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(coordinatore: Prof. Sergio Ledda)

FINE NEEDLE ASPIRATION CYTOLOGY (FNAC) AS A USEFUL TECHNIQUE TO EVALUATE SEASONAL VARIATIONS OF SPERMATOGENESIS IN CERVIDS: RELATIONSHIPS WITH HISTOLOGY AND SPERM QUALITY

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Ai miei più cari affetti

Contents

1. Objectives and aims of the study

This study was aimed at addressing the question whether Fine Needle Aspiration Cytology (FNAC) may be used as a reliable method to evaluate spermatogenesis and its seasonal variation in wild deer. No previous studies have been carried out about this topic and only qualitative correlations have been performed between cytological pattern and open testicular biopsy, the gold standard method for spermatogenesis assessment (Mehrotra and Chaurasia, 2007; Han *et al.*, 2006; Meng *et al.*, 2001; Batra *et al.,* 1999; Yadav *et al.,* 1997; Craft *et al*., 1997). Furthermore, due to the annual pattern of recrudescence and involution of spermatogenesis, deer could be used as an optimal model system to investigate mechanisms that regulate testicular activity (Klonish *et al.*, 2006).

To the best of my knowledge, this is the first study where FNAC has been applied in wild deer and data are still lacking in spermatogenesis both in Iberian red deer (*Cervus elaphus hispanicus,* Helzheimer 1909) and Sardinian red deer (*Cervus elaphus corsicanus,* Erxleben 1777).

Reproductive biology of Iberian red deer stags has been deeply investigated (Domínguez-Rebolledo *et al.,* 2011; Esteso *et al.,* 2009, 2006 and 2003; Garde *et al.* 1998; Gomendio *et al.*, 2007; Malo *et al.,* 2009, 2006, 2005a and 2005b; Martínez *et al.,* 2008; Martinez-Pastor *et al.,* 2005) although no studies have been carried out regarding spermatogenesis and its seasonal

variations. So far, studies carried out on red deer spermatogenesis have been only performed on post-mortem samples (Hochereau-de Reviers *et al.*, 1978).

Data about Sardinian red deer are even more lacking and no studies have been published about the reproductive biology of this endangered subspecies.

Developed by Posner in the early 1900s as a diagnostic tool in human infertility, Fine Needle Aspiration Cytology has been recently adopted in veterinary medicine as a minimally invasive method to evaluate and quantify spermatogenesis (Stelletta *et al.*, 2011; Santos *et al.*, 2010; Gouletsou *et al.*, 2010; Romagnoli *et al.*, 2009; De Souza *et al.*, 2004; Leme and Papa, 2000). Due to its low invasiveness, FNAC could be performed repeatedly *in vivo* without severe consequences on reproductive performances (Gouletsou *et al*., 2010; Westlander, 2001; Leme and Papa, 2000). Thus, FNAC could be used to monitor spermatogenesis of the same individual throughout its whole reproductive cycle; this advantage is quite important in species where sexual competition is extremely developed (Malo *et al.,* 2009, 2006, 2005a and 2005b; Gomendio *et al.*, 2007 and 2006; Clutton-Brock *et al.,* 1982) and drastically changes of reproductive activity are known to occur during the year.

Furthermore, I aimed to explore which kind of relationships occur between germ cells, Sertoli cells and sperm quality on the basis of the hypothesis that fully active spermatogenesis during the breeding season should be related with high sperm quality.

To achieve these objectives, I have collected samples post-mortem from Iberian red deer stags during the breeding season and out of the breeding season. Histological analyses were

performed, according to the Johnsen's score, in order to evaluate whether FNAC is a reliable method for quantifying spermatogenesis. Then, testicular cytological patterns were correlated with sperm analysis in order to evaluate their relationships.

Finally, preliminary data are reported about reproductive biology and the application of FNAC *in vivo* on Sardinian red deer, an endemic and endangered subspecies of Sardinia and Corse.

2. Introduction

2.1 A comparative overview of the male reproductive system across the animal kingdom

The male reproductive system is composed of the gonads, the testes, and the accessory organs (epididymides, ductuli deferentes, accessory sex glands and penis).

The testes are the sexual organs of the male reproductive system with endocrine and spermatogenic functions. In the accessory organs, the spermatozoa undergo maturation processes and are transported and stored.

Apart from members of the Afrotheria (e.g., Proboscidea, Sirenia, Hyracoidea and Afrosoricida), in which the testes remain close to the kidneys, in other mammals caudal migration of testes takes place late in foetal life (Setchell and Breed, 2006) or after birth like in marsupials (Renfree and Shaw, 2001).

Testes locations vary widely amongst mammals: in some species they remain near the bladder close to the dorsal abdominal body wall (e.g., whales, Order Cetacea; nine-banded armadillo and giant anteater, Superorder Xenartha), in others they move close to the ventral abdominal body wall (e.g., most Laurasiatheria such as hedgehogs, Family Erinaceidae; insectivorous bats, Sub-Order Microchiroptera; and the true seals, Family Phocidae), although in most insectivores (e.g., shrews, Family Soricidae; and moles, Family Talpidae) the testes are located in shallow

coelomic evaginations, the cremaster sacs, in the posterior region of the abdominal cavity (Setchell and Breed, 2006).

By contrast, in artiodactyls, carnivores and primates, testes are located outside the abdominal cavity, in the scrotum. This adaptation was likely developed because the spermatogenesis normally happens at temperature lower than the body temperature. In fact, a slight increase of scrotal temperature can induce disruptions of spermatogenesis like azoospermia or oligospermia by increasing germ cell apoptosis (Cai *et al.,* 2010; Setchell, 1988). For this reason, cryptorchidism is frequently associated with infertility.

Apart from marsupials, in mammals the process of gonadal differentiation takes place during the early phases of embryogenesis. The primordial germ cells (PGCs) migrate from the vitelline sac into the genital ridge. Then, on the basis of chemotactic factors they differentiate into testes or ovaries. In marsupials and placental mammals, this process depends on the Y linked-SRY gene (sex determining region of the Y chromosome (Sinclair *et al.*, 1990). Expression of the SRY gene results in the formation of the testes in males (XY); on the contrary, its absence determines the development of ovaries in females (XX).

The testes are even, egg-shaped and capsulated organs. This organ is composed of the parenchyma and the stroma. The first is composed of the seminiferous tubules which could be distinguished as tubules contortus and rectus. The length of the seminiferous tubules is established during the foetal development and is correlated with the mitotic activity of the immature Sertoli cells (Foresta, 1993). On the other side, the stroma is composed of the tunica

albuginea and the mediastinum which are made of connective tissue. The tunica albuginea is extremely resistant and cannot be stretched. At the caudal pole of the testis, the tunica penetrates into the organ and forms the mediastinum. The latter divides the parenchyma into lobules.

As the female gonads, the main three cell types of the testis are:

- Germinal cells, originating from the ectoderm;
- Supporting cells, originating from the celomatic epithelium;
- Interstitial cells originating from the mesenchyma.

2.2 Spermatogenesis and the cycle of the seminiferous epithelium

Spermatogenesis is a complex process that takes place in the seminiferous tubules of the testis and consists of the development of haploid cells from stem cells called spermatogonia (Hess *et al.*, 2008).

Profound differences exist amongst the vertebrates in how germ cell development and maturation is accomplished. These differences can be broadly divided into two distinct patterns: one present in anamniotes (fish and amphibian) and the other in amniotes (reptiles, birds and mammals). For anamniotes, spermatogenesis occurs in spermatocysts which, for most species, develop within seminiferous lobules. Cysts are produced when a Sertoli cell becomes associated with a primary spermatogonium. Mitotic divisions of a primary spermatogonium produce a cohort of secondary spermatogonia that are enclosed by Sertoli cell which forms the wall of the

cyst. With spermatogenic progression a clone of isogenic spermatozoa is produced, which are released, by rupture of the cyst, into the lumen of the seminiferous lobule. Following spermiation, the Sertoli cell degenerates. For anamniotes, therefore, there is no permanent germinal epithelium, since spermatocysts have to be replaced during successive breeding seasons. By contrast, spermatogenesis in amniotes does not occur in cysts but in the seminiferous tubules that possess a permanent population of Sertoli cells and spermatogonia which act as a germ cell reservoir for succeeding bouts of spermatogenic activity (Pudney, 1995).

In many mammals this process takes place within the seminiferous tubule throughout the reproductive life span of the male (Kerr *et al.*, 2006) while in others it is interrupted or subdivided into a series of distinct phases based on environmental cues that are transduced into hormonal signals stimulating or inhibiting spermatogenesis (e.g., seasonal breeders) (Lincoln, 1989). In others, unique processes occur such that a single wave of spermatogenic development is seen within the testis, subsequent to which the animal is sterile; such a process occurs in marsupial mice, *Antechinus strudii* and *swainsonii*, wherein the stem cells, after a burst of mitotic activity, differentiate and proceed through the spermatogenic process, resulting in a single wave of reproductive activity (Kerr *et al.*, 1983).

The seminiferous epithelium consists of germ cells that form concentric layers penetrated by a single type of somatic cells first identified by Enrico Sertoli in 1865 (Hess *et al.*, 2008).

In any given area within the seminiferous epithelium a well-defined series of events occurs which follows each other in a precise, orderly sequence. These events occur at extremely well-

timed intervals with respect to one another, and give rise to a precise number of cellular associations, each consisting of one or two generations of spermatogonia, spermatocytes and spermatids.

Spermatogenesis implies different main events (Foresta, 1993):

- Presence of a pool of stem germ cells that can renew themselves by mitotic process;
- Production of haploid germinal cells by two successive meiotic divisions;
- Production of highly specialised cells by morphological and structural changes.

Spermatogenesis is a species-specific process and it can be influenced both by intrinsic and extrinsic factors. This process starts from the puberty and develops through highly timed and organised steps that can be distinctly classified in stages and phases (Hess *et al.*, 2008).

The stages of the seminiferous epithelium were first described by Leblond and Clermont in 1952 according to the cellular association observed in histological tubule cross-sections and the cycle of the seminiferous epithelium was defined as the sequence of events that occur from the disappearance of a give cellular association to its appearance in the same area of the seminiferous epithelium.

Nevertheless, the number of stages varies according to the species and the criteria adopted for the classification. In addition, spermatogenesis is a continuous process so further transitional stages can be observed.

In humans and several primate species, more than one spermatogenic stage is observed per tubule cross-section whereas in other species (e.g. rodents) only one stage is seen within single

tubules. Stages are identified on the basis of meiotic phase of primary spermatocytes and the changes observed in the Golgi region of spermatids and that can be visualised by the periodic acid-Schiff's reaction (PAS). In the mouse XII stages have been identified (Leblond and Clermont, 1952) whereas VIII in the rat (Roosen-Rungen and Giesel, 1950). In red deer, the relative frequencies of stages of seminiferous epithelium cycle are similar to that described in ram and bull although seasonal changes are more pronounced (Hochereau-de Reviers *et al.*, 1978).

Phases of the seminiferous epithelium are classified according to the mitotic and meiotic activity of germ cells. The main phases of the spermatogenesis are:

- Spermatocytogenesis which involves mitotic cell division of spermatogonial cells (2n). With about ten subsequent mitotic divisions and last with one spermatogonia B that give rise to two primary spermatocytes at preleptotene stage.
- Meiosis
- Spermiogenesis

The latter could be further divided into: Golgi, capping, acrosomal and maturation phases. These steps are useful for the identification of specific stages in the cycle of the seminiferous epithelium (Hess and de França, 2008).

In mammals, the time it takes to produce a single spermatozoon under normal conditions ranges from 30 to 78 days across species (Hess and de França, 2008). Although, the duration of spermatogenesis has been considered to be constant in a given species, recent study has

demonstrated that differences exist among breeds or strains of the same species (Russel *et al.*, 1990). Injection of tritiated thymidine, a very specific precursor for DNA, is classically used to determine the duration of spermatogenesis. So far, in red deer, the duration of spermatogenesis has not been determined and in the only study about testicular histology in this species (Hochereau-de Reviers MT *et al.*, 1978) stages of seminiferous epithelium were distinguished the eight stages described by Roosen-Runge and Giesel (1950) for the rat.

2.3 Cytology of the seminiferous epithelium

2.3.1 Sertoli Cell

With the myoid cells, Sertoli cells are the only somatic cells present in the tubule seminiferous and with the germ cells constitute the seminiferous epithelium. Their name came from the first Italian scientist, Enrico Sertoli, who first described them in 1865 when he was a new university graduate at the age of 23.

