and Tail) corresponds to the sum of pixels intensity. Mean intensity-Mean int (Comet, Head and Tail) corresponds to the mean intensity of pixels. r-values are significant at least at $P<0.05$.

4.3. Relationship among alkaline Comet assay measures

There was a correlation between the alkaline dimension measures of tail and head area, comet height, head diameter, comet and tail length, comet area (range r= 0.48 to 0.97; $P<0.05$) and the intensity values (Table 13.4.). Comet and tail length were correlated to tail area (r= 0.95 and 0.88; respectively, $P<0.05$), comet length with comet area (r= 0.95; $P<0.05$) and tail length with tail area (r= 0.72; $P<0.05$). Comet length had no correlation with %T-DNA ($P>0.05$), whereas tail length was positively correlated to % T-DNA (r= 0.71; $P<0.05$). The correlation between comet and tail length with tail moment and Olive moment was high (range r= 0.72 and -0.97; respectively, $P<0.05$). There was a correlation between tail length with %T DNA (r= 0.71; $P<0.05$). Tail area was correlated to tail intensity (r= 0.95; $P<0.05$), tail moment and Olive moment (r= 0.81 and 0.82; respectively, $P<0.05$). Tail intensity was correlated to Olive moment and tail moment (r= 0.69 and 0.67; respectively, $P<0.05$). %T-DNA was correlated to tail moment and Olive moment (r= 0.87 and 0.84; respectively $P<0.05$), whereas the %H-DNA was negatively correlated to tail moment and Olive moment (r= -0.87 and -0.84; respectively, $P<0.05$). Tail moment and Olive moment were highly correlated to each other (r= 0.99; $P<0.05$).

Table 13.4.: Correlation coefficients among alkaline Comet measures.

		Alkaline																	
		Comet					Head				Tail					Moment	
	Alkaline	length	area	height	Mean int	intensity	diameter	area	Mean int	intensity	%DNA	length	area	Mean int	intensity	%DNA	tail	Olive
Comet	length		0.95	0.88	0.78	0.93	0.46	0.53	0.73	0.63		0.86	0.95	0.78	0.96		0.72	0.76
	area			0.97	0.84	0.96	0.61	0.69	0.73	0.70		0.72	0.94	0.86	0.97		0.58	0.61
	height				0.82	0.91	0.59	0.68	0.69	0.63		0.65	0.92	0.87	0.93		0.54	0.55
	Mean int					0.67	0.76	0.97	0.85			0.50	0.71	0.92	0.78		0.54	0.55
	intensity									0.48			0.45	0.18	0.65	-0.48		
Head	diameter							0.96	0.65	0.89	0.58			0.67	0.48	-0.58		
	area								0.71	0.93	0.54			0.76	0.58	-0.54		
	Mean int									0.87			-0.60	0.92	0.78			
	intensity										0.48		0.45	0.81	0.65	-0.48		
	% DNA											-0.71	-0.51			-1	-0.87	-0.84
Tail	length												0.88	0.5	0.81	0.71	0.96	0.97
	area													0.74	0.95	0.51	0.81	0.82
	Mean int														0.88			
	intensity																0.67	0.69
	% DNA																0.87	0.84
Moment	tail																	0.99

Comet measures are grouped as Comet (length, area, height, Mean int, intensity), Head (diameter, area, Mean int, % DNA), Tail (length, area, height, Mean int, intensity, % DNA), Moment (tail and Olive). Intensity (Comet, Head and Tail) corresponds to the sum of pixels intensity. Mean intensity-Mean int (Comet, Head and Tail) corresponds to the mean intensity of pixels. r-values are significant at least at $P<0.05$.

4.4. Relationship among neutral and alkaline Comet assay measures

There was no correlation between the neutral Comet assay measures of comet and tail length, % DNA (head or tail), moment (tail or Olive) and any of the alkaline Comet assay measures ($P>0.05$). Comet and tail area, comet height head diameter and head area were correlated (range from alkaline comet area with neutral head area $r= 0.45$ to 0.74 alkaline comet area with neutral comet height; $P<0.05$; Table 14.4.). The intensity measures of the alkaline with the neutral ranged from $r= 0.45$ ($P<0.05$) between alkaline and neutral head intensity, to $r= 0.78$ between alkaline and neutral comet intensity. The alkaline tail moment was correlated to neutral head intensity ($r= 0.45$; $P<0.05$) and alkaline Olive tail moment was correlated to neutral tail intensity ($r= 0.45$; $P<0.05$). Alkaline tail moment and Olive tail moment were correlated to the neutral comet area ($r= 0.52$ and 0.53; respectively, $P<0.05$) and neutral tail area ($r= 0.47$ and 0.48; respectively, $P<0.05$).

Table 14.4.: Correlation coefficients between neutral and alkaline Comet measures.

		Neutral								
		Comet				Head			Tail	
Alkaline		area	height	Mean int	intensity	diameter	area	Mean int	area	Mean int	intensity
Comet	length	0.69	0.72	0.65	0.78			0.69	0.65	0.59	0.70
	area	0.65	0.74	0.68	0.74	0.59	0.45	0.75	0.57	0.56	0.60
	height	0.61	0.71	0.68	0.69	0.67	0.50	0.76	0.50	0.53	0.52
	Mean int	0.58	0.67	0.65	0.72	0.44		0.69	0.55	0.60	0.63
	intensity	0.65	0.74	0.69	0.78	0.49		0.73	0.60	0.62	0.68
Head	diameter		0.45								
	area		0.57	0.57	0.50			0.57		0.49	
	Mean int	0.57	0.65	0.61	0.72			0.61	-0.58	0.62	0.68
	intensity	0.45	0.61	0.57	0.62			0.54	0.43	0.56	0.58
	% DNA										
Tail	length	0.63	0.56	0.49	0.67			0.54	0.59	0.46	0.61
	area	0.67	0.66	0.60	0.70	0.57	0.46	0.68	0.58	0.48	0.58
	Mean int	0.56	0.66	0.66	0.69	0.55		0.71	0.49	0.58	0.57
	intensity	0.66	0.71	0.67	0.76	0.55		0.73	0.59	0.57	0.64
	% DNA										
Moment	tail	0.52			0.49				0.47		
	Olive	0.53			0.51				0.48		0.45

Comet measures are grouped as Comet (area, height, Mean int, intensity), Head (diameter, area, Mean int), Tail (area, Mean int, intensity). Intensity (Comet, Head and Tail) corresponds to the sum of pixels intensity. Mean intensity-Mean int (Comet, Head and Tail) corresponds to the mean intensity of pixels. r-values are significant at least at least at $P<0.05$.

4.5. Relationship between SCSA, SBH and Ghosts in neutral Comet assay

There was a correlation between COMP-α_t and neutral Ghosts ($r= 0.83$; $P<0.05$), whereas there was no correlation between COMP-α_t and SBH ($P>0.05$). Mean-α_t, SBH ($r= 0.58$; $P<0.05$) and neutral Ghosts ($r= 0.60$; $P<0.05$) were correlated, as SD-α_t with SBH ($r= 0.58$; $P<0.05$) and neutral Ghosts ($r= 0.79$; $P<0.05$). There was no correlation between neutral Ghosts and SBH ($P>0.05$).

4.6. Relationship between neutral Ghosts, neutral and alkaline Comet measures

Neutral Ghosts were correlated to neutral head area, head diameter and head intensity ($r= -0.83$; -0.59 and -0.54; respectively, $P<0.05$). Neutral Ghosts were not correlated to any of the alkaline Comet measures ($P>0.05$).

4.7. Relationship between SBH, neutral and alkaline Comet measures

SBH was not correlated to any of the neutral Comet measures ($P>0.05$). SBH was correlated to alkaline %H-DNA and %T-DNA ($r= 0.51$ and $r= -0.51$, respectively, $P< 0.05$).

5. Relationship among age, body condition score, scrotal circumference, sperm morphology and sperm motility measures

Age was positively correlated with scrotal circumference (r= 0.50; P<0.05) and among morphologic categories with premature germ cells (r= 0.48; P<0.05). Age was not correlated with any of the sperm motility measures (P>0.05). Body condition score (BCS) was not associated with any of the morphologic and motility values (P>0.05). For the bull BSE classification system, morphologically normal sperm were inversely correlated to primary and secondary abnormalities (r= -0.82 and -0.53; respectively, P<0.05) and among the individual morphologic categories (stallion classification system), morphologically normal sperm were correlated to bent midpieces (r= -0.54; P<0.05) and premature germ cells (r= -0.50; P<0.05). There was no correlation between normal sperm and total and progressive motility (respectively P>0.05), whereas there was a correlation between normal sperm with lateral head amplitude (ALH), curvilinear velocity (VCL), average path velocity (VAP), % rapid (RAP) and beat cross frequency (BCF), (r= 0.68; 0.61; 0.54; 0.51 and -0.58; respectively, P<0.05). Primary abnormalities were not correlated with any of the individual morphologic categories (stallion classification system) and either with total and progressive motility (P>0.05). There was a correlation between primary abnormalities and beat cross frequency (BCF) and lateral head amplitude (ALH), (r= -0.52 and -0.48; respectively, P<0.05).