"Io non penso che le cellule ramificate siano destinate a produrre spermatozoi, sebbene ciò non possa essere escluso categoricamente, ciononostante io penso che la funzione delle cellule ramificate sia legata alla formazione degli spermatozoi";

"I do not think that the branched cells I described are destined to produce spermatozoa, although it is not possible to deny it categorically, but I think that the function of the branched cells is linked to the formation of spermatozoa" Enrico Sertoli, 1865 from a translation of Setchell.

Sertoli cell functions include providing structural support and nutrition to the developing germ cells, phagocytosis of degenerating germ cells and residual bodies, release of spermatids at spermiation and production of a host of proteins that regulate and/or respond to pituitary hormone release that influence mitotic activity of spermatogonia (Johnson *et al.*, 2008). Furthermore, Sertoli cells forms the blood testis barrier that resides in tight junctions located between adjacent Sertoli cells and divides the seminiferous tubule in two compartments: the basal and the adluminal compartment. In the first, spermatogonia and early preleptotene primary spermatocytes are present whereas in the last secondary spermatocytes, spermatids and spermatozoa. Given that spermatocytes and spermatids first appear at puberty, these cells are not recognised by the immune system as self, but rather as foreign cells (Johnson *et al.*, 2008).

Sertoli cells are columnar in shape, their nuclei exhibit a variety of shape but they are usually oval or pear-shaped with a large and distinctive nucleolus (Johnson *et al.*, 2008).

In cytological smears, Sertoli cell appear mainly as naked nuclei. Because of their typical and constant morphology, they are quite easily identified. They are large (30-35 μm in diameter); nucleus appears round or oval, (22 μm in diameter) with finely granulated chromatin and usually

one single nucleolus (Schenck and Schill, 1988). Cytoplasm is slightly basophilic, may be vacuolated and contain spermatozoa.

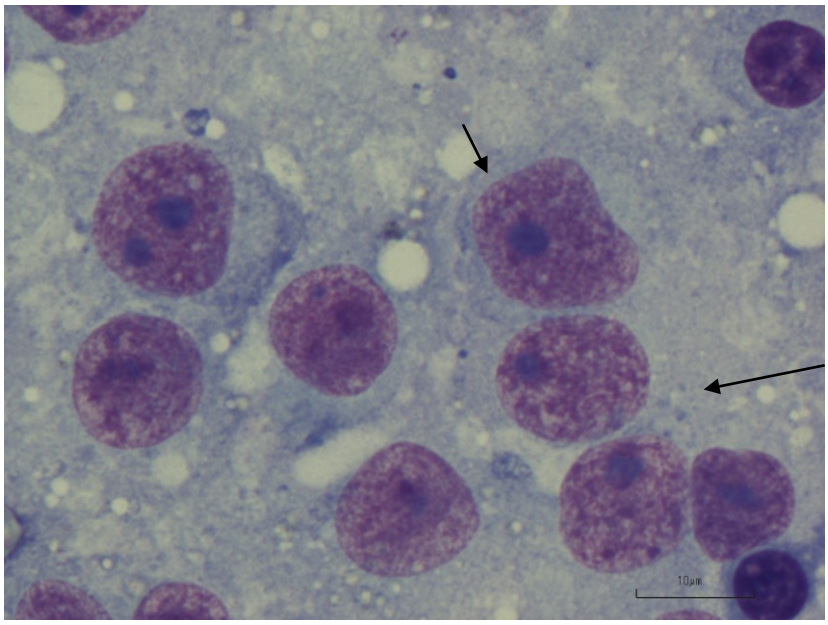

Figure 1. Sertoli cells in Iberian red deer (arrows). Scale bar= 10μm

2.3.2 Spermatogonium

Spermatogonia are diploid cells (2n) that can renew and differentiate into spermatocytes which undergo to meiosis. The mechanisms that regulate the differentiation and the renewal of this stem cell still remain unclear (Kerr *et al.*, 2006). On the basis of their morphological characteristic, three different types of spermatogonia have been identified in human: Spermatogonia A Dark, Spermatogonia A Pale and Spermatogonia B.

Spermatogonia A Dark are the stem cell which can divide by mitotic process in Spermatogonia A Dark or in more differentiated Spermatogonia A Pale.

In well-studied laboratory rodents, such as rats and mice, spermatogonia can be classified as undifferentiated Type A Spermatogonia [Spermatogonia A single (A_s), Spermatogonia A paired (A_{pr}) and Spermatogonia A aligned (A_{al})]; Differentiated Type A Spermatogonia that can be distinguished as A_1, A_2, A_3 and A_4; Intermediate Spermatogonia (int) and Type B Spermatogonia (Hess and França, 2008).

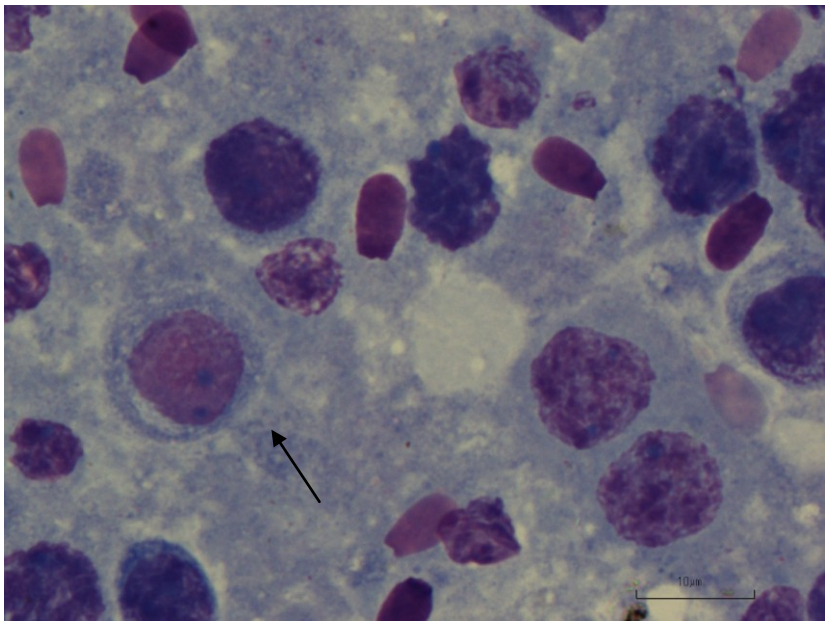

Figure 2. Spermatogonium of Iberian red deer (arrow) Scale bar= 10µm

In cytological samples spermatogonia are difficult to find because they are scarce compared with more differentiated counterparts, such as spermatocytes and spermatids (Santos *et al.*, 2010).

They usually appear as naked nuclei with fine and homogeneous chromatin. The nucleus is usually eccentric; it can be bigger than the nucleus of Sertoli cell. They can show one or more nucleoli. In human, the cellular diameter is about 15-18 μm (Foresta, 1993; Papić *et al.*, 1986); besides in canine species they are about 18-20 μm (Santos *et al.*, 2010). The cytoplasm is scarce and less basophilic compared to other spermatogenic cells. In dog the oval-shaped nucleus display a special feature: parachromatin condensation with semilunar shape (Santos *et al.*, 2010). In cytological samples, the subtypes of spermatogonia cannot be distinguished on the basis of their morphology.

2.3.3 Primary spermatocyte

Primary spermatocytes are the largest spermatogenic cells. They appear round or oval with clumped chromatin and with a prominent nucleus. Nucleoli are rare but occasionally there is a rather conspicuous nucleolus, which is eccentrically located between coarse chromatin threads (Schenck *et al.*, 1988). The cytoplasm is scanty and less basophilic compared to spermatogonia.

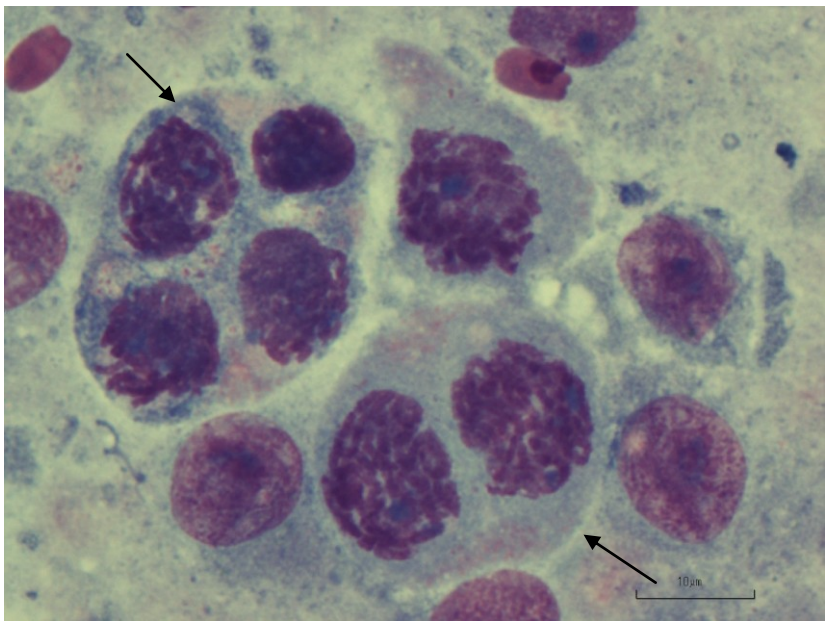

Figure 3. Multinucleated primary spermatocytes in Iberian red deer (arrows) Scale bar= 10µm

Multinucleated spermatocytes can be occasionally observed. This could be due to an increase in testicular temperature that can rapidly induce failure of the primary spermatocyte to differentiate leading to multinucleated cell formations (De Souza *et al.*, 2004).

The prophase of the primary spermatocyte could be distinguished as Preleptotene, Leptotene, Zygotene and Pachytene.

In the pachytene spermatocyte, where the crossing over occurs, they reach the biggest cellular and nuclear volume.

During metaphase the chromosomes appear along the equatorial axis and, finally, during anaphase and telophase they migrate to the opposite poles of the cell originating two secondary spermatocytes.

2.3.4 Secondary spermatocyte

Secondary spermatocytes are the rarest spermatogenic cells. Their rare occurrence is generally due to the fact that their lifetime is extremely short (24-48h) (Pàpic *et al.*, 1988). Cellular diameter is 13-14 μm in human (Foresta *et al.*, 1993), smaller than those of primary spermatocytes but with a totally different chromatin structure (uniformly, fine granular pattern similar to round spermatids).

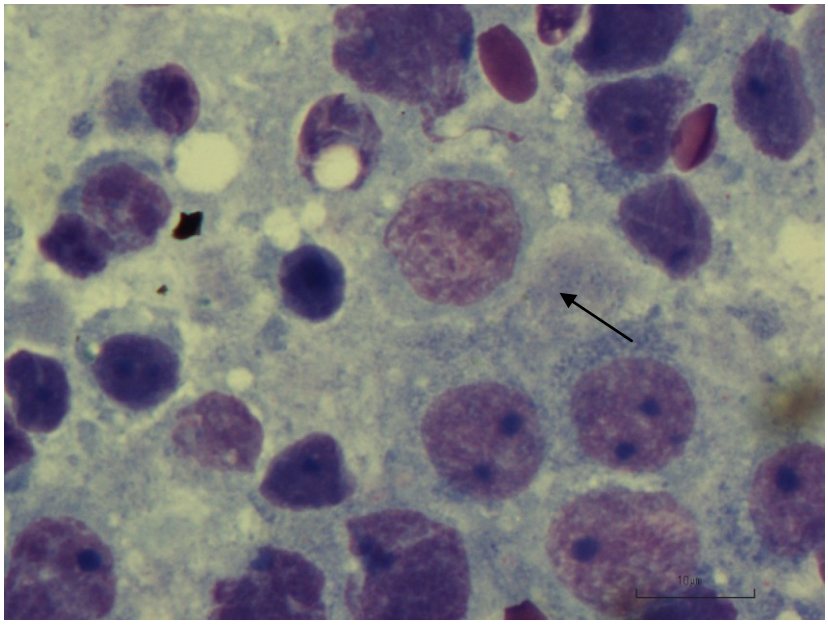

Figure 4. Secondary spermatocyte in Iberian red deer (arrow) Scale bar= 10μm

A scanty, faintly basophilic cytoplasm surrounds the nucleus, which is usually centrally located (Schenck and Schill, 1988).