Secondary abnormalities were correlated among individual morphologic categories with abnormal midpiece ($r= 0.49$; $P<0.05$) and among motility values with average path velocity (VAP), curvilinear velocity (VCL) straight line velocity (VSL), ($r= -0.70$; -0.64 and -0.58; respectively, $P<0.05$).

5.1. Relationship among individual sperm morphologic categories (stallion classification system) and motility measures

Normal sperm were correlated with all motility values (Table 15.4.), except with beat cross frequency (BCF), straightness (STR) and linearity (LIN) and among individual morphologic categories were inversely correlated with premature germ cells, coiled and bent tails, bent midpieces and proximal droplets ($r= -0.74$; -0.68; -0.50; -0.48 and -0.45; respectively, $P<0.05$). Any of the individual morphologic categories were correlated to STR and LIN ($P>0.05$). Abnormal heads were not correlated with any of the motility values ($P>0.05$) and among individual morphologic categories were correlated to proximal droplets and abnormal midpieces ($r= 0.73$; 0.56; respectively, $P<0.05$). Abnormal acrosomes and midpieces and distal droplets were not correlated to any of the motility values ($P>0.05$). Detached heads were correlated with coiled tails ($r= 0.61$; $P<0.05$) and among the motility values to total and progressive motility ($r= -0.65$ and -0.62; respectively, $P<0.05$). There was a

correlation between detached heads and % rapid (RAP), average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL), ($r = -0.59$; -0.49; -0.49 and -0.49; respectively, $P<0.05$), whereas there was no correlation between detached heads and lateral head amplitude (ALH), beat cross frequency (BCF), straightness (STR) and linearity (LIN); ($P>0.05$). Proximal droplets were correlated to premature germ cells ($r = 0.56$; $P<0.05$), but not with any of the motility values ($P>0.05$). Distal droplets and abnormal midpieces were not correlated with any morphologic abnormality and motility value (respectively $P>0.05$). Bent midpieces were correlated to bent tails ($r = 0.55$; $P<0.05$) and beat cross frequency (BCF, $r = 0.75$; $P<0.05$). Coiled tails were correlated to premature germ cells ($r = 0.47$; $P<0.05$) and most of the sperm motility values (range- $r = -0.64$ to -0.48 $P<0.05$). Premature germ cells were correlated to most of the motility values (range $r = -0.79$ to -0.60; $P<0.05$).

Table 15.4.: Correlation coefficients between individual sperm morphologic categories (stallion classification system) and motility values.

Individual morphology	Morphology						Motility							
	PD	AM	BM	BT	CT	PGC	Total	Progressive	RAP	VAP	VSL	VCL	ALH	BCF
Normal	-0.45		-0.48	-0.50	-0.68	-0.74	0.81	0.75	0.79	0.72	0.60	0.74	0.67	
AH	0.73	0.56												
DH					0.61		-0.65	-0.62	-0.59	-0.49	-0.49	-0.49		
PD						0.56								
BM				0.55										0.75
BT					0.50									
CT						0.47	-0.64	-0.60	-0.63	-0.52	-0.48	-0.51		
PGC							-0.77	-0.78	-0.79	-0.72	-0.60	-0.75	-0.73	

The individual morphologic categories are abbreviated as follows: AH-abnormal head, DH-detached head, PD-proximal droplet, AM-abnormal midpiece, BM-bent midpiece, BT-bent tail, CT-coil tail and PCG- premature germ cells. The motility values are abbreviated as % rapid (RAP), average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL), lateral head amplitude (ALH), beat cross frequency (BCF). r-values are significant at least at $P<0.05$.

5.2. Relationship among motility values

Sperm motility values were correlated with each other (Table 16.4.; $P<0.05$).

Table 16.4.: Relationship among sperm motility values.

		Motility						
		Progressive	RAP	VAP	VSL	VCL	ALH	LIN
Motility	Total	0.90	0.99	0.87	0.74	0.90	0.77	
	Progressive		0.89	0.81	0.83	0.81	0.60	0.53
	RAP			0.90	0.77	0.92	0.80	
	VAP				0.90	0.98	0.82	
	VSL					0.83	0.54	0.60
	VCL						0.88	
	ALH							
	BCF							
	STR							0.67
	LIN							

The motility values are abbreviated as % rapid (RAP), average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL), lateral head amplitude (ALH), beat cross frequency (BCF), straightness (STR) and linearity (LIN). r-values are significant at least at $P<0.05$.

5.3. Relationship between sperm motility and the neutral Comet assay

Neutral Ghosts were correlated with most motility values (range r= –0.80 to –0.60; P<0.05; Table 17.4.). Motility values were correlated to the head Comet measures and among those to head area (range from total and progressive motility with head area r= 0.68 to beat cross frequency-BCF r= –0.50; respectively, P<0.05). BCF was correlated to comet, head and tail intensity (r= -0.72; -0.53 and -0.51; respectively, P<0.05) and to comet, tail and head area (r= -0.72; -0.63 and -0.50; respectively, P<0.05). Linearity was correlated with %H-DNA and %T-DNA (r= 0.56 and -0.56; respectively, P<0.05), to tail intensity and Olive tail moment (r= -0.49 and -0.47; respectively, P<0.05).

Table 17.4.: Relationship among motility values, neutral Ghosts (N-Ghosts) and neutral Comet assay.

		Neutral Comet																
		Comet					Head				Tail					Moment	
Motility	N-Ghosts	length	area	height	Mean int	intensity	diameter	area	Mean int	intensity	%DNA	length	area	Mean int	intensity	%DNA	tail	Olive
Total	-0.77							0.68										
Progressive	-0.76							0.68										
RAP	-0.77							0.67										
VAP	-0.80						0.54	0.65		0.56								
VSL	-0.75						0.46	0.59		0.45								
VCL	-0.76						0.50	0.64		0.54								
ALH	-0.60		0.51				0.45	0.53		0.51								
BCF		-0.58	-0.72	-0.68	-0.45	-0.72		-0.50	-0.49	-0.53		-0.56	-0.63	-0.72	-0.51		-0.48	
STR																		
LIN											0.56				-0.49	-0.56		-0.47

Comet measures are grouped as Comet (length, area, height, Mean int, intensity), Head (diameter, area, Mean int, % DNA), Tail (length, area, height, Mean int, intensity, % DNA), Moment (Tail and Olive). Intensity (Comet, Head and Tail) corresponds to the sum of pixels intensity. Mean intensity-Mean int (Comet, Head and Tail) corresponds to the mean intensity of pixels. The motility values are abbreviated as % rapid (RAP), average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL), lateral head amplitude (ALH), beat cross frequency (BCF), straightness (STR) and linearity (LIN). r-values are significant at least at $P<0.05$.

5.4. Relationship between sperm motility and the alkaline Comet assay

There was no correlation between motility values and the alkaline Comet assay measures ($P>0.05$), except a correlation between linearity (LIN) and alkaline head mean intensity ($r= -0.55$; $P<0.05$).

5.5. Relationship between sperm motility and SCSA

Motility values were correlated to Mean-α_t, SD-α_t and COMP-α_t (ranging from $r= -0.82$ to -0.42 %; respectively, $P<0.05$; Table 18.4.). Motility values were not correlated to Mode-α_t ($P>0.05$). BCF, STR and LIN were not correlated to any of the SCSA measures ($P>0.05$).

Table 18.4.: Relationship between sperm motility values and SCSA.

Motility	SCSA		
	Mean-α_t	SD-α_t	COMP-α_t
Total	-0.45	-0.65	-0.81
Progressive	-0.54	-0.71	-0.78
RAP	-0.42	-0.69	-0.82
VAP		-0.77	-0.81
VSL		-0.70	-0.69
VCL		-0.73	-0.81
ALH		-0.63	-0.70

The motility values are abbreviated as % rapid (RAP), average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL), lateral head amplitude (ALH), beat cross frequency (BCF), straightness (STR) and linearity (LIN). SCSA measures are abbreviated as Mean-α_t, Standard Deviation-α_t (SD-α_t), Cell outside main population (COMP-α_t). r-values are significant at least at $P<0.05$.

5.6. Relationship between sperm motility and SBH

There was no correlation among motility values and SBH ($P>0.05$).

5.7. Relationship between sperm morphology (stallion classification system) and neutral Comet assay

Among individual morphologic categories (stallion classification system) % normal sperm were correlated to neutral Ghosts and among the neutral Comet measures to head area (r= -0.75 and 0.68; respectively, P<0.05; Table 19.4.). Abnormal heads, acrosomes and midpieces, proximal and distal droplets, and bent tails were not correlated to N-Ghosts nor to any of the neutral comet measures (P>0.05). There was a correlation between detached heads and neutral Ghosts (r= 0.53; P<0.05) and among the neutral Comet measures between detached heads and comet, head and tail mean intensity (r= 0.62; -0.59 and 0.56; respectively, P<0.05). Detached heads were correlated to head area (r= -0.56; P<0.05). There was no correlation between proximal and distal droplets with either neutral ghost either the neutral Comet measures (respectively P>0.05). There was a correlation between bent midpieces with tail length, comet length and tail intensity (r= -0.56; -0.46 and -0.47; respectively, P<0.05). Coiled tails were correlated to neutral Ghosts and head area (r= 0.78 and -0.57; respectively, P<0.05). Premature germ cells were related to neutral Ghosts (r= 0.62; P<0.05), whereas there was no correlation with any of the neutral Comet measures (P>0.05). The % normal sperm in the BSE classification system (chapter 2.1.c) were correlated to neutral ghosts (r= -0.61; P<0.05) and among the neutral measures to length (comet and tail r= 0.72; 0.65; respectively, P<0.05), area (comet and tail r= 0.67; 0.65;

respectively, $P<0.05$), intensity (tail and comet r= 0.61; 0.52; respectively), %H- and T-DNA (r= -0.58; 0.58; respectively, $P<0.05$) and moment (tail and Olive r= 0.55; 0.50; respectively $P<0.05$). Primary abnormalities were not correlated to neutral ghosts ($P>0.05$), whereas they were correlated among the neutral comet measures to length (comet and tail r= -0.72; -0.67; respectively, $P<0.05$), area (comet and tail r= -0.61; respectively, $P<0.05$), intensity (tail and comet r= -0.62 and -0.48; respectively, $P<0.05$), %H- and T-DNA (r= 0.57 and -0.57; respectively, $P<0.05$) and tail moment (r= -0.52; $P<0.05$). There was no correlation between primary abnormalities and Olive tail moment ($P>0.05$). Secondary abnormalities were correlated to neutral Ghosts (r= 0.51; $P<0.05$), whereas they were not correlated with any of the neutral Comet measures (respectively, $P>0.05$).

Table 19.4.: Relationship among morphology and neutral Comet assay measures.

Sperm morphology			Neutral																	
			Comet					Head					Tail					Moment		
		N-Ghosts	length	area	height	Mean int	intensity	diameter	area	Mean int	intensity	%DNA	length	area	Mean int	intensity	%DNA	tail	Olive	
Individual	Normal	-0.75							0.68											
	DH	0.53				0.62			-0.56	0.59					0.56					
	BM		-0.46										-0.56			-0.47				
	CT	0.78							-0.57											
	PGC	0.62																		
Bull BSE	Normal	-0.61	0.72	0.67			0.52					-0.58	0.65	0.65		0.61	0.58	0.55	0.50	
	Primary		-0.72	-0.62			-0.48					0.57	-0.67	-0.61		-0.62	-0.57	-0.52		
	Secondary	0.51																		

Comet measures are grouped as Comet (length, area, height, Mean int, intensity), Head (diameter, area, Mean int, % DNA), Tail (length, area, height, Mean int, intensity, % DNA), Moment (Tail and Olive). Intensity (Comet, Head and Tail) corresponds to the sum of pixels intensity. Mean intensity-Mean int (Comet, Head and Tail) corresponds to the mean intensity of pixels. Neutral ghosts are abbreviated as N-Ghosts. Morphology includes stallion classification system (individual morphologic categories), abbreviated as follows: DH-detached head, BM-bent midpiece, CT-coil tail and PCG- premature germ cells and the bull BSE classification system (normal, primary and secondary abnormalities). r-values are significant at least at $P<0.05$.

5.8. Relationship between sperm morphology and alkaline Comet assay

Among individual morphologic categories (stallion classification system) detached heads were correlated to alkaline comet length and tail mean intensity (r= 0.47; r= 0.44; respectively, $P<0.05$). Abnormal midpieces were correlated to head area, %H- and T-DNA (r= -0.51; -0.46; 0.46; respectively, $P<0.05$). In the BSE classification system, there was no correlation among % normal, primary and secondary abnormalities with alkaline Comet measures, except between secondary with head mean intensity (r= -0.48; $P<0.05$).

5.9. Relationship between sperm morphology, SCSA and SBH

There was no correlation among the individual morphologic categories either among BSE classification system with SBH (respectively $P>0.05$). Among the individual morphologic categories (stallion classification system) % normal was correlated to SCSA measures (ranged from normal with COMP-α_t r= -0.85 to normal with Mean-α_t r= -0.62; respectively, $P<0.05$; Table 20.4.). In the BSE classification system % normal were correlated to SD-α_t and COMP-α_t (respectively r= -0.50 and -0.49; respectively, $P<0.05$). Abnormal heads, acrosomes, proximal and distal droplets, and bent

midpieces were not correlated to any of the SCSA measures (respectively $P>0.05$). There was a correlation between detached heads with the Mode-α_t ($r= -0.45$; $P<0.05$). There was a correlation between abnormal midpieces and SD-α_t ($r= 0.45$; $P<0.05$) and between bent tails and COMP-α_t ($r= 0.46$; $P<0.05$). Coil tails were correlated to COMP-α_t, SD-α_t, Mean-α_t ($r= 0.63$; 0.55 and 0.48; respectively, $P<0.05$). There was a correlation between premature germ cells with COMP-α_t, SD-α_t, Mean-α_t ($r= 0.82$; 0.76 and 0.53; respectively, $P<0.05$). In the BSE classification system primary abnormalities were not correlated to any of the SCSA measures ($P>0.05$), whereas secondary abnormalities were correlated to SD-α_t and COMP-α_t ($r= 0.60$ and 0.50; respectively, $P<0.05$). There was no correlation among the other abnormalities with SBH nor with SCSA measures ($P>0.05$).

Table 20.4.: Relationship between sperm morphology and SCSA measures.

Sperm Morphology		SCSA			
		M-α_t	SD-α_t	COMP-α_t	Mode-α_t
Individual	Normal	-0.62	-0.74	-0.85	
	DH				-0.45
	AM		0.45		
	BT			0.46	
	CT	0.48	0.55	0.63	
	PGC	0.53	0.76	0.82	
Bull BSE	Normal		-0.50	-0.49	
	Secondary		0.60	0.50	-0.51

Measures are abbreviated as follows: SBH-Sperm Bos Halomax (), M-α_t-Mean-α_t, SD-α_t -Standard Deviation-α_t, COMP-α_t -Cell outside main population, Mo-α_t -Mode-α_t. Morphology includes stallion classification system (individual morphologic categories), abbreviated as follows: DH-detached head, AM-abnormal midpiece, BT-bent tail, CT-coil tail and PCG- premature germ cells and the bull BSE system (normal, primary and secondary abnormalities). r-values are significant at least at $P<0.05$.

6. Discussion

This study used a software program to objectively describe and measure comet size rather than subjectively through visual estimation (e.g., the presence or absence of a comet shape). The software allowed measurement of comet size and fluorescence intensity, which, in turn, allows determination of assay repeatability.

Previous studies have not described the day-to-day (inter-assay) repeatability of Comet assays. In our study, to measure inter-assay variability, a control sample was included in each batch of samples processed (i.e., only 10 samples could be placed in the electrophoresis chamber at a time). In general, for both assays, *dimension* measures (comet and tail length, comet height, head diameter and head area) had the lowest variability, while *intensity* measures were highest; and neutral had less variability than alkaline Comet assay measures. For both assays, inter-assay repeatability for *intensity* measures was also less than for the *dimension* measures while the percent DNA in the tail (% T-DNA) was the most repeatable measure, consistent with previous study (Collins et al., 2008). The composite measures (tail and Olive moments) had less variability in the neutral compared to the alkaline Comet assay (20, 19 vs. 47, 35; respectively). Between replicates (i.e., slides) assay (intra-assay) repeatability for the study bulls (n=20) was higher, and similar to that previously reported (Hughes et al., 1997), than inter-assay repeatability for the control sample; however, Hughes et al. (1997) did not measure inter-assay variability. Thus, one slide (i.e.,

50 sperm nuclei) rather than two may be satisfactory to evaluate the DNA quality using the Comet assay. These results suggest that most variability occurs among days due to sample processing (reagent preparation) rather than slide preparation. Further, it appears that neutral Comet assay *dimension* measures are more repeatable than *intensity* and moment (tail/Olive) measures. The moment measures exhibit variability primarily because they incorporate *intensity,* a highly variable measure, into their value. It is unclear which Comet assay (neutral or alkaline) (Singh et al., 1989; Haines et al., 1998; Zee et al., 2009; Enciso et al., 2011; Ribas Maynou et al., 2012a) may be more appropriate for evaluation of sperm DNA quality. In this study, the appearance and the length of the neutral and alkaline comet tails showed marked differences. The neutral comets are elongated compared to the rounder shaped alkaline comets (Figures 4.4. and 5.4.), suggesting that sperm DNA remains in a more compact form in the alkaline Comet resulting in fewer actual comet shapes. However, the head diameter and area are smaller in the alkaline compared to the neutral Comet, suggesting that less DNA remains following processing. Therefore, sperm DNA that is more "defragmented" prior to alkaline processing may result in the production of smaller fragments due to the alkaline conditions, that may not be visualized (Simon and Carrell, 2013) and may actually visually disappear, not form a comet and thus not be evaluated. Therefore, the actual image of the alkaline comet may not represent 100% of the sperm DNA prior to processing, but rather only the