2.3.5 Spermatids

Spermatids originate from the last meiotic division of spermatocytes and, through the spermiogenesis, develop into spermatozoa.

Recognizable stages spermiogenesis: (phase A) Golgi phase, (phase B) cap phase, (phase C) acrosomal phase and (phase D) maturation phase (Foresta, 1993; Santos *et al.*, 2010).

Thus, on the basis of their distinct morphology, in cytological smears spermatids are generally distinguished as round spermatids (corresponding to phases A and B of spermiogenesis) and elongated spermatids (corresponding to phases C and D of spermiogenesis) (Foresta, 1993; Santos *et al.*, 2010; Stelletta *et al.*, 2011).

Round spermatids have a much smaller nucleus than do secondary spermatocytes. Nucleus is round or slightly oval, with uniformly fine granular chromatin, with rare nucleoli.

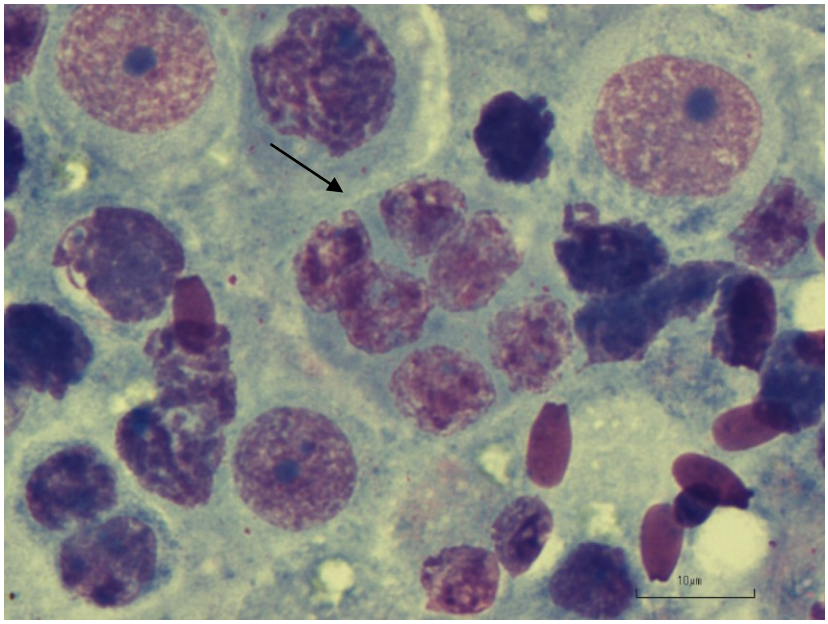

Figure 5. Multinucleated round spermatids in Iberian red deer (arrow) Scale bar= 10µm

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Elongated spermatids are characterised by more elongated and darker nuclei, as the chromatin became more clumped as maturation progressed (Santos *et al.*, 2010). Multinucleated forms are quite frequently observed in normal testicular smears both in human and animals (Schenck and Schill, 1988; Santos *et al.*, 2010).

At least 90% of testicular giant cells originate from clumped spermatids while others originate from spermatocytes, indicating that they have lost contact with Sertoli cells (Schenck and Schill, 1988).

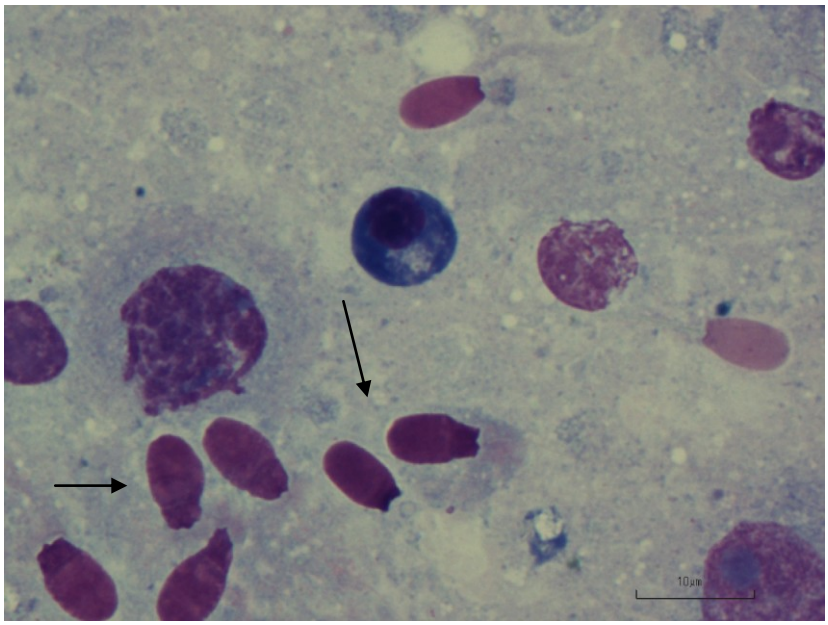

Figure 6. Elongated spermatids in Iberian red deer (arrows) Scale bar= 10µm

2.3.6 Spermatozoa

Spermatozoa are one of the most specialised cells. Human spermatozoa were first described in 1677 by Anton van Leeuwenhoek, soon after inventing the microscope. In a letter dated November 1677 and addressed to Lord Brounker, Secretary of The Royal Society, he mentioned that "I had seen such a multitude of live animalcules more than a million, having the size of a grain of sand and moving in a space …those animalcules were smaller than the red blood cells. They had a round body, foam in the front, terminated in a point at the back; they were equipped with a tail with five to six times the body length. They progressed in a snake-like motion helped by their tail" (Karamanou *et al.*, 2010) .In cytological smears, they are distinguished on the basis of the oval-shaped head and the presence of the tail.

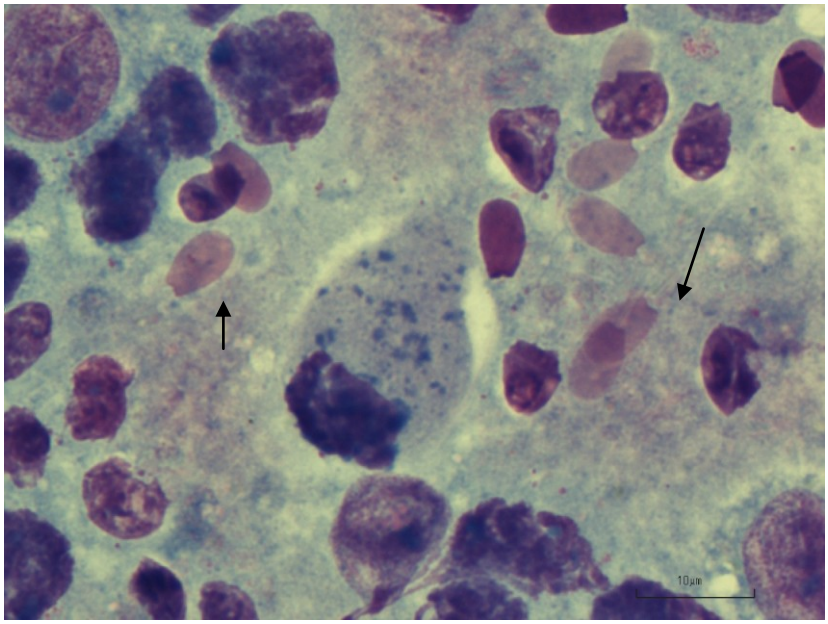

Figure 7. Spermatozoa of Iberian red deer (arrows) Scale bar= 10µm

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Spermatozoa of Iberian red deer are 8.570±0.406 μm length (range: 7.370–9.810) and 4.873±0.229 width μm (range: 4.130–5.590) (Malo *et al.*, 2006).

2.4 Methods for collecting testicular tissue: Fine Needle Aspiration Cytology (FNAC)

Assessment of spermatogenesis is an important component in the evaluation of male fertility.

Currently, the main techniques for obtaining testicular parenchyma *in vivo* are open biopsy and puncture biopsy. Nevertheless, all these methods are fairly invasive and *in vivo* they can provoke severe and irreversible damage to reproductive efficiency.

Besides, Fine Needle Aspiration Cytology (FNAC) proved to be a quick, reliable and minimally invasive technique to evaluate the spermatogenesis (Mehrotra *et al.,* 2007).

Developed by Posner (1904) in the early 1900s, Fine Needle Aspiration Cytology of the testis has been mainly regarded as a diagnostic tool in human infertility. Only recently, this method has been adopted in veterinary medicine to evaluate spermatogenesis (Stelletta *et al.*, 2011; Santos *et al.*, 2010; Gouletsou *et al.*, 2010; Romagnoli *et al.*, 2009; De Souza *et al.*, 2004; Leme and Papa, 2000).

On the basis of its minimal invasiveness, FNAC could be repeatedly *in vivo* on the same individual without affecting reproductive activity (Gouletsou *et al.*, 2010; Westlander, 2001; Leme and Papa, 2000). Thus, FNAC could be used to monitor spermatogenesis on the same

individual and during its whole reproductive cycle; this advantage is quite important in species where sexual competition is extremely pronounced and drastically changes of reproductive activity occur during the year.

So far, studies carried out on red deer spermatogenesis have been mainly performed on post-mortem samples (Martinez-Pastor *et al.*, 2005; Hochereau-de Reviers *et al.*, 1978). Repeated observations on spermatogenic functions could be relevant in deer where sexual selection and sperm competition are extremely developed (Clutton-Brock *et al.,* 1982).

Correlation between 82% and 100% was found between FNAC and histology although the reliability was only qualitative and based on pathological categories (Mehrotra *et al.,* 2007; Han *et al*., 2006; Craft *et al.*, 1997, Meng *et al.*, 2001; Yadav *et al.*, 1997).

According to our experience, FNAC proved to be a reliable method even to diagnose pathological cases. In fact, we have observed a case of Sertoli cell-only pattern in Iberian red deer. The testis affected showed lower weight (34.26 gr) compared to the other (48.10 gr) and only Sertoli cells were observed in cytological smears (**Figure 8** and **Figure 9**); this result was confirmed by histological diagnosis (Johnsen's score: 2.02). The contralateral testis showed reduced spermatogenesis within the range of the same period both in cytological (SI: 3.27 compared to mean±SD of the same period: SI: 16.04±5.04; range 3.15-26.17) (**Figure 11**) and histological analysis (Johnsen's score: 7.78 compared to mean±SD Johnsen's score of the same period: d 7.77±0.29). Furthermore, epididymal motile and viable sperm were recovered from this testis.

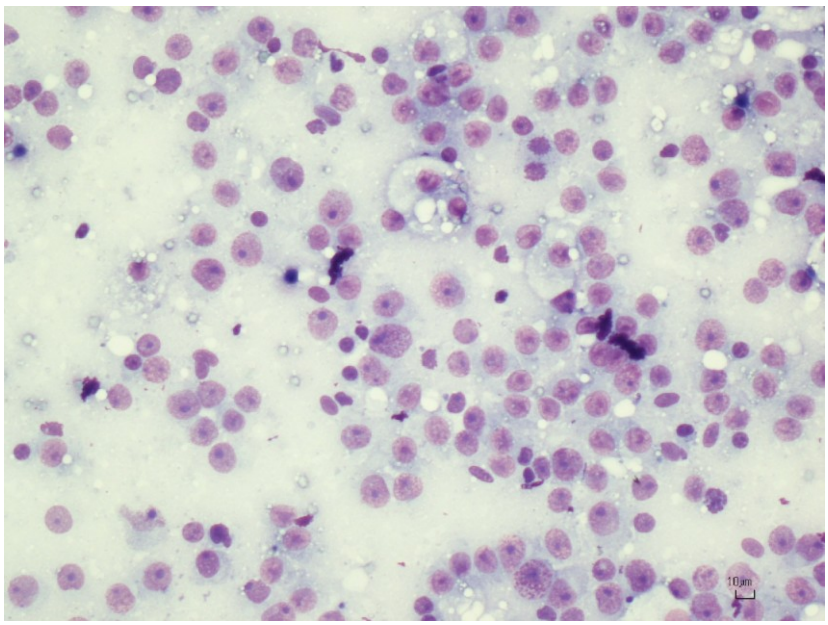

Figure 8. Sertoli cell-only pattern in a testis of Iberian red deer (200X) Scale bar= 10µm