remaining DNA that is more "resistant" to the alkaline conditions. The concept of "lost" DNA is also supported by the lack of "ghost" images in the alkaline Comet assay which suggests the conditions may dissolve this DNA and prevent it from being imaged. This finding is similar to a previous study in which irradiated human lymphocytes, exhibited highly "defragmented" DNA, caused by apoptosis, but were not identified under alkaline conditions (Czene et al., 2002). It is unclear whether "Ghosts" are a result of apoptosis or necrosis. However, Fairbairn et al. (1995) suggested that both the neutral and alkaline Comet assays should be able to detect apoptotic DNA since it is characterized by double-stranded breaks. Yasuhara et al. (2003) suggested that the neutral Comet assay could distinguish between apoptosis (longer comet tail and small head) and necrosis (shorter comet tail and larger head- in rat Jurkat cells). In another study, it was suggested that ghosts were an expression of apoptosis, as well as a high degree of DNA "damage" and necrosis (Rat-1 cells exposed to irradiation and to hydrogen peroxide), (Kumaravel et al., 2009). Ersson and Möller (2011) suggested that Ghosts were not scored because the software was not able to define the head measures. However, in our study, comet measures associated with a ghost image (Figure 5.4., image 6b; Table 2.4.) include a small head diameter and area, low head intensity, low percent of head DNA, and a high percent of tail DNA, as well as high tail and Olive tail moments. There were more "Ghosts" in the group with poorer compared to higher sperm quality (i.e., 17 vs.

3%). The ghosts were correlated with detached heads, coiled tails and premature germ cells, all sperm abnormalities that represent either immature sperm forms (coiled tails and premature germ cells) or sperm associated with prolonged sperm storage in the extracurrent reproductive tract (detached heads). Ghosts were also correlated with the SCSA measures, Mean-α_t, SD-α_t, and COMP-α_t, all of which measure DNA quality. Interestingly, in the poor sperm quality group, the percent COMP-α_t (39%) was twice the percent Ghosts (17%) suggesting that Ghosts may reflect a more "damaged" level of DNA, while the SCSA identifies a less "damaged" DNA construct as well as the more "damaged" sperm. Combined these resulted suggest that Ghosts represent a highly decondensed DNA form (i.e., immature or highly damaged) that is very susceptible to decondensing conditions and can be identified in the neutral Comet and the SCSA, but because of the strong alkaline conditions of the alkaline Comet assay, cannot be identified because of their complete dissolution.

In addition to the high pH the alkaline Comet assay detects alkaline labile sites, abasic sites generated by spontaneous base loss (purines or pyrimidines) and converted to single-stranded breaks under strong alkaline conditions (Muriel et al., 2004). Therefore, the alkaline may produce more DNA breaks than the neutral Comet assay thereby resulting in the potential of losing more DNA during processing. As previously reported by Singh et al. (1989) the high level of human sperm DNA migration in alkaline conditions may be

due to the presence the alkaline labile site, which, under normal conditions, may play a protective role before and during fertilization, and may represent the starting point for DNA decondensation in the embryo. In the current study, "Ghosts" were negatively correlated with neutral Comet measures (i.e., head diameter, area, intensity); however, they were not correlated with any of the alkaline Comet measures, therefore the neutral Comet assay may be more appropriate for the evaluation of sperm DNA since it can identify these ghost forms.

This study also compared the ability of four different DNA assays to detect changes in DNA quality between two groups of bulls selected based on their initial percent of morphologically normal sperm and motility values (low vs. high). Most SCSA measures (Mean-α_t, SD-α, COMP-α_t), and percent neutral Ghosts identified differences in DNA quality between the two groups; however, the SBH values were not different. In addition, neutral Comet values (comet area, head diameter, area, and intensity) were higher in the group with higher sperm quality whereas no alkaline Comet measure was different between the two groups. Most SCSA measures (Mean-α_t, SD-α, COMP-α_t) were inversely correlated with neutral Comet head measures (diameter, area, and intensity) suggesting that a decrease in head dimension is associated with a decline in DNA quality; however, there was no association with alkaline Comet assay measures. SBH was correlated ($r= 0.58$) to the Mean-α_t and SD-α_t, but not the COMP-α_t or the percent of neutral

Ghosts. This is in contrast to a previous study on human sperm (Chohan et al., 2006), in which the SCDt (Halotest in humans) was correlated ($r= 0.86$; $P<0.001$) to the DNA fragmentation index (% DFI, same as COMP-α_t). Another study (García-Macías et al., 2007) in bulls reported that SCSA and SBH predicted small changes in fertility (i.e., non-return rate-NNR) comparing the DNA quality of 60 bulls divided in 3 fertility groups, even though there was no correlation between SBH and SCSA parameters. Differences among studies may be due to variation among individuals, operator subjectivity, especially for the SBH and the Comet assays, which are more subjective measures, as they evaluate microscopically few hundreds cells, requiring well trained operators and longer time compared to the flow cytometer that is a more robust technique, based on the ability of the flow cytometer to analyze thousands cells in few minutes.

Future studies should be conducted in order to identify the relationship of the Comet assays and the SBH with fertility in bulls. Currently in bulls the SCSA may (Karabinus et al., 1990; Ballachey et al., 1988) or may not (Hallap et al., 2005; Nagy et al., 2013) be correlated with fertility. The objectives of future studies would be to determine the relationship between the Comet assays, the SBH and fertility, in order to determine the usefulness of those assays in animal husbandry.

Historically, sperm quality is evaluated using sperm motility and morphologic features. The two groups of bulls in this study were

determined based on sperm morphology to create different levels of sperm quality that would allow testing of DNA quality among DNA assays. Two sperm morphology classification systems (bull and stallion) were compared. Both systems identify morphologically normal sperm, but the bull system places abnormalities into broad categories (i.e., primary, secondary) based on the origin of the abnormality (testis, epididymis), whereas the stallion classification identifies specific abnormalities (i.e., abnormal head, abnormal midpiece). Using the bull system there was no difference in the percent of normal sperm or the abnormality type between the two bull groups; whereas, in the stallion system percent normal sperm were higher in the high sperm quality group, which was also accompanied by an increase in specific abnormalities. The latter finding is not surprising since the two groups were originally classified using the stallion system. It is however interesting that the bull system did not discriminate the two groups based on the percent normal sperm. This result is likely due to the fact that two different individuals evaluated each sperm sample, one familiar with the bull system, the other familiar with the stallion classification system. Total sperm motility was higher in the high compared to the low sperm quality group (90 vs. 23%) and, combined with the stallion classification system for morphology indicated that the two groups were in fact different with respect to traditional sperm quality features.

We also considered body condition score, age and the scrotal circumference of those bulls. The lack of correlation between BCS and sperm quality was probably due to the good body condition of the group. The age was correlated to an increase in scrotal circumference.

Most individual morphologic features were correlated with sperm motility values assessed by CASMA, while the bull classification system (% normal, primary and secondary) showed few correlations with motility values. Specifically using the individual classification system, morphologically normal sperm were correlated with most motility values, whereas individual abnormalities such as detached heads, coiled tails and premature germ cells were negatively associated to motility values (Table 15.4.), suggesting that a high incidence of those abnormalities in a sperm sample can reduce sperm motility. CASMA motility values were correlated to the DNA quality assays, which is similar to a previous study on human sperm that reported a weak correlation between neutral Ghosts (visually scored as cells with high DNA damage) and % motile sperm ($r= -0.17$; $P<0.01$), (Trisini et al., 2004). In our study the neutral Ghosts were correlated to most motility measures and neutral head area. Further, the SCSA measures, COMP-α_t, SD-α_t and Mean-α_t were all correlated to the motility measures (Table 18.4.), while there was no correlation between motility and any of the alkaline Comet measures, except for the alkaline head intensity. This is in contrast with a study on human

sperm (Simon and Lewis, 2011a), in which a weak negative correlation between progressive motility and DFI% measured by the alkaline Comet has been reported (r^2= -0.21; P<0.05). Neutral Ghosts were negatively correlated with % normal sperm, for both classification systems, and were positively correlated with detached heads, coiled tails and premature germ cells (Table 19.4.) suggesting that these abnormalities are representative of sperm with abnormal DNA quality. Premature germ cells and coiled tails were also associated with COMP-α_t, Mean-α_t and SD-α_t (Table 20.4.), whereas they were not associated to any of the alkaline measures. This is in contrast to a previous study (Irvine et al., 2000) that reported a relationship in a group of fertile and infertile men between traditional features of sperm concentration, motility and morphology and % T-DNA and Olive tail moment, using the alkaline Comet assay (pH-12.3). This is in accordance with a previous study (Hughes et al., 1996) that used the alkaline Comet assay (pH-13) to compare sperm DNA from a group of fertile and infertile men. No difference was found in the baseline level of DNA "damage", while a difference was found when sperm were exposed to irradiation with X-rays, increasing the damage in sperm from infertile but not from fertile men. The lack of a standardized protocol and differences among species and among evaluation methods make the results between studies difficult to compare. Therefore, especially within a species, it is essential to compare assays of DNA quality across a wide range of known sperm quality variation. While

it cannot be assumed that wide variation in conventional sperm quality variables such as motility and morphology will also indicate similar variation in DNA quality, it was the initial approach that was taken in this study. Following the initial screening using sperm morphology and sperm motility the bull sperm samples were evaluated using the Sperm Chromatin Structure Assay (SCSA), a test routinely used and validated in our laboratory. The SCSA subsequently confirmed that we had a population of sperm samples that would exhibit variability in DNA quality (Table 9.4.) that could then be measured using the neutral and alkaline Comet assays as well as the SBH.