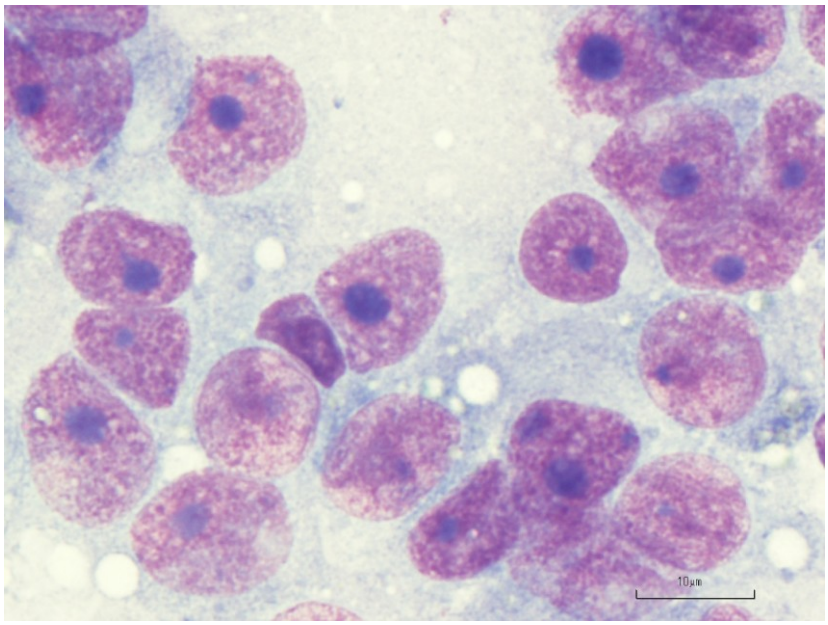

Figure 9. Sertoli cells at higher magnification (1000X) Scale bar= 10µm

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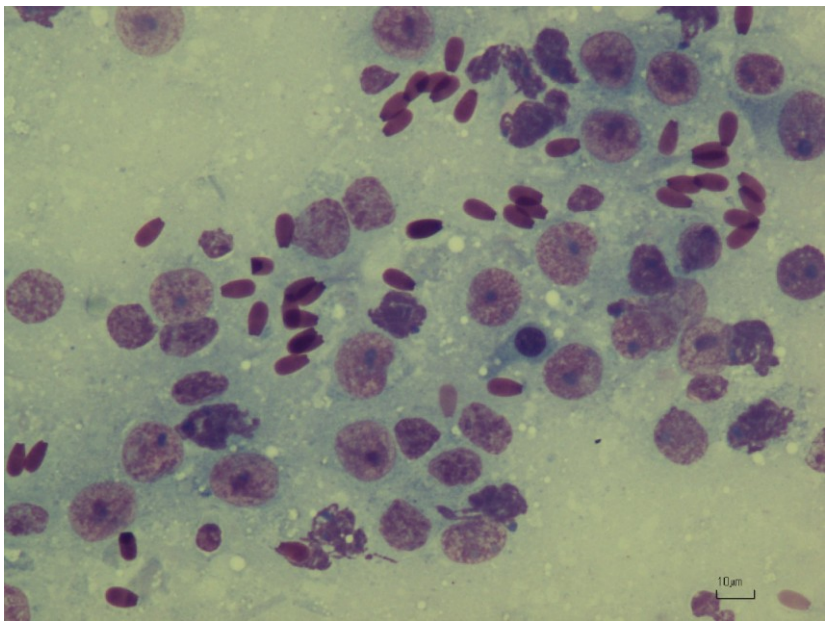

Figure 10. Testicular cytological pattern in the contralateral testis of Iberian red deer with Sertoli cell-only(400X). Scale bar= 10μm

Only recently, FNAC has been applied in domesticated species like dog (Romagnoli *et al.*, 2009; Santos *et al.*, 2010; Gouletsou *et al.*, 2010 and 2011; De Souza *et al.*, 2004), stallion (Leme and Papa, 2000) and alpaca (Stelletta *et al.*, 2011) in order to evaluate spermatogenesis and the proportion of testicular cells. Furthermore, several indexes that quantify testicular functions could be determined on the basis of cytological pattern. In fact, Sertoli index and Spermatic index are the main indexes that quantify spermatogenic and spermiogenic activity, respectively. Futhermore, several ratios could be assessed such as germ cell:Sertoli cell and round spermatids: primary spermatocyte, which indicate Sertoli cell workload and germ cell loss, respectively. Apart from this several advantages, cellular associations and tubule diameter could be not evaluated on the cytological smears.

Finally, in cytological smears cellular details could be more appreciated and this allows easily morphometric evaluations.

3. Seasonal Pattern of Spermatogenesis in Iberian Red Deer (*Cervus elaphus hispanicus*, Helzheimer 1909) Revealed by Fine Needle Aspiration Cytology (FNAC)

3.1 Introduction

Several species limit their breeding behaviour to restrict periods of the year when reproductive success is more likely and, as a consequence of this adaptation, drastically changes in the male reproductive tract have been described (Knobil and Neill's, 2006). Indeed, during the involution phase of the masculine gonad, testicular mass could decrease between 10 % and 95 % depending on the species (Young *et al.*, 2001).

In mature male seasonal breeders, the annual cycle of testicular growth and involution involves significant changes in structure and function of both tubular and interstitial compartments (Klonish *et al.*, 2006). Increasing in relative testis size are assumed to reflect increased investment in sperm production, since there is evidence for a correlation between testis and the number of sperm produced per unit time (Amman, 1970; Møller, 1989).

In roe deer (*Capreolus capreolus*) (Klonish *et al.*, 2006; Roelants *et al.*, 2002), viscacha (*Lagostomus maximus maximus*) (Muñoz *et al.*, 2001) and cat (*Felis catus*) (Stornelli *et al.*, 2009) germ cells, Sertoli cells and Leydig cells all display significant morphological and physiological alterations due to seasonal changes. The main changes in the testicular structure

involve the reduction of the tubule diameter, arrest of spermatogenesis with spermatogonia and Sertoli cells as the only epithelial components (Klonish *et al.*, 2006; Schön *et al.*, 2004; Roelants *et al.*, 2002).

Iberian red deer *(Cervus elaphus hispanicus,* Helzheimer 1909*)* are seasonal breeders with a short breeding season which starts in early September and lasts to mid-October (Martinez-Pastor *et al.*, 2005). As shown in several seasonally breeding species, gonads reveal annual cycles of testicular involution and recrudescence on the basis of photoperiodical, behavioural, genetic and hormonal influences (Malpaux, 2006). Follicle-stimulating hormone (FSH) and androgens are the two major endocrine factors that regulate spermatogenesis (O'Donnell *et al.*, 2006). In Iberian red deer, Testosterone (T) levels remain low until July and then begin to rise and reach a peak in September when the breeding season starts; thereafter T levels start to decline (Malo *et al.*, 2009). Androgens are proved to be determinant in triggering the spermatogenesis and increasing the testicular mass (Klonish *et al.*, 2005); therefore, in seasonal breeders, they show the same annual pattern (Malo *et al.*, 2009). About a three-fold increase of testicular mass was observed in red deer (*Cervus elaphus*) during the breeding season compared to the non-breeding season with significant differences in the number of germ cells (Hochereau-de Reviers and Lincoln, 1978). Nevertheless, all the previous studies on spermatogenesis have been mainly performed on histological samples. On the contrary, Fine Needle Aspiration Cytology (FNAC) proved to be a quick, reliable and minimally invasive technique to evaluate the spermatogenesis (Mehrotra *et al.,* 2007). Testicular cytology was first proposed more then a century ago as a

diagnostic tool in human infertility (Posner *et al.*, 1904). A correlation between 82% and 100% was found between FNAC and histology even if the reliability was only qualitative (Mehrotra *et al.* 2007; Han *et al.*, 2006; Craft *et al.*, 1997). Only recently, FNAC has been applied in animal breeding in species like dog (Romagnoli *et al.*, 2009; Santos *et al.*, 2010, Gouletsou *et al.*, 2010 and 2011), stallion (Leme and Papa, 2000) and alpaca (Stelletta *et al.*, 2011). Moreover, FNAC could be performed repeatedly in vivo without severe consequences on the reproductive performances (Gouletsou *et al.*, 2010, 2011; Westlander, 2001; Leme and Papa, 2000).

Quantitative studies of the population relationships of the seminiferous epithelium and the testicular sperm reserve in wild animals is not only valuable in establishing physiological standards, but also provides information for establishing protocols for assisted reproduction programs for the endangered species (De Azevedo *et al.*, 2010).

Therefore, in this study we proposed FNAC as a technique to investigate and detect the cyclic changes of spermatogenesis in Iberian red deer, a seasonal breeder model. For the first time to our knowledge, this minimally-invasive technique has been applied to evaluate the seasonal changes of spermatogenesis.

3.2 Materials and Methods

All the reagents were purchased from Sigma-Aldrich (Madrid, Spain) unless otherwise indicated.

Testes, with no testicular pathology, were collected from 53 adult stags (age > 4.5 years, weight > 130 kg) of Iberian red deer *(Cervus elaphus hispanicus)* exposed to natural photoperiod from the end of November 2010 till the end of February 2011 in the south of Spain (Comunidades Autónomas de Castilla-La Mancha and Andalucía). Samples were collected from 7 different game reserves.

Samples were collected post-mortem from animals legally culled and hunted in their natural habitat. Hunting was in accordance with the harvest plan of the game reserve, made following Spanish Harvest Regulation which conforms to European Union Regulation. Testes, within the scrotal sac, were cut with a knife and transported to the laboratory in a plastic bag at room temperature (20-25°C). Testes were removed from the scrotal sac and isolated. Weight of each testis was recorded (Balance EK-400H, A&D Company, Tokyo, Japan) after removing the epididymis and the spermatic cord with a surgical blade. Samples were processed between 6-10 hours after death of animals.

In order to evaluate the effects of seasonal changes on spermatogenesis, samples were divided in three groups: Group **BS** (Breeding season, post-rutting; from late November to late December, N=20), Group **TP** (Transition period; from early January to early February, N=17) and Group **NB** (non-breeding season; late February, N=16). Each testis was analyzed separately.

3.2.1 Fine needle aspiration cytology (FNAC)

Fine Needle Aspiration Cytology (FNAC) was performed by inserting a 20 G needle, previously connected to a 5 ml syringe, in the testicular parenchyma at the cranial side, opposite to the epididymis. Constant suction and gentle in-out movements were applied. After few seconds, the needle was slowly removed from the testis and the content was flushed with air onto a glass slide and gently smeared. This procedure was repeated twice for each testis. The smears were air dried for at least 24 h.

Then, the slides were stained with Hemacolor (Merck, Darmstadt, Germany), a rapid Romanowsky-type stain. Briefly, each smear was embedded in solution 1 (5 immersions of 1"), and then in solution 2 (6 immersions of 1"). After that, the smear was washed abundantly in a buffer solution (according to Weise, pH 7.2) and embedded in the final solution 3 (7 immersions of 1"). Finally, it was washed again abundantly in a buffer solution (according to Weise, pH 7.2) and left air-dried for at least 24 h.

Each smear was finally cleared in Xylol (Sigma-Aldrich, Gillingham,UK) and mounted with DPX (Fluka, Buchs, Switzerland) .

The smears were examined under oil at 1000X magnifications using light microscopy (Microscope Nikon Eclipse 80i). At least two hundred consecutive spermatogenic and Sertoli cells were counted in each smears and classified as follows: Sertoli cells, spermatogonia, primary spermatocytes (I), secondary spermatocytes (II), round spermatids (ab), elongated spermatids

(cd) and spermatozoa. Proportion of each cell type was determined. Moreover, testicular cytological indexes were determined as follows (Papić *et al.*, 1986; Han U *et al.*, 2006):

- Sertoli cell index (SEI), was the percentage of Sertoli cells to 100 spermatogenic cells as index of spermatogenic activity;
- Spermatic index (SI) was the percentage of spermatozoa to 100 spermatogenic cells as index of spermiogenic activity;
- Sperm-Sertoli index (SSEI) was calculated from the ratio of spermatozoa to Sertoli cells;
- The ratio of the number of spermatids ab/ primary spermatocytes was assessed as index of meiotic activity (MI) and germ cell loss.
- Germ cells: Sertoli cell ratios were evaluated as index of Sertoli cells functionality and workload.

3.2.2 Histology

One fragment of testicular tissue (around 1 cm^3) was isolated from the tunica albuginea and mediastinum and fixed in modified Davidson's fixative solution for 24-48 h, then stored in ethanol: water (70:30. v/v) until analysis (Howroyd *et al.*, 2005; Latendresse *et al.,* 2002). Subsequently, the samples were embedded in paraffin, cut into 4-µm sections and stained with haematoxylin and eosin (H&E). Hystological samples were classified according to a slightly modified Johnsen's score (1970). Briefly, 50 tubules were counted for each testis at 500X

magnification and a score from 1 to 10 were assigned to each seminiferous tubule. Only round seminiferous tubules were assessed in order to evaluate one cytological association. Only the testes of 27 stags were adequate for histological analysis (n=11 in Group BS, n=7 in Group BSI and n=9 in Group TP).

Number of Sertoli cells was evaluated in 10 round randomly selected tubules at 500X magnification for each testis. Only both testes of 17 deer were adequate for this analysis: Group **BS** (n=6), Group **TP** (n=3) and Group **NB** (n=8).

Score 10	Complete spermatogenesis, germinal epithelium well organised;
Score 9	Complete spermatogenesis; germinal epithelium disorganised
Score 8	Complete spermatogenesis; less than 15 spermatozoa present;
Score 7	No spermatozoa but many spermatids;
Score 6	Less than 10 spermatids present;
Score 5	No spermatozoa or spermatids but many spermatocytes;
Score 4	Only few (less than 5) spermatocytes;
Score 3	Spermatogonia are the only germ cell present;
Score 2	Only Sertoli cells present;
Score 1	No cells in tubule sections.

Table 1. Slightly modified Johnsen's score

3.2.3 Statistical analysis

All statistical analyses were performed using the SPSS 17.0 statistical software package (SPSS Inc, Chicago, IL, USA). For each variable descriptive statistics are reported. Kolmogorov-Smirnov test and Levene's test were used to check data normality and homogeneity of variance, respectively. Spermatocytes II percentage were not normally distributed even if square root-transformed.

One-way ANOVA was performed in order to compare means between groups. Tukey post-hoc test was applied in case of homogeneity of variance, otherwise Dunnet-T3 post-hoc test. Pearson correlation was used to evaluate correlations of cytological analysis. Differences were considered significant at $p< 0.05$.

3.3 Results

3.3.1 Comparisons of Groups: testicular weight and cytological pattern of spermatogenesis

Our results showed that testicular weight is significantly lower out of the breeding season ($p<0.001$).

In all three groups, round spermatids were the most frequent cells whereas secondary spermatocytes the rarest. Differences of Sertoli cells and spermatogenic cells percentages are shown in **Table 2.**

	Group BS (N=20)			Group TP (N=17)			Group NB (N=16)		
	Mean ± SD	**CV (%)**	**Min-Max**	**Mean ± SD**	**CV (%)**	**Min-Max**	**Mean ± SD**	**CV (%)**	**Min-Max**
Testicular weight (gr)	**44.68±17.09^{NB***}**	38.25	23.20-81.87	**40.00±6.42^{NB***}**	16.06	30.90-52.97	**29.08±6.29$^{BS***/TP***}$**	21.64	17.05-44.40
Sertoli cells (%)	**13.12±7.70$^{TP*/NB*}$**	58.68	3.17-29.07	**9.35±4.85^{BS*}**	51.94	2.15-21.31	**8.89±3.95^{BS*}**	44.41	3.15-19.65
Spermatogonia (%)	**1.62±1.22**	75.14	0.00-5.02	**1.51±0.95**	62.88	0.00-4.52	**1.55±1.00**	65.20	0.00-4.31
Spermatocytes I (%)	**14.68±4.44^{TP*}**	30.24	4.56-24.55	**16.91±2.63^{BS*}**	15.56	11.79-22.28	**16.53±2.98**	18.04	9.46-24.40
Spermatocytes II (%)	**0.26±0.38**	145.32	0.00-1.75	**0.29±0.54**	185.91	0.00-2.03	**0.31±0.43**	137.03	0.00-1.40
Spermatids ab (%)	**35.56±5.85**	16.46	23.40-47.49	**36.71±6.73**	18.32	24.38-55.60	**34.93±6.80**	19.46	23.65-51.80
Spermatids cd (%)	**14.61±5.07$^{TP**/NB***}$**	34.69	5.91-26.65	**18.22±4.76^{BS**}**	26.15	5.81-28.69	**21.75±7.51^{BS***}**	34.52	9.55-37.44
Spermatozoa (%)	**20.15±6.12$^{/NB*}$**	30.39	7.56-32.53	**17.01±6.40**	37.62	5.43-29.78	**16.04±5.04^{BS*}**	31.45	3.15-26.17

Table 2. Testicular weight, Sertoli cells and germ cells percentages during the breeding season (BS), transition period (TP) and non breeding season (NB). Superscripts indicate significant differences between groups: *p<0.05; **p<0.01 and ***p<0.001.

Significant differences were found in SEI and SI indexes between groups **BS** and **TP** and between **BS** and **NB** whereas MI and SSEI did not show any significant difference (**Table 3**). Frequency of distribution of SEI and SI in the three groups is reported in **Figure 1**.

Total germ cells:Sertoli cell ratio did not show statistically differences between groups ($p>0.05$). Mean ± SD of germ cells:Sertoli cell was 9.76±6.50 in Group **BS** (n= 40); 13.44±9.24 in Group **TP** (n=34) and 12.84±6.89 in Group **NB** (n=32). Germ cells: Sertoli cell ratios are reported in **Figure 2.**

	Group BS (N=20)			Group TP (N=17)			Group NB (N=16)		
	Mean ± SD	**CV (%)**	**Min-Max**	**Mean ± SD**	**CV (%)**	**Min-Max**	**Mean ± SD**	**CV (%)**	**Min-Max**
SEI (%)	**16.06±11.06^{TP*,NB**}**	68.86	3.27-40.99	**10.63±6.18^{BS*}**	58.16	2.20-27.08	**9.96±4.93^{BS**}**	49.50	3.26-24.46
SSEI	**2.40±1.98**	82.77	0.28-8.86	**2.68±2.31**	86.02	0.34-9.29	**2.25±1.43**	63.67	0.58-6.86
SI (%)	**23.03±6.29^{TP**,NB**}**	27.31	9.90-35.05	**18.67±6.79^{BS**}**	36.37	6.45-31.12	**17.59±5.34^{BS**}**	30.39	3.30-27.86
MI	**2.72±1.32**	48.66	1.31-9.08	**2.25±0.63**	27.93	1.13-3.62	**2.21±0.79**	35.58	1.20-5.48

Table 3. Testicular cell indexes during the breeding season (BS), transition period (TP) and non breeding season (NB). Superscripts indicate significant differences among groups: $p<0.05$; $**p<0.01$.

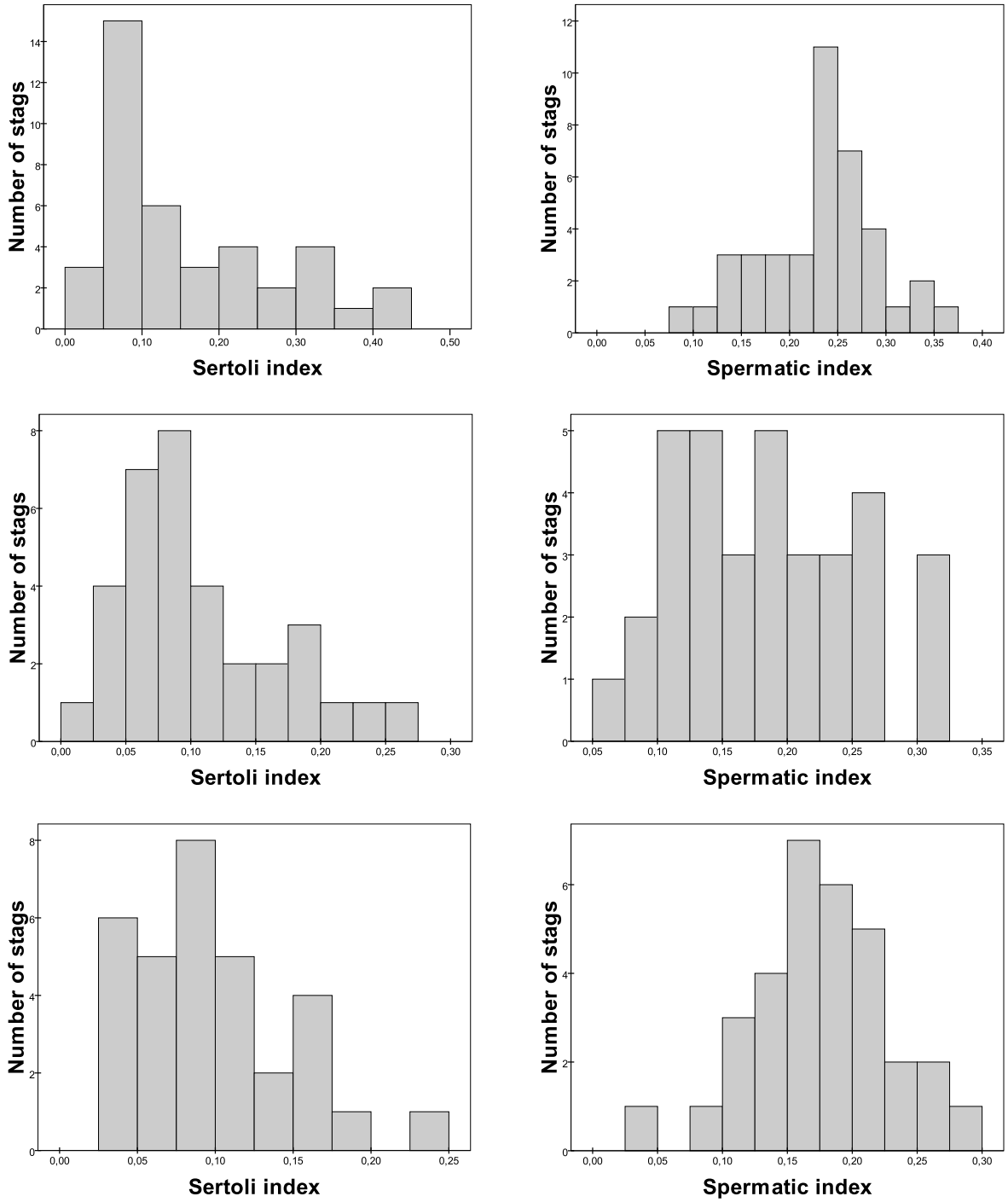

Figure 1. Frequency distribution of Sertoli index and Spermatic index during the breeding season (**BS**), transition period (**TP**) and non breeding season (**NB**).