However, in this group of study, among the assays tested, the alkaline Comet assay and the SBH showed no difference between groups of bulls with high and low sperm quality. Also, the SBH had no correlation either with motility or morphology. In conclusion, this is the first study to compare neutral and alkaline Comet assay, the SCSA and the SBH, in a group of bulls selected on traditional features of sperm motility and sperm morphology. Among the sperm DNA quality tests the SCSA and the neutral Comet assay, scored visually by identifying neutral Ghosts and by using a software, were the assays able to distinguish between the high and low sperm quality groups. The alkaline Comet assay and the SBH were not able to differentiate between groups with different sperm quality and they were not correlated to sperm motility and morphology, because the alkaline assay may actually dissolve part of the DNA, and therefore

no comet is visualized. The SBH, similar to the alkaline Comet assay, may dissolve DNA or the assay conditions may not be sufficient to relax the DNA and allow discrimination between sperm of greater and lesser DNA quality. The Comet software provided objective image measures and allowed for the determination that the neutral head *dimension* measures were correlated to sperm motility and morphology, as well as SCSA measures. Ghosts were able to differentiate between the groups with different sperm quality and considering the correlation with SCSA measures, as well as with sperm motility and morphology, researchers should take into account those cells with a high migration of DNA as an important component of the neutral Comet assay. For the Comet assay standardization would be beneficial, in order to allow comparisons of results from different laboratories. In this group of bulls the head measures were more reliable than the tail measures. This may be different among species, reflecting differences in the DNA condensation process typical for each species. Future studies should be conducted to determine a possible correlation between neutral Comet assay and fertility, in order to investigate the benefit of this assay in practice.

CHAPTER 5

EXPERIMENT 3

Sperm DNA quality in Miniature Horse stallions following unilateral orchiectomy

Abstract

Unilateral orchiectomy may interfere with thermoregulation of the remaining testis caused by inflammation surrounding the incision site, thus altering normal spermatogenesis and consequently sperm quality. A previous study reported no transient negative effect on sperm quality from the remaining testis following unilateral orchiectomy (UO) in Miniature Horse stallions [McCormick JD, Valdez R, Rakestraw PC, Varner DD, Blanchard TL, Cavinder CA, et al. Effect of surgical technique for unilateral orchiectomy on subsequent testicular function in Miniature Horse stallions. Equine Vet J 2012; 44 (S43): 100-104].

In the present study, using sperm samples from the previous study, two measures of sperm DNA quality (neutral Comet assay and the Sperm Chromatin Structure Assay-[SCSA]) were compared at pre-UO (0 d) and at 14, 30, 60 days post-UO to determine if sperm DNA changed following a mild testis stress (i.e., unilateral orchiectomy). The percent DNA in the comet tail was higher at 14 and 60 d compared to 0 d ($P<0.05$) post-UO. All other comet tail measures (i.e., length, moment, migration) were higher at all time periods post-UO compared to 0 d ($P<0.05$). Two SCSA measures (Mean-α_t, Mode-α_t) increased at 14 d post-UO ($P<0.05$), while two measures

(SD-α_t and COMP-α_t) did not change ($P>0.05$). This study identified a decrease in sperm DNA quality using both the neutral Comet assay and the SCSA, which was not identified using traditional measures of sperm quality.

1. Introduction

In domestic mammals, normal spermatogenesis is a temperature dependent process, ensured by maintaining the testes below body temperature (Setchell BP., 1977).

Increased testicular temperature following scrotal insulation or elevated ambient temperature may alter spermatogenesis, resulting in a decrease in sperm quality of men (Mieussett et al., 1987), bulls (Brito et al., 2003; Vogler et al., 1993), stallions (Freidman et al., 1991; Blanchard et al., 1996) and boars (Malmgren and Larsson, 1984). In addition, clinical conditions such as varicocele, spermatic cord torsion, as well as a variety of genetic, metabolic and environmental factors have been associated with chromatin abnormalities in men (Aitken et al., 2013), bulls (Rahman et al., 2011), and stallions (Love and Kenney, 1999). The effect of heat stress on stallion sperm quality has been studied previously using models that insulate the scrotum, revealing an associated decline in sperm motility, morphology, and DNA quality, as well as total sperm numbers (Freidman et al., 1991; Blanchard et al., 1996; Love and Kenney, 1999). In contrast, physical exercise during hot

weather was modeled to study the effects of moderate to strenuous exercise on sperm quality (Mawyer et al., 2012; Rosenberg et al., 2013). In both studies, despite an increase in both body and scrotal temperatures, sperm quality (i.e., sperm motility, morphology, DNA quality, and total sperm number) was not affected. These findings suggest that the thermoregulation mechanisms that control testis temperature are able to compensate to training and ambient conditions to maintain sperm quality. Combined, these results suggest that environmentally induced testis stress can affect many sperm quality measures (e.g., insulation) or none (e.g., exercise). Previous models in mice (heat stressed) (Paul et al., 2009) and bulls (irradiated sperm) (Fatehi et al., 2006) as well as clinical data in humans (Saleh et al., 2003) indicate that sperm with abnormal DNA are capable of fertilizing, but lack the ability to maintain pregnancy with embryos usually lost prior to the morula stage.

In horses, early embryonic death (EED) that occurs following ultrasonographic diagnosis (i.e., >12-14 post-ovulation) is usually attributed to mare factors such as age and reproductive status; however, pregnancy failure prior to this time is often assumed to be caused by fertilization failure. Therefore, it is of particular interest to study changes in sperm DNA in otherwise normal sperm (based on classical measures of sperm morphology and motility). A previous complementary study found no effect of UO on sperm motility, viability, morphology, and total sperm numbers at 14, 30, or 60 days post-UO (McCormick et al., 2012), but DNA quality was not

evaluated.

The aim of this study, using semen samples from the previous complementary study, was to evaluate sperm DNA quality using two different assays (neutral Comet assay and SCSA) to determine if DNA quality was changed following a mild scrotal stress (i.e., scrotal swelling following UO) even though traditional sperm quality measures did not change.

2. Materials and Methods

Semen was collected from 9 mature reproductively normal Miniature Horse stallions after unilateral orchiectomy, using a Miniature Horse mare for sexual stimulation, a breeding phantom for mounting and a 33-cm length Missouri-model artificial vagina (Missouri-Style AV-C26716N- Nasco, Ft. Atkinson, Wisconsin, USA), and processed as previously reported (McCormick et al., 2012). For the neutral Comet assay and the SCSA, semen was evaluated once before the surgery and twice at each of 3 time periods post-UO: at 14, 30 and 60 days following unilateral orchiectomy.

2. 1. Neutral Comet assay (single cell gel electrophoresis)

The protocol for the Comet assay was modified from a previous study (Tice et al., 2000). Raw semen was diluted in 1 mL

cryovial tube with Dulbecco's phosphate-buffered saline (DPBS) to obtain a concentration of 0.5 million sperm/mL of DPBS and flash-frozen at -80°C. Frozen samples were thawed in a water bath at 37°C for 30 s, pipetted thoroughly to reduce sperm clumps and then placed on microscope slides (CometSlide™ 2 well/slide; Trevigen Inc. Gaithersburg, MD). In addition, a sperm sample of a control stallion was included in each trial to monitor technique and interassay repeatability. Slides were maintained in a horizontal position. Two-hundred and fifty (250) µL of low melting point agarose (LMPA; Trevigen Inc. Gaithersburg, MD) was melted in a commercial microwave at 50% power for 20 s, then transferred to microcentrifuge tubes (1.5 mL) with snap caps (VWR International, LLC Radnor, PA, USA) and floated in a water bath at 37°C. Two and six-tenths (2.6) µL of the sperm/DPBS dilution (containing approximately 1300 sperm) were added to each microcentrifuge tube containing LMPA at 37°C and vortexed for 10 s. Seventy-five (75) µL of semen/LMPA mixture were quickly pipetted onto each Comet slide well (~750 sperm per slide). The slides were placed in a refrigerator for 5 min. Slides were then set in a rack (CometSlide™ Rack System Trevigen Inc. Gaithersburg, MD) and immersed in a cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris HCl, 1% Triton X-100, 20 mM Dithiothreitol, pH 10) for 1 h in the refrigerator, followed by immersion in the same lysis solution with the addition of 0.1 mg/mL Proteinase K (Proteinase K from Tritirachium album, MP Biomedicals, LLC, CA; USA) for 2.5 h at

37°C. The solutions were freshly made before the sample processing and stored, respectively, in the refrigerator or in the incubator. Once removed from the second lysis solution, slides were washed 3 times with distilled water at 20 min intervals and then placed in an electrophoretic horizontal unit (Fisher Scientific Electrophoresis Systems FB-SBR-2025) and kept for 1 h in chilled electrophoresis solution (500 mM NaCl, 0.1 M Tris-base, 1 mM EDTA, 0.2% DMSO), q.s. to 1.6 L with distilled water. The pH was adjusted to 9. The osmolarity was 967±0.49 mOsm/L (range 845-1020). Electrophoresis was performed at 32 V (0.7 V/cm), 0.44-0.52 A, for 30 min. Slides were rinsed drop - wise 3 times at 5-min intervals with a neutralization buffer (0.4 M Tris-base, pH 7.5), then dehydrated with cold 70% ethanol and left overnight at room temperature, in the dark. Slides were stained with 40 µL of diluted propidium iodide (PI, final concentration 0.38 µM) from a stock concentration of 9.6 µM PI in 1 mL Tris-EDTA buffer (10 mM Tris HCl and 1mM EDTA), incubated for 5 min in the dark and scored with a fluorescent microscope (Olympus BX51; Olympus America, Inc., Melville, NY, USA) at magnification X 200, connected to a computer with Image Analysis Software, Comet Assay IV Version 4.11 (Perceptive Instruments Ltd., Suffolk, UK). One hundred (100) sperm/slide were counted (50 sperm/well).