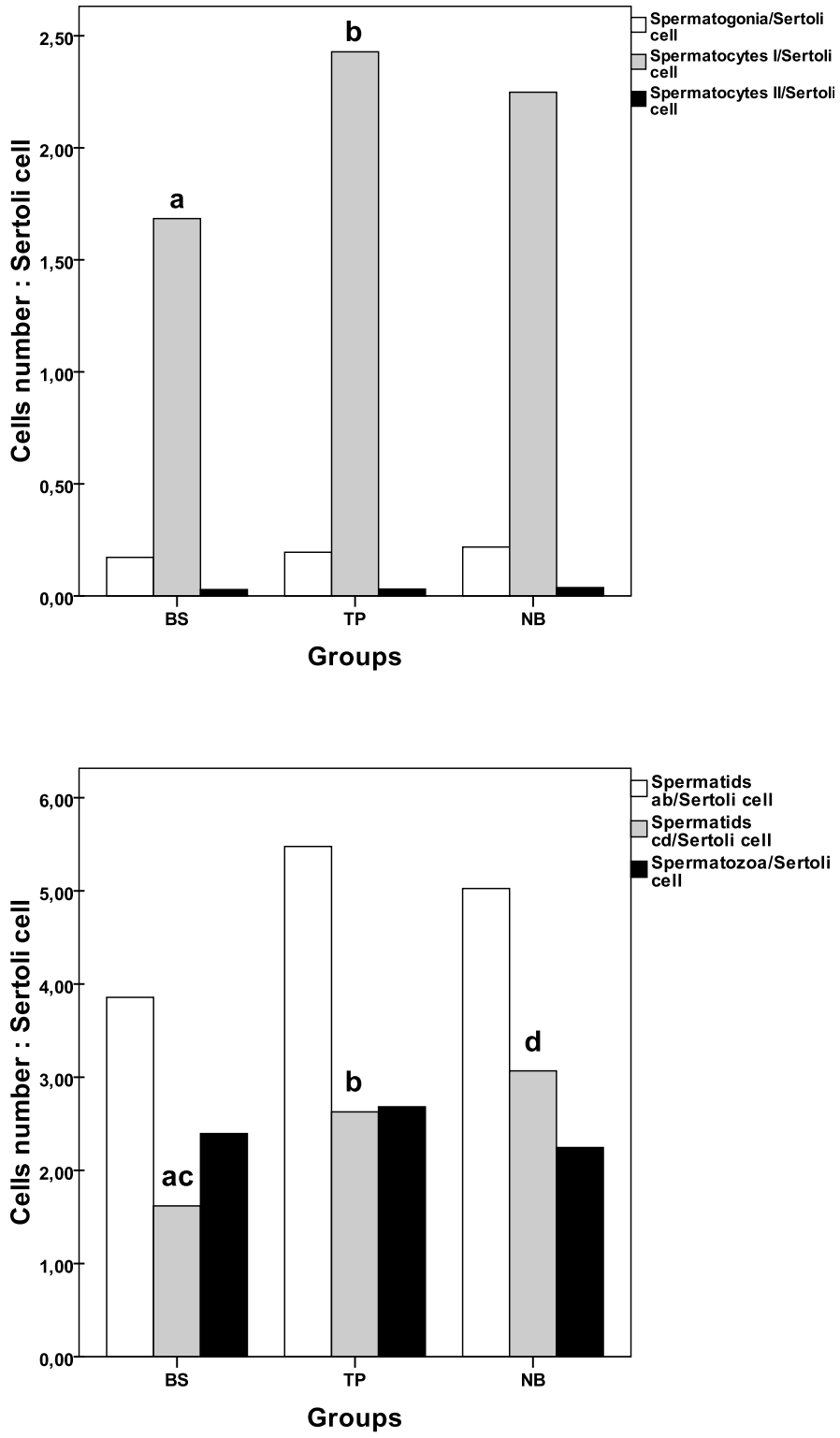

Figure 2. Germ cells: Sertoli cell ratio during the breeding season (**BS**), transition period (**TP**) and non breeding season (**NB**). Significant differences are reported as follows: $^{a\text{-}b}$ $p<0.05$; $^{c\text{-}d}$ $p<0.01$.

3.3.2 Histological analyses

Johnsen's score was 8.19±0.17 (min-max: 7.92-8.56) in Group **BS**, 7.82±0.27 (min-max: 7.46-8.24) in Group **TP** and 7.77±0.29 in Group **NB** (min-max: 7.18-8.28). Statistical differences were found between Group **BS** and **TP** and between Group **BS** and **NB** ($F_{18,018}$=0.000 p<0.001 in both cases).

Besides, Johnsen's score did not differ significantly between **TP** and **NB** (p=0.901).

Significant decrease was observed for score 10 and score 9 between groups **BS** and **NB** ($F_{4.603}$=0.015 p=0.030; $F_{3.210}$=0.049 p=0.037). Besides, the number of tubules with score 7 significantly increased between Group **BS** and **NB** ($F_{5.001}$=0.010 p=0.014). Data are shown in **Table 4**.

Mean number of Sertoli cells per tubule cross-section was 7.78±2.62 in Group **BS** (min-max: 2.7-12.5) (n=5); 3.23±0.52 in Group **TP** (min-max: 2.6-3.8) (n=3) and 2.58±0.77 in Group **NB** (min-max: 1.7-4.8) (n=8). Significant differences were found between Group **BS** and **TP** ($F_{35.388}$=0.000 p=0.001) and between Group **BS** and **NB** ($F_{35.388}$=0.000 p<0.001). **TP** e **NB** groups did not differ significantly (p=0.101).

	Group BS (N=11)			Group TP (N=7)			Group NB (N=9)		
	Mean ± SD	**CV (%)**	**Min-Max**	**Mean ± SD**	**CV (%)**	**Min-Max**	**Mean ± SD**	**CV (%)**	**Min-Max**
Score 10	**6.05±4.58^{NB*}**	75.80	1-16	**3.21±2.81**	87.30	0-8	**3.00±2.28^{BS*}**	75.84	0-7
Score 9	**16.58±5.54^{NB*}**	33.22	7-26	**12.64±8.45**	66.87	2-26	**11.61±6.43^{BS*}**	55.35	1-25
Score 8	**9.32±3.51**	37.67	2-16	**10.21±4.59**	24.05	2-19	**10.50±3.35**	31.81	3-17
Score 7	**17.09±3.73^{NB*}**	21.81	11-22	**21.21±5.10**	24.05	13-30	**22.00±6.77^{BS*}**	30.79	11-36
Score 6	**0.55±0.67**	123.01	0-2	**1.71±1.59**	92.74	0-5	**1.94±2.01**	103.57	0-8
Score 5	**0.27±0.46**	167.14	0-1	**0.71±0.99**	139.23	0-3	**0.72±0.96**	132.68	0-3
Score 4	**0.05±0.22**	469.04	0-1	**0.07±0.27**	374.17	0-1	**0.17±0.51**	308.70	0-2
Score 3	**0±0.00**		0-0	**0.07±0.27**	374.17	0-1	**0.06±0.24**	424.26	0-1
Score 2	**0±0.00**		0-0	**0.14±0.36**	254.20	0-1	**0±0.00**		0-0
Score 1	**0±0.00**		0-0	**0±0.00**		0-0	**0±0.00**		0-0

Table 4. Descriptive statistics of Johnsen's scores during the breeding season (BS), transition period (TP) and non breeding season (NB). Superscripts indicate significant differences between groups; * $p<0.05$.

3.3.3 Correlations between testicular cell percentages and indexes within groups

A negative correlation was observed between spermatids ab and spermatids cd in all three groups (r=-0.506 p=0.001; r=-0.430 p=0.011 and r=-0.667 p<0.001 in Group **BS**, **TP** and **NB**, respectively). Sertoli cell percentage and SEI were negatively correlated with spermatozoa percentage in Group **BS** (r=-0.546 p<0.001 and r=-0.531 p<0.001; **Figure 3**) and this relation became less strong and significant in Group **TP** (r=-0.426 p=0.012 and r=-0.417 p=0.014, respectively) and Non significant in Group **NB** (r=-0.198 p=0.277 and r=-0.210 p=0.249, respectively).

We observed a negative correlation between the proportion of Sertoli cell and spermatids ab only in Group **BS** (r=-0.383 p=0.015, **Figure 3**). Non significant correlations were found in the other groups (r=-0.305 p=0.079; r=-0.215 p=0.238 in Group **TP** and **NB**, respectively).

None relationship was found between testicular weight and testicular cells proportions.

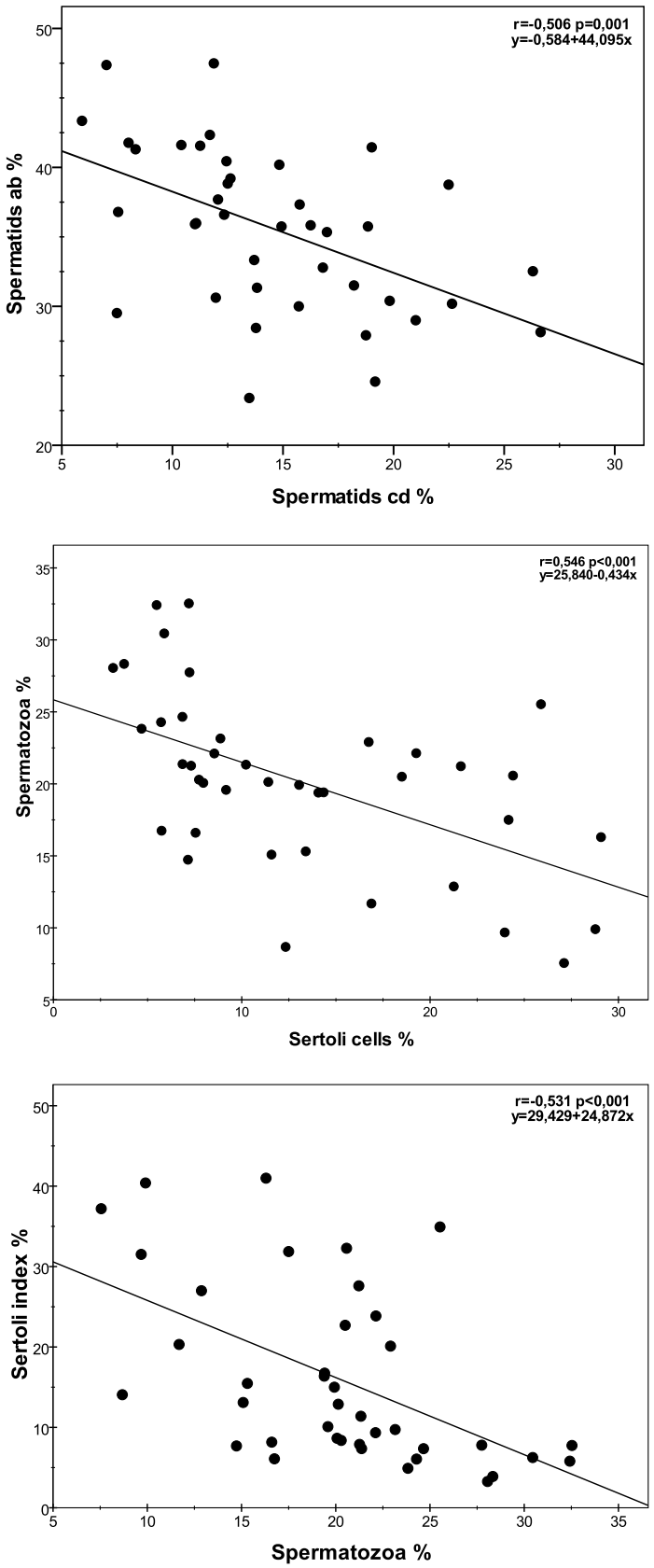

Figure 3. Negative relationship between testicular cells during the breeding season (Group BS).

3.4 Discussion

Our results showed that FNAC is a useful method to evaluate spermatogenesis and detect the seasonal changes of testicular cells percentages and indexes in Iberian red deer. For the first time to our knowledge, this minimally invasive technique has been used to investigate the seasonality of spermatogenesis using a seasonal breeder model.

The involution of spermatogenesis in Iberian red deer is characterized by a decrease of the testicular mass and a decline of the number of spermatozoa in agreement with data obtained in roe deer by flow cytometric analysis (Blottner *et al.*, 1996; Goeritz *et al.*, 2002). Spermatic index (SI) in Iberian red deer during post-rutting season (23.03±6.29) is lower than that observed by our group in Sardinian red deer (SI: 31.84) and fallow deer (29.40±4.70) during the rutting (data not published) and compared to what observed in human (SI 34.8±13.3) (Foresta *et al.*, 1993), horse (SI: 31.5±8.5) (Leme and Papa, 2000), dog (SI: 26.6±3.8) and alpaca (27.28±13.83 and 20.63±14.47 in the right and in the left testis, respectively) (Stelletta *et al.*, 2011).

Decline of spermatozoa and increase of elongated spermatids proportions are observed during the non breeding season. Similar pattern was described by histological analysis where Johnsen's score and the number of seminiferous tubules with spermatozoa (scores 10 and 9) decline significantly during the non breeding season with a current increase of the number of tubules where all germ cells except spermatozoa were present (score 7).

Non significant differences were found in round spermatids:primary spermatocyte ratios between the breeding and non breeding season and this result, with the current increase of elongated spermatids percentage, suggests that the involution of spermatogenesis involves firstly the spermiogenesis (the last phase of germ cells development), without affecting the meiotic activity. This finding is also supported by the higher spermatids cd: Sertoli cell ratio and the stronger negative correlation between round spermatids and elongated spermatids out of the breeding season.

Contrary to what observed by Hochereau-de Reviers *et al.* (1978), we did not observed any variations of spermatogonial divisions and meiosis maybe because in this study samples were collected during autumn and spring when seasonal changes are supposed to be more pronounced. Total number of germ cells per Sertoli cell ratio did not vary among groups and this result confirms that the number of germ cells per Sertoli cell is related to the number of functional Sertoli cells (Griswold, 1998) and highlights the harmonic involution of the seminiferous epithelium. In fact, the supporting capacity of the Sertoli cell in each species is the decisive factor for the initial population of primary spermatocytes (Sharpe, 1994). Besides, higher ratios of primary spermatocytes and elongated spermatids per Sertoli cell were observed out of the breeding season when Sertoli cell number is lower. In teratospermic cats, Neubauer *et al.* (2004) found fewer Sertoli cells per tubule cross-section cats and higher spermatogenic cells:Sertoli cell ratios compared to normospermic subjects.