The following comet measures were recorded: head length (µ) = horizontal distance from the start of the head to the end of the head; head intensity (%) = expressed as a percentage of the comet's total

intensity and corresponding to % DNA in head; tail intensity (%) = (100-Head%DNA) and corresponding to % DNA in tail; tail length (μ) = the horizontal distance from the center of the head (start of tail) to the end of the tail; tail migration (μ) = the horizontal distance from the end of the head to the end of the tail; tail moment (μ) = product of the proportion of tail intensity and the displacement of tail center of mass relative to the center of the head. This last measure is even called Olive tail moment, defined as a combined measure of tail length and tail intensity (Olive et al., 1990), product of a physical quantity and its directed distance from an axis (Ashby et al., 1995).

2. 2. Sperm Chromatin Structure Assay (*SCSA)*

The SCSA was performed as previously described by (Love and Kenney, 1998). Briefly, frozen semen samples were thawed in a 37°C water bath and 2 - 7 μL aliquot of semen was diluted to 200 μL in a buffer solution (0.186 g disodium EDTA, 0.790 g Tris HCl, 4.380 g NaCl in 500 mL deionized water, pH 7.4). This was mixed with 400 μL of acid-detergent solution (2.19 g NaCl, 1.0 mL of 2 N HCl solution, 0.25 mL Triton-X, qs. 250 mL deionized water). After 30 s, 1.2 mL of the acridine orange (AO) solution was added (3.8869 g citric acid monohydrate, 8.9429 g Na_2HPO_4, 4.3850 g NaCI, 0.1700 g disodium EDTA, 4 μg/mL acridine orange stock solution-1 mg/mL, qs. 500 mL water, pH 6.0). The sample was then allowed to

equilibrate for 30 s on the flow cytometer. The cell flow rate was 100-200 cells/s. Sperm from a control stallion (i.e., good sperm quality based on a low percent COMP-α_t) was used to standardize instrument settings prior to analysis. The flow cytometer was adjusted such that the mean green fluorescence was set at 500 channels (FL-1 ~ 500) and mean red fluorescence at 150 channels (FL-3 @ 150). Five thousand cells were evaluated and data were stored in List-Mode and subsequently analyzed using WinList software (Verity Software House, Topsham, ME, USA).

2. 3. Statistical analysis

Data were analyzed using a mixed model repeated measures ANOVA. Means were separated by Least- Squares procedure (SAS institute, Inc. Corp., Cary, NC, USA).

3. Results

3. 1. Inter-assay repeatability

For the control sample, there were no differences among days (P>0.05; Table 1.5.) in any of the Comet assay measures.

Table 1.5.: Inter-assay repeatability for frozen-thawed sperm from a single control ejaculate measured by neutral Comet assay (mean ± SD).

Measure		Pre-UO[a]	Post-UO		
			14 days	30 days	60 days
Head	Length (μ)	51 ± 7	49 ± 8	48 ± 8	48 ± 7
	% DNA (%)	66 ± 27	67 ± 27	63 ± 28	70 ± 28
Tail	Length (μ)	58 ± 21	66 ± 27	67 ± 28	65 ± 27
	Migration (μ)	33 ± 23	42 ± 30	43 ± 31	40 ± 30
	Moment (μ)	9 ± 9	12 ± 15	13 ± 17	11 ± 15
	% DNA (%)	35 ± 28	32 ± 27	33 ± 28	30 ± 28

Within rows, values are not different (P>0.05).
[a] UO = unilateral orchiectomy

3. 2. Effect of unilateral orchiectomy on sperm DNA quality using the neutral Comet assay

There was no effect of UO on head length (P>0.05, Table 2.5.). The % DNA in the head was greater pre-UO compared to the 14 and 60 d samples (P<0.05), but similar to the 30 d samples (P>0.05). The tail length, moment, and migration were less in the pre-UO samples compared to all the post-UO samples (P<0.05). The

% DNA in the tail (reciprocal of % DNA in the head) was less in the pre-UO samples than either the 14 or 60 d samples ($P<0.05$), but similar to the 30 d samples ($P>0.05$).

Table 2.5.: Comet assay measures (mean ± SD) before and after unilateral orchiectomy (UO).

Measure		Pre-UO	Post-UO		
			14 days	30 days	60 days
Head	Length (μ)	49 ± 7	46 ± 7	48 ± 7	47 ± 6
	% DNA (%)	68 ± 22^{b}	60 ± 23^{a}	63 ± 23^{ab}	58 ± 21^{a}
Tail	Length (μ)	57 ± 6^{a}	82 ± 12^{b}	76 ± 13^{b}	79 ± 10^{b}
	Migration (μ)	33 ± 6^{a}	59 ± 14^{c}	52 ± 14^{b}	55 ± 11^{bc}
	Moment (μ)	9 ± 3^{a}	16 ± 6^{b}	13 ± 6^{b}	15 ± 6^{b}
	% DNA (%)	32 ± 9^{a}	40 ± 10^{b}	36 ± 13^{ab}	42 ± 13^{b}

^{a,b,c} Superscripts within rows are different at $P<0.05$.

3. 3. Effect of unilateral orchiectomy on DNA quality using the SCSA

The SD-α_t and COMP-α_t were similar among all time periods ($P>0.05$, Table 3.5.). The Mean-α_t and Mode-α_t were higher 14 days post-UO compared to pre-UO ($P<0.05$), but similar at 30 days post-UO ($P>0.05$) and decreased by 60 days post-UO ($P<0.05$).

Table 3.5.: Sperm Chromatin Structure Assay (SCSA) measures (mean ± SD) before and after unilateral orchiectomy (UO).

		Post-UO		
Measure	**Pre-UO**	**14 days**	**30 days**	**60 days**
Mean-α_t	204 ± 7^{b}	218 ± 17^{c}	202 ± 10^{b}	190 ± 8^{a}
Mode-α_t	201 ± 6^{b}	212 ± 14^{c}	198 ± 10^{b}	185 ± 8^{a}
SD-α_t	31 ± 5	34 ± 10	33 ± 9	37 ± 8
COMP-α_t (%)	8 ± 4	9 ± 5	7 ± 2	7 ± 2

[a,b,c] Superscripts within rows are different ($P<0.05$).

4. Discussion

Elective unilateral orchiectomy is a relatively common procedure to remove an abnormal testis, such as that resulting from trauma or neoplasia. It is unclear how soon following orchiectomy a stallion can return to breeding service due to the local inflammation and heat produced by the surgical procedure; therefore, it is often assumed that a minimum of one spermatogenic cycle (~ 60 days) should be allowed for sperm quality to return to pre-UO levels. In a previous complementary study, compared to pre-UO levels, sperm quality (i.e., motility, morphology, viability) did not change at 14, 30, or 60 days post-UO (McCormick et al., 2012). These results suggested that the degree of scrotal inflammation surrounding the surgical site following UO represented a "mild" testis stress that was not sufficient to elicit a decline in traditional sperm quality measures; however, DNA quality was not evaluated. Other investigators have reported early embryonic loss following testis stress induced by heat (Zhu and Setchell, 2004), irradiation (Ahmadi and Ng, 1999), as well as various clinical conditions (Ribas-Maynou et al., 2012b), indicating that sperm with damaged DNA have the capability to fertilize but lack the ability to maintain embryonic development. The current study provided a model to measure sperm DNA quality following a mild testis stress that did not alter traditional sperm quality measures (i.e., motility, morphology, viability) to determine if DNA quality could be altered in the face of otherwise normal sperm quality.

A previous study in mice described a mild scrotal heat stress in which motile sperm had damaged DNA (Banks et al., 2005). A study involving men revealed changes in sperm motility and morphology associated with increased scrotal temperature resulting from a sedentary lifestyle (Hjollund et al., 2000). More pronounced testis stress in bulls (Karabinus et al., 1997) and stallions (Love and Kenney, 1999) was associated with a decline in DNA quality.