The number of round spermatids per primary spermatocyte was 2.72±1.32 during post-rutting indicating a cell loss of 32% on the average during the two meiotic divisions; similar data was observed in gerbil (Segatelli *et al.*, 2004), goat (França *et al.*, 1999), cat (Neubauer *et al.*, 2004) and human (Roosen-Runge, 1973) although in these studies only spermatocytes in prophase were considered. In addition, round spermatids per primary spermatocyte ratio decreases without significant differences between breeding and no breeding season.

A negative correlation was found between spermatozoa and Sertoli cells percentages and SEI index in all three groups but it was higher during the post-rutting period. This result might be due to the fact that higher Sertoli percentage implies higher volume occupied by these cells in the seminiferous tubule and a strong negative correlation of volume occupancy of Sertoli cells with sperm production (Russell *et al.*, 1990). By contrast, both in humans and horses, the number of Sertoli cells evaluated in histological sections is related to the level of spermatogenesis as measured as daily sperm production per testis (Johnson *et al.,* 2008; Johnson, 1986; Johnson and Thompson 1983; Johnson *et al.*, 1984).

In agreement with previous studies on FNAC, SEI showed a high degree of variation both inter-males and inter-species suggesting the high variability of this parameter: Sardinian red deer (6.53, data no published), fallow deer (6.93±1.80, data not published), horse (20.9±17) (Leme and Papa, 2000), dog (4.2±0.8) (Santos *et al.*, 2010), alpaca (39.31±27.17 in the right testis and 45.75±24.79 in the left testis) (Stelletta *et al.*, 2011) and human (30.0±19.5) (Foresta *et al.*,

1993). Currently, the reason of such variability is not cleared but recent studies have shown that Sertoli cell number is determined during the foetal life (Kotsampasi *et al.*, 2008). In particular, undernutrition during pregnancy do not affect testis weight neither the onset of puberty but it has profound effects on Sertoli cells number and this factor could influence the reproductive potential as later as in adulthood (Kotsampasi *et al.*, 2008).

Interestingly, the decrease of SEI index and Sertoli cells percentage suggest seasonal fluctuations of the Sertoli cells population ad this finding is supported by the decrease Sertoli cells number per tubule cross-section.

Sertoli cells are the only somatic cell in the seminiferous epithelium and they have been considered to be numerically stable in adulthood (Steinberger and Steinberger, 1977). Nevertheless, it has been demonstrated that in seasonal breeders such as horse (Johnson and Thompson, 1983; Johnson *et al.*,1986; Johnson *et al.*, 1991; Johnson and Thompson, 2008), viscacha (Muñoz *et al.*, 2001), cat (Stornelli *et al*., 2009) and Soay ram (Hochereau-de Reviers *et al.*, 1985), Sertoli cells number is not fixed but fluctuates with season. In the stallion, it was found that the number of Sertoli cells increase approximately 48% between the onset and the middle of the breeding season, even if the number of Sertoli cells/gram of parenchyma remains unchanged. In red deer as well, unless the differences were statistically significant, Hochereau-de-Reviers and Lincoln (1978) found that the number of Sertoli cells increases by approximately 36% during the breeding season.

Furthermore, recent studies have demonstrated that in adult djungarian hamster Sertoli cells are not terminally differentiated and they regain their proliferative activity and junction protein organization after stimulation by exogenous FSH (Tarulli *et al.*, 2006). Thus, it is likely that seasonal variation of FSH could affect Sertoli cell number and junction proteins even in Iberian red deer with profound effects on spermatogenesis.

In fact, important seasonal ultrastuctural and morphological changes of Sertoli cells have been reported in the viscacha (*Lagostomus maximus maximus*) where a decrease of the number of Sertoli cells per tubule cross-sections during the involution phase was associated with changes of length and organizations of the inter-Sertoli tight junctions (Muñoz *et al.*, 2001). In man the formation of inter-Sertoli tight junctions is related to the functional maturation of Sertoli cells which occurs during puberty and involves loss of proliferative ability and expression of functions not present at their immature state (Sharpe *et al.*, 2003). The phase of Sertoli cells maturation with loss of their proliferative ability differs according to species even in the same order (Sharpe *et al.*, 2003).

On the contrary, in other species like hamster (Sinha Hikim *et al.*, 1988) and brown hare (Strbenc *et al.*, 2003) Sertoli cells persist as a stable population and show a minimal turnover indicating negligible rates of proliferation and apoptosis.

Theoretically, assuming that Sertoli cells do not vary with season, SEI should increase with declining of spermatogenesis. In fact, in human, progressive increase of Sertoli cell percentage and reduction in spermatozoon percentage, SI and SSEI have been reported with increasing

severity of reduction in spermatogenesis (Han *et al.,* 2006; Batra *et al.,* 1999). On the contrary, we observed that out of the breeding season SEI and Sertoli cell percentage decrease simultaneously with SI and spermatozoa percentage suggesting that SEI is not likely a good index of spermatogenic activity in the species where Sertoli cell population fluctuates with season.

In conclusion, FNAC showed to be a reliable tool to evaluate the seasonal changes of spermatogenesis in Iberian red deer and, for the first time to our knowledge, it proved to be useful to detect the cyclic fluctuations of testicular germ cells and Sertoli cells. Indeed, our results confirmed that, as shown in other seasonal breeding species, Sertoli cell number is not fixed but fluctuates with season in Iberian red deer. Moreover, because of its low invasiveness (Gouletsou *et al.*, 2010, 2011; Westlander, 2001; Leme and Papa, 2000), FNAC could be applied *in vivo* without affecting the reproductive performance. At this purpose, FNAC could be used to investigate spermatogenesis even in endangered species where reproductive problems due to inbreeding or effects of environmental pollution are quite frequent.

4. Seasonal Changes of Epididymal Sperm Parameters of Iberian Red Deer and its Relationships with Spermatogenesis Evaluated by Fine Needle Aspiration Cytology (FNAC)

4.1 Introduction

In seasonal breeders, the reproductive activity is associated with profound changes in spermatogenesis and ejaculate characteristics. Seasonal variation of reproduction in mammals is an adaptation to annual changes in the environment and minimizes the energy cost of their reproductive efforts (Goeritz *et al.*, 2003).

As it shown in several deer species, sperm quality, semen volume and percentage of morphologically normal spermatozoa reach highest values during the rutting season which is the period of maximal sperm production and highest libido (Umapathy *et al.*, 2007; Gizejewski, 2004; Goeritz *et al.*, 2003; Blottner *et al.*, 1996; Gosch and Fisher, 1989). In Cervids, where sexual dimorphism is highly pronounced, spermatogenesis is strictly correlated to the antler cycle: in fact, it is at minimum at antler casting, starts at velvet shedding and reaches the maximum at hard antlers (Gosch and Fisher, 1989). Moreover, Malo *et al.* (2005) found that antler size and complexity are honest indicators of sperm production and of sperm velocity in Iberian red deer.

Profound differences of reproductive cycle and strategies exist among cervids (Asher, 2010) with photoperiod being the main environmental parameter controlling reproduction (Malpaux, 2006): while some species exhibit high seasonal pattern in cool temperate climes such as *Capreolus capreolus* (Goeritz *et al.*, 2003; Blottner *et al.*, 1996) others, living in equatorial regions, like *Cervus eldi thamin* are aseasonal (Monfort *et al.*, 1993).

Besides seasonality, several factors affect sperm quality, like epididymal transit, seminal plasma composition and environmental factors, but spermatogenesis is the first and the most important. Moreover, it is widely accepted the sperm motility, viability and morphology change depending on the season and all of these parameters could affect fertility both *in vivo* and *in vitro.*

In *Cervus elaphus,* Gizejewski (2004) found that the lowest percentage of major defects of sperm (< 5%), was recorded during the mating season whereas a higher percentage (>10%) was recorded during the pre- and post-mating season. In roe deer, for example, the most prominent sperm defect observed during the recrudescence phase (April) was detached heads whereas tail defects during the involution phase (December) (Goeritz *et al.*, 2003). In *Cervus eldi thamin,* sperm abnormalities were predominant out of the breeding season: abnormal acrosome, retained cytoplasmic droplet and detached heads were the most common defects during the recrudescence phase whereas abnormal acrosome and distal droplet were the most common during the regression phase.

In Iberian red deer, breeding season could be divided in rut (mid-September to mid-October) and post-rut (mid-October to mid-December) which is the phase when sperm quality was higher despite the lower quantity collected (Martinez-Pastor *et al.*, 2005).

Malo *et al.* (2005) found that in Iberian red deer the three sperm velocity parameters (VCL, VSL and VAP) and, to a lesser extent, the proportion of normal sperm showed significant associations with fertility. Moreover, Malo *et al.* (2005) examined semen traits and male fertility and found a negative association between midpiece size and swimming velocity and these differences among males were mainly determined by sperm swimming velocity and by the proportions of morphologically normal spermatozoa. Furthermore, it has been demonstrated that sperm motility and percentage of morphologically normal spermatozoa affect the fertility and sex-ratio at birth (Gomendio and Roldan , 2008, 2007, 2006; Malo *et al.*, 2005 and 2006).

The aim of the study was to evaluate seasonal effects on sperm characteristics and how these parameters are associated with spermatogenesis evaluated by Fine Needle Aspiration Cytology (FNAC) technique.

4.2 Materials and Methods

4.2.1 Fine Needle Aspiration Cytology (FNAC)

Fine needle aspiration cytology (FNAC) was performed as shown in experiment 1. In this case, summa of both testes was considered in order to relate the testicular cells proportions and indexes to the content of both epididymides.

4.2.2 Sperm collection

All the reagents were purchased from Sigma-Aldrich (Madrid, Spain) unless otherwise indicated. Samples were collected from 48 adult stags (age > 4.5 years, weight > 130 kg) of Iberian red deer *(Cervus elaphus hispanicus,* Helzheimer 1909*)* exposed to natural photoperiod from the end of November 2010 till the end of February 2011 in the south of Spain (Comunidades Autónomas de Castilla-La Mancha and Andalucía). Samples were collected from 7 different game reserves. Samples were collected post-mortem from animals legally culled and hunted in their natural habitat. Hunting was in accordance with the harvest plan of the game reserve, made following Spanish Harvest Regulation which conforms to European Union Regulation. Testes, within the

scrotal sac, were cut with a knife and transported to the laboratory in a plastic bag at room temperature (20-25°C). Testes were removed from the scrotal sac and isolated.

Sperm was collected from the cauda of epididymis by repeated longitudinal and transverse cut and place it in 0.5 ml of Salamon modified solution (Fernandez- Santos *et al.*, 2005).

In particular, the base solution (Fraction A) was composed of: TRIS (2.7 % w/v), fructose (1%), citric acid (1.4%), glycerol (0%, v/v), and egg yolk (20%, v/v) with pH=6.8 and osmolality of 300 mOsm/Kg (Esteso *et al.*, 2006).

The contents of both epididymides were pooled because previous studies demonstrated that no differences were found between them (Garde *et al.*, 1998).

Weight of each testis was recorded (Balance EK-400H, A&D Company, Tokyo, Japan) after removing the epidydimis and the spermatic cord with a surgical blade.

Samples were processed between 6-10 hours after death of animals.

4.2.3 Sperm concentration

Samples were put on a glass tube, previously weighted. The weight of the semen was obtained deducting the initial weight of the tube.

Sperm concentration was assessed shortly after collection using a Makler chamber (Sefi Medical Instruments, Haifa, Israel).

4.2.4 Sperm motility evaluation

Percentage of individual motile spermatozoa and quality of motility (QM) were assessed (using a scale from 0 to 5) with a Makler chamber (5 µl of sample) previously warmed at 37°C. Sperm motility index (SMI) was calculated according to the formula (Kikuchi *et al.*, 1998):

[% individual motility + (quality of motility x 20)] x 0.5

CASA (Computer Assisted Sperm Analysis) analysis was carried out as described by Martinez *et al.* (2006). Briefly, samples were diluted in Fraction A at 20-30*10^6 spz/ml. Then, a drop of 5 µl was placed on a Makler chamber and examined with a phase contrast microscope (Nikon Labophot-2; negative contrast optics 10X magnifications) with a warming stage at 37°C.