The effect of heat stress in stallions may be of concern when ambient temperature is high or when exposed to rigorous training regimens or episodes of fever that elevate testis temperature. Some studies have failed to demonstrate an adverse effect of extreme exercise or elevated environmental temperature on sperm quality (Mawyer et al., 2012; Rosenberg et al., 2013). Scrotal insulation for 24-48 hours, however, results in a significant reduction in sperm quality (Love and Kenney, 1999; Freidman et al., 1991).

It is of particular interest to determine if DNA quality can decline even though traditional measures of sperm quality remain normal. The Comet assay has been suggested as a more sensitive method of measuring sperm DNA quality (Collins et al., 1997) and it has been compared to other sperm DNA assays including the TUNEL assay, Sperm Chromatin Dispersion Test and the SCSA (Pérez-Cerezales et al., 2012; Simon et al., 2014; Ribas-Maynou et al., 2013). Previous studies have compared the Comet assay and SCSA in humans (Larson et al., 2001; Aravindan et al., 1997), mice (Pérez-Cerezales et al., 2012), and bulls (Boe-Hansen et al., 2005)

using high and low quality sperm samples from fertile and subfertile subjects; however, none have used a model for mild testis stress in which traditional sperm quality features are not affected.

This current study compared the results of two DNA assays using the same ejaculates from a previous study (McCormick et al., 2012) to determine if a "mild" testis stress could be detected by either of the assays. It seems unlikely that DNA quality would be reduced in a model in which sperm motility, morphology, or viability were not affected; nevertheless, lower DNA quality was detected by both assays in the current study. Post-UO, all comet tail measures increased (i.e., % tail DNA, length, migration, moment) and these changes persisted through the 60 days evaluation period. Increases in comet tail measures are consistent with a decline in DNA quality due to electrophoretic movement of DNA from the head to the tail region of the comet. The transfer of DNA from the head to the tail is caused by an increase in DNA strand breaks thus releasing DNA to migrate. Previous studies in human (Hughes et al., 1997), boar (Fraser and Strzeżek, 2007b), and bull (Boe-Hansen et al., 2005) have demonstrated similar Comet assay changes associated with a decline in DNA quality, but this is the first study to report similar changes in stallion sperm.

A decrease in DNA quality was also detected by the SCSA (i.e., increased Mean-α_t and Mode-α_t) at 14 days after UO, but an increase in either the SD-α_t or the % COMP-α_t was not evident. The degree of change detected in the Mean-α_t is consistent with the level

of change observed in a previous study in which reduced DNA quality was associated with a decline in fertility (i.e., seasonal pregnancy rate and per cycle pregnancy rate; Love and Kenney, 1998). Other studies in stallions (Love and Kenney, 1999) and bulls (Karabinus et al., 1997) revealed a similar decline in DNA quality after 48 h of scrotal insulation. In these two studies, the change in the Mean-α_t occurred at the same time interval (i.e., 14 days post insulation) as the current study. The Mean-α_t and Mode-α_t represent a shift in the majority of the sperm population rather than a subset of the sperm population, which is represented by changes in the SD-α_t and the % COMP-α_t. In this study, the SD-α_t and % COMP-α_t did not change following UO. These results suggest that the scrotal inflammation was not sufficient to cause a decline in DNA quality of a subset of sperm, but instead affected the entire sperm population.

The Comet assay results indicate that, similar to the SCSA results, DNA quality declines by 14 d post-UO. In contrast to the SCSA results, DNA changes detected by the Comet Assay persisted through the 60-d evaluation period. Combined, these results suggest that both assays detected an initial decline in DNA quality at 14 days post-UO, but only the Comet assay was able to detect a change in DNA integrity at 30 and 60 days after UO. These differences may be explained by the differences in the assays. The SCSA primarily detects changes in the exposed toroid-linker region of the sperm chromatin, but is unable to identify changes in the tightly compact protamine-DNA donut regions because of the inability of the

acridine orange to intercalate in these regions (Shaman and Ward, 2006). The Comet assay, however, is able to extract protamines and histones and decondense the entire DNA complex. While previous studies indicate that reduced DNA quality, measured by the SCSA, is related to a decline in fertility, it is unknown how the change in DNA quality in the current study may affect fertility. Previous studies in men indicate that the neutral Comet assay identifies double-stranded DNA breaks associated with the nuclear matrix that can result in early pregnancy loss (Ribas-Maynou et al., 2012b; Morris et al., 2002). Interestingly, these changes were not able to detect infertile patients (Ribas-Maynou et al., 2014). Therefore, in the current study, changes detected by the Comet assay that persist thru the 60 day evaluation period have the potential to affect early embryonic loss even though other aspects of sperm quality, including SCSA measures, are normal. However, the effect of DNA "damage" on fertility in stallions, as measured by the neutral Comet assay, remains unknown.

High DNA fragmentation, as measured by SCSA, has been associated with pregnancy failure in humans following in vitro fertilization and intra-cytoplasmic sperm injection (Larson et al., 2000). In contrast, in aging stallions with testicular dysfunction, a decline in sperm motility and sperm morphology preceded a decline in DNA quality (Blanchard et al., 2013). The current study introduced a model for a mild testis stress that results in a decline in

DNA quality as measured by two assays (i.e., Comet and SCS assays), but without changes in traditional sperm quality features.

The incidence of early embryonic death in horses is estimated between 5-24% and is primarily attributed to mare factors (Vanderwall DK, 2008); however, the stallion also contributes to EED (Allen et al., 2007; Morris and Allen, 2002). Others have reported higher EED rates in mares bred to certain stallions (Allen et al., 2007; Morris and Allen, 2002). It is unclear what may cause these embryonic losses, but reasons could range from genetic (Kenney et al., 1990) to non-genetic (Blanchard et al., 1994). Transient causes of EED due to the stallion could arise from "mild" stress to the DNA occurring over a short time period and this may manifest as unexplained EED.

General Conclusions

The growth of ART in animal husbandry pushes researchers to improve methods to evaluate sperm functions. Besides the classical features of sperm motility, viability and morphology, more recently the evaluation of sperm DNA has resulted in the development of different assays, which measure different aspects of DNA integrity. The overall objective of this thesis was to increase the knowledge about sperm DNA integrity and about different sperm DNA assays. In these studies the neutral Comet assay protocol has been modified from an alkaline version for somatic cells (Tice et al., 2000) with the addition of DTT; thus, the modified protocol has been applied to sperm of different species (buffalo, bull and stallion). Several previous studies have evaluated comet images visually, by subjectively determining whether a comet is present or not. This was the first study to objectively describe the comet images by using a software, which measure comet size and intensity. Even though the sperm from the three species differ in sperm head dimensions and in protamine composition, the modified protocol of the Comet assay was easily applied to the three species. In the current study the use of Comet software allowed objective image measures and was a useful tool in evaluating the comet images.

The results in IMB bulls highlighted a correlation between neutral comet measures of Olive and tail moments, %DNA in head and tail and tail area with fertility in vivo. This was the first study to evaluate the relationship between Comet measures and fertility in

vivo in IMB bulls. The SCSA was considered in this study as the reference assay to which the other DNA assays were compared, as it has long been considered valuable in measuring sperm DNA integrity. The flow cytometer analyzes thousands of sperm in a few minutes and its relationship to fertility has been reported for many species. In this study, the SD-α_t was correlated to fertility in vivo in IMB bulls. Among the other assays that measure DNA integrity the Comet assay and the SBH may identify changes in DNA integrity that the SCSA may not measure. For the IMB bulls the SCSA, the Comet and the SBH were not correlated to each other and this may be due to the differences among the assays and/or to the narrow range of variation in sperm quality among the individuals analyzed. In fact, in bulls, in which a broader range of variation in sperm quality was selected, based on sperm motility and morphology, the SCSA measures, Mean-α_t, SD-α_t, COMP-α_t, were inversely correlated with neutral comet head measures (diameter, area, and intensity) suggesting that a decrease in head dimension is associated with a decline in DNA quality. In addition, lower DNA quality was detected by both the neutral Comet assay and the SCSA in stallions exposed to a mild in vivo stress such as unilateral orchiectomy. The study on bulls was the first to compare neutral and alkaline Comet assays, the SCSA and the SBH in sperm of this species. Among these tests, the SCSA and the neutral Comet assay were able to distinguish between the high- and low-sperm quality groups, while the alkaline Comet assay and the SBH assay were not, nor they were

correlated to sperm motility and morphology. The alkaline Comet assay and the SBH may dissolve sperm with poorer DNA quality completely so that it cannot be evaluated or the assay conditions may not be sufficient to decondense the DNA and allow discernment between sperm of greater and lower DNA quality. The neutral Comet assay is more valuable than the alkaline Comet assay for assessing sperm DNA quality in bulls because it readily identifies a decrease in comet area, head diameter, head area, and head intensity, as well as an increase in % Ghosts in association with lower sperm DNA quality. A unique feature of the neutral Comet assay was the presence of Ghosts, which appeared to be related to sperm quality. Ghosts have DNA that highly migrates to the tail region such that the Comet head and tail are completely separated, assuming a ghost-like appearance. Those were more prevalent in the group of bulls with lesser sperm quality. Ghosts, visually scored, were also able to differentiate between the groups with different sperm quality and considering the correlation with SCSA measures, as well as with sperm motility and morphology, researchers should take into account those cells with a high migration of DNA as an important component of the neutral Comet assay.

Furthermore, the study on the stallions aimed to evaluate sperm DNA integrity on individuals exposed to a mild stress in vivo, represented by scrotal swelling following unilateral orchiectomy (UO). Both the neutral Comet and the SCS assays detected an initial decline in DNA quality at 14 days post-UO, but only the Comet

assay was able to detect a change in DNA integrity at 30 and 60 days after UO.

Overall, the neutral Comet assay was a valuable tool to measure sperm DNA integrity and the results of this thesis evidenced its relationship with fertility in IMB bulls, its ability to distinguish between high and low sperm quality groups in bulls, and its ability to identify subtle changes in stallions exposed to a mild heat stress. Future studies should be conducted using large comparative trials on different species to confirm and investigate on the relationship between the neutral Comet assay and fertility in vivo and in vitro. Those studies should be addressed to clarify the practical application of this assay that could be beneficial to improve our understanding on sperm nuclear function and to identify subfertile individuals, which may need in vitro processing strategies to increase their fertility potential.

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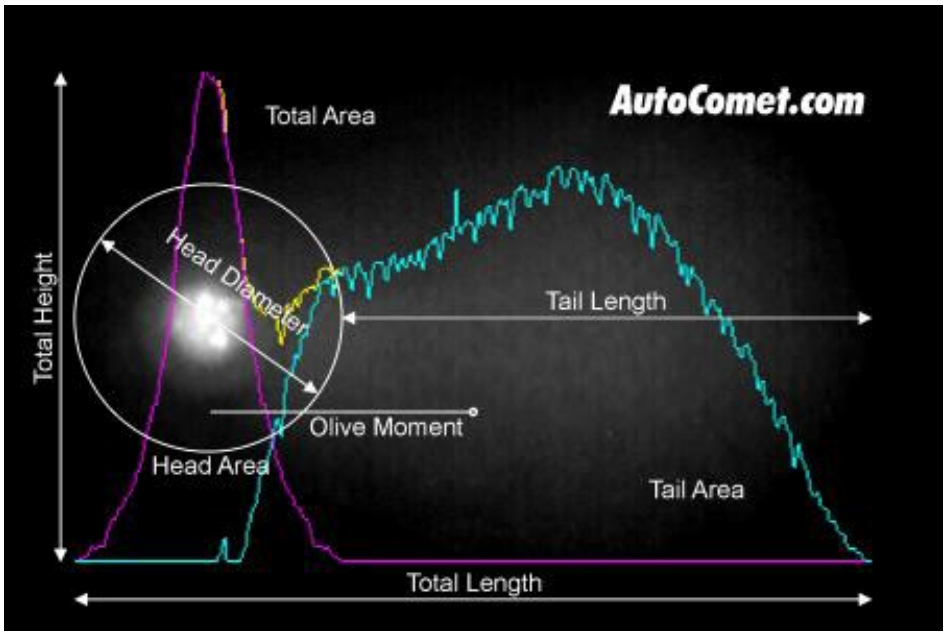
APPENDIX A: COMET MEASURES

Figure 4x: Graphic representation of the comet measures. CometScore tutorial, ©2013 Tritek Corp.



Comet measures are described in the user manual as follows:

A Comet is defined as all adjoining pixel in a designated shape, where each pixel intensity corresponds to the amount of DNA at that location. It must be oriented from the left (head) to the right (tail).

Comet Length (µm): number of pixels in horizontal direction converted to microns in Comet when the software is calibrated (corresponding to Total Length in Fig 4x);

Tail Length (µm): Head Diameter subtracted from Comet Length, expressed in microns;

Comet Height (μm): number of pixels converted to microns in vertical direction in Comet (corresponding to Total Height in Fig. 4x);

Comet Area (μm²): number of pixels in Comet converted to μm²;

Head Diameter (μm): number of pixels converted to microns in horizontal direction in Head;

Head Area (μm²): number of pixels converted to microns in Head;

Tail Area (μm²): number of pixels in Tail converted to μm²;

Total Comet Intensity: sum of pixel intensity values in Comet;

Mean Comet Intensity: mean intensity of pixels in Comet;

Total Head Intensity: sum of pixel intensity values in Head;

Mean Head Intensity: mean intensity of pixels in Head;

Total Tail Intensity: sum of pixel intensity values in Tail;

Mean Tail Intensity: mean intensity of pixels in Tail;

%DNA in Head (%H-DNA): Total Head Intensity divided by Total Comet Intensity (multiplied by 100);

%DNA in Tail (%T-DNA): Total Tail Intensity divided by Total Comet Intensity (multiplied by 100);

Tail Moment: %DNA in Tail multiplied by Tail Length x 0.031 (constant factor related to the microscope);

Olive Tail Moment (OTM): summation of Tail Intensity profile values multiplied by their relative distances to the Head Center, divided by Total Comet Intensity.

APPENDIX B: COMET ASSAY - SPERM REPORTS

Author	pH	Techniques	Electrophoresis	Treatment	Species	Scoring method	Results
Singh; 1989	N sp.	A	25 V/10'	Sperm vs lymphocytes or mouse bone marrow cells	H, M	V (measured from photomicrographs	Abundant ssDNA in sperm (alkaline sensitive sites)
Hughes; 1996	13	A	25V/10'	untreated vs treated with X-rays and hydrogen peroxide	H	S	Untreated: no differences between fertile and infertile men. Treated: sperm DNA resistant to damage.
Hughes; 1997	13	A	25 v/10'	Assay reproducibility	H	S	Low repeatability among days vs high between slides. %H DNA preferred parameter
Aravindan; 1997	10	A	125mV/30'	Comet, SCSA and TUNEL	H	V (4 levels)	Strong correlations among the three assays
Haines; 1998	N sp.	N vs A	25V/20'	in vitro irradiation	H, M	S	Extensive DNA migration both species in unirradiated sperm after A.
Steele; 1999	N sp.	A	25V/10'	testicular and epididymal sperm	H	S	Testicular sperm DNA less damaged than epididymal
Linfor; 2002	N sp.	A	20V/15'	Fresh, cooled, frozen	E	V (tail or not)	Fresh 25%damage; almost 100% comets in frozen-thawed 5 cycles

Author	pH	Techniques	Electrophoresis	Treatment	Species	Scoring method	Results
Zilli; 2003	>12	A	20V/15'	fresh vs frozen	Sb	S	Higher %T DNA and OM in frozen than fresh
Boe-Hansen; 2005	8	N, SCSA	25 V/20'	Sex sorted vs conventional semen	B	S	Conventional sperm: higher TM and DFI. NO correlation between assays (data not shown)
Fraser; 2007a	8	N	12 V/1h	fresh vs frozen	Bo	V (tail or not)	> damage in frozen-thawed vs fresh
Slowinska; 2008	8.4	N	20V/10'	fresh vs frozen	B	S	low fragmentation of DNA in frozen
Zee; 2009	N=N sp.; A= 12.5	N vs double (N and A same slide)	N= 20 V/12', A= 20 V/4'	Comet vs SCDt	Ko	Densitometric analysis	Same sperm DNA fragmentation for the 3three assays. Abundant ssDNA in koala sperm
Kumar; 2011	9	N	24 V/1h	fresh vs frozen	Bu	V (4 levels)	Damage 11% fresh vs 37.25% Frozen-Thawed
Enciso; 2011	N=7.5; A= N sp.	N and A same slide (double) vs Halosperm	N= 20 V/12', A= 20 V/4'	Density gradient centrifugation vs swim up	H	V (7 levels)	DGC more useful to select sperm than SUP to eliminate dsDD
Mukhopadhyay; 2011	9	N, 8-OHdG	18V/30'	fresh vs frozen	B, Bu	V (tail or not)	Damage frozen vs fresh
Ribas-Maynou; 2012a	N sp.	N vs A	N= 20 V/12' 30sec, A= 20 V/4'	Different fertility groups, in 5 fertile samples oxidative and enzymatic damage induced	H	V (4 levels)	A sensitivity= ssDNA (oxidative damage) and N =dsDNA (enzymatic damage)

Author	pH	Techniques	Electrophoresis	Treatment	Species	Scoring method	Results
Ribas-Maynou; 2013	N sp.	N, A, SCSA, TUNEL, SCDt	N= 20 V/12' 30sec, A= 20 V/4'	Different assays in fertile and infertile	H	V (4 levels)	N no power in predicting fertility; A, TUNEL,SCDt and SCSA able to distinguish infertile.
Ribas-Maynou; 2014	N sp.	N vs A	N= 20 V/12' 30sec, A= 20 V/4'	fresh vs frozen	H	V (4 levels)	No effect of freezing in N, 10% increase in A

Previous reports about the Comet assay on sperm. Note: N sp.: not specified; techniques-A-alkaline Comet; N-neutral Comet. Species-H-human; E-equine; M-mouse; B-bull, Bu-Buffalo, Bo-Boar, Ko-Koala and Sb-Sea bass. Scoring method: V-visual; S-software.

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