Analysis was carried out using the Motility Analyzer v. 7.4 (Hamilton Thorne Research).

A spermatozoon was considered motile when VCL >10 µm/s and progressive if VCL >25 µm/s and STR >80%. At least, 250 cells were analysed.

The software set-up configuration was as follows: Frames acquired: 20; Frame rate: 25/s; Minimum contrast: 10; Minimum size: 9; Lo/Hi size gates 0.9/2.1; Lo/Hi intensity gates 0.4/1.6; non-motile head size: 9; non-motile brightness: 10; medium VAP value: 25; low VAP value:10; slow cells motile: NO; threshold STR: 80. Thus, the image acquisition rate was 25 frames/s and the acquisition time was 0.8 s. A total of 10 parameters were evaluated: **MT** (Total Motility),

MP (Progressive Motility), **VAP** (Average Path Velocity; µm/s) which corresponds to the average velocity of sperm track; **VSL** (Velocity Straight Line; µm/s) which corresponds to the average sperm velocity taking into account the first and the last point of trajectory; **VCL** (Velocity Curvilinear line; µm/s) which corresponds to the average sperm velocity considering point to point the sperm track; **ALH** (Amplitude Lateral Head Movement; µm); **BCF** (Beat Cross Frequency; Hz) which express the beat frequency of the tail; **WOB** (percentage of lateral head displacement; %); **STR** (Straightness; %) which is the VSL/VAP ratio; **LIN** (Linearity; %) which is the VSL/VCL ratio.

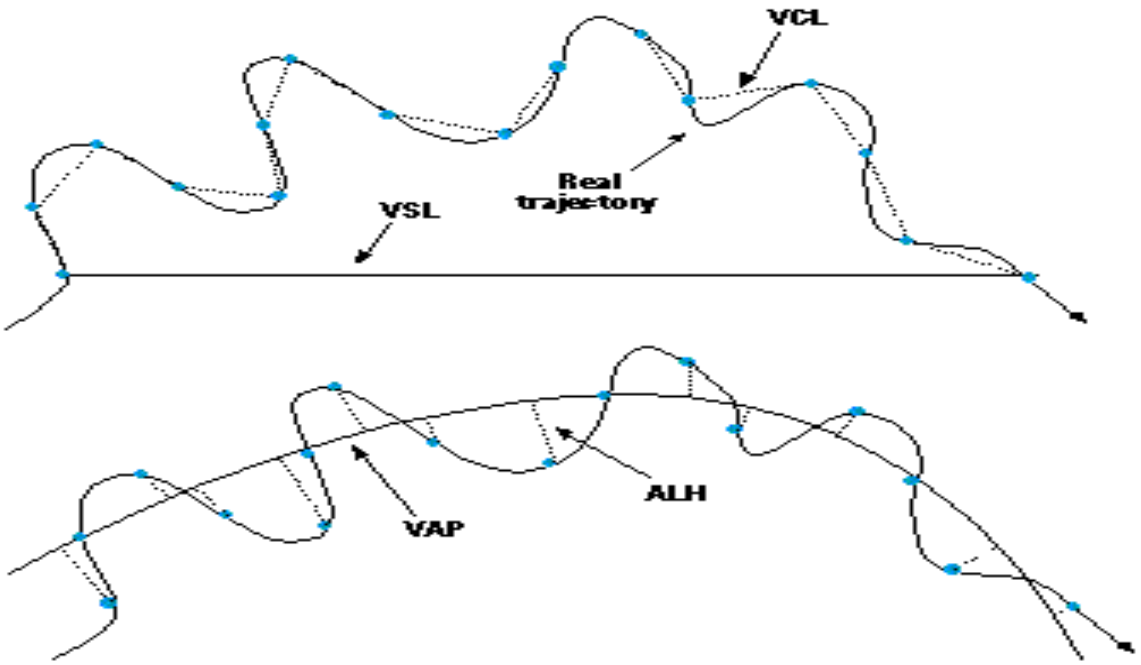

Figure 1. Schematic representation of the main kinetics parameters assessed by CASA system (from Tablado *et al.*, 1996).

4.2.5 Sperm viability

Sperm viability was evaluated as shown by Domíniguez-Rebolledo *et al.* (2011).

Briefly, the monomeric cyanine nucleic acid stain YO-PRO-1 was used to assess sperm viability. Samples were diluted down to 10^6 spermatozoa / ml in flow cytometry PBS with 0.1 μM YO-PRO-1 and 10 μM PI. After 20 min in the dark, the samples were run through a flow cytometer. Labelling cells with the apoptotic marker YO-PRO-1 yielded three subpopulations: viable (unstained: YO-PRO-1- / PI-), apoptotic-like membrane changes (YO-PRO-1+/ PI) and non-viable (membrane damaged: PI+). Hoechst 33342 was included at 5.1 μM.

4.2.6 Sperm morphometry

Microscopic slides were prepared from each fresh (upon dilution) sample by placing 5μl of the sperm on a slide and smearing it. Then, smears were air-dried and stained using Panoptic (Panreac, Barcelona, Spain) (Alvarez *et al.*, 2008).

Stained slides were used to perform ASMA using the morphometry module of a commercially available system (Sperm-Class Analyzer, version 99 CASMA system, Microptic, Barcelona). The machine was equipped with a Nikon (Labophot-2, Tokyo, Japan) microscope with a 60X bright-field objective and a Sony video camera (CCD AVC-D7CE, Sony Corporation, Tokyo, Japan).

The morphometric dimensions were acquired for 150-160 images and that assured that a minimum of 145 of sperm head were analysed properly.

A total of eight parameters were evaluated:

- **Head length** (L expressed in μm);
- **Head width** (W expressed in μm);
- **Head Area** (A, expressed in μm^2);
- **Head Perimeter** (P, expressed in μm).

On the basis of the previous of these parameters, other four parameters were evaluated:

- **Fun1** (Ellipticity: L/W);
- **Fun2** (Circularity: $4\Pi A/P^2$)
- **Fun3** (Elongation: (L-W)/(L+W))
- **Fun4** (Regularity: ΠLW/4A)

4.2.7 Sperm abnormalities

Sperm abnormalities were evaluated on 150 spermatozoa stained with Panoptic. Percentage of sperm abnormalities were assessed as follows:

- **Decapitated heads;**
- **Head abnormalities;**

- **Proximal droplets;**
- **Tail defects;**
- **Total sperm abnormalities.**

Only proximal droplets were considered because distal droplet is a normal occurrence epididymal spermatozoa of ruminants (Cooper, 2005).

4.2.8 Statistical analysis

All statistical analyses were performed using the SPSS 17.0 statistical software package (SPSS Inc, Chicago, IL, USA). For each variable descriptive statistics are reported. Kolmogorov-Smirnov test and Levene's test were used to check data normality and homogeneity of variance, respectively. One-way ANOVA was performed in order to compare means between groups. Tukey post-hoc test was applied in case of homogeneity of variance, otherwise Dunnet-T3 post-hoc test. Pearson correlation was used to evaluate correlations between sperm parameters and cytological analysis. Differences were considered significant at $p< 0.05$.

4.3 Results

4.3.1 Testicular cell percentages and indexes assessed by FNAC

Percentage of spermatogenic cells and Sertoli cells described the same pattern shown in Chapter 3. Testicular cell percentages and indexes are reported in Table 1 and 2, respectively. Not statistical differences were reported for any parameter between this data (n= 47) and data reported in Chapter 3 (n=53) ($p > 0.05$).

	Group BS (N=17)			Group TP (N=16)			Group NB (N=14)		
	Mean ± SD	**CV (%)**	**Min-Max**	**Mean ± SD**	**CV (%)**	**Min-Max**	**Mean ± SD**	**CV (%)**	**Min-Max**
Testicular weight (gr)	**83.53±27.14**	32.49	47.49-124.66	**80.34±12.62**	15.70	62.86-98.82	**58.56±12.56**	21.45	36.71-81.50
Sertoli cells (%)	**14.12±7.74**	54.82	3.47-28.09	**8.88±4.17**	46.96	2.37-18.14	**8.38±3.31**	39.50	3.34-14.44
Spermatogonia (%)	**1.57±0.90**	57.32	0.00-3.15	**1.43±0.56**	39.16	0.41-2.15	**1.49±0.81**	54.36	0.21-3.61
Spermatocytes I (%)	**14.63±4.16**	28.43	5.95-20.76	**16.84±1.96**	11.64	13.12-19.48	**16.70±2.60**	15.57	12.58-23.25
Spermatocytes II (%)	**0.29±0.26**	89.66	0.00-0.86	**0.20±0.34**	170	0.00-1.29	**0.27±0.26**	96.30	0.00-0.66
Spermatids ab (%)	**35.50±5.24**	14.76	25.97-44.43	**37.13±4.93**	13.28	27.23-44.14	**35.21±6.02**	17.10	28.44-44.94
Spermatids cd (%)	**14.66±4.56**	31.10	7.05-21.10	**17.98±3.65**	20.30	12.53-26.50	**21.49±6.52**	30.34	10.55-36.14
Spermatozoa (%)	**19.23±4.15**	21.58	11.95-28.20	**17.53±5.11**	29.15	8.45-24.78	**16.46±4.02**	24.42	9.09-22.45

Table 1. Descriptive statistics of Sertoli cells and germ cells percentages during breeding season (BS), transition period (TP) and non breeding season (NB) (n=47)

	Group BS (N=17)			Group TP (N=16)			Group NB (N=14)		
	Mean ± SD	**CV (%)**	**Min-Max**	**Mean ± SD**	**CV (%)**	**Min-Max**	**Mean ± SD**	**CV (%)**	**Min-Max**
SEI (%)	**17.39±11.10**	63.83	3.60-39.08	**9.97±5.19**	52.06	2.43-22.17	**9.28±4.01**	43.21	3.99-16.88
SSEI	**2.09±1.86**	89.00	0.43-8.13	**2.69±2.16**	80.30	0.47-9.15	**2.24±0.92**	41.07	1.12-3.48
SI (%)	**22.27±3.65**	16.39	16.62-29.37	**19.18±5.47**	28.52	10.33-29.13	**18.00±4.38**	24.33	9.57-24.40
MI	**2.67±1.14**	42.70	1.47-6.61	**2.24±0.46**	20.54	1.58-3.00	**2.18±0.63**	28.90	1.22-3.57

Table 2. Descriptive statistic of testicular cell indexes during breeding season (BS), transition period (TP) and non breeding season (NB) (n=47

4.3.2 Epididymal sperm concentration and total number of spermatozoa recovered

Significant differences in sperm concentration were found between Group **TP** and Group **NB**. However, the total number of spermatozoa collected did not differ statistically between groups ($p > 0.05$) (**Table 3).**

4.3.3 Sperm motility

Percentage of motile sperm, quality of motility and SMI greatly differ between groups. All the parameters were the highest during the breeding season and lowest during the non breeding season (**Table 4**).

MT, MP and BCF did not differ statistically between groups. All the remaining parameters (VAP, VSL, VCL, ALH and WOB), except for STR, were statistically higher during the breeding season (**Table 5**).

4.3.4 Sperm viability

Viable spermatozoa ($yopro^-/ip^-$) were significantly higher during the breeding season. Not viable sperm did not statistically differ between groups ($yopro^+/ip^+$) ($p > 0.05$). Besides, apoptotic cells ($yopro^+/ip^-$) were significantly higher in Group BS and Group TP compared to NB (**Table 6**).

4.3.5 Sperm morphometry

Sperm head length, width, perimeter, area and circularity were significantly higher during the breeding season compared to the non breeding season (**Table 7**). Non significant differences were found for the remaining parameters ($p > 0.05$).

4.3.6 Sperm abnormalities

Non significant differences were found between groups. A slightly increase of the percentage of sperm tail defects and total sperm abnormalities were observed from the breeding season to the non breeding season but they were statistically significant ($p > 0.05$) (**Table 8**).

	Group BS (N=17)			Group TP (N=15)			Group NB (N=14)		
	Mean±SD	**CV(%)**	**Min-Max**	**Mean±SD**	**CV(%)**	**Min-Max**	**Mean ± SD**	**CV(%)**	**Min-Max**
Concentration (10^6spz/ml)	**1301.28±756.69**	58.15	276-2712	**1736.47±908.06**^{NB*}	52.29	276-3144	**980.93±519.24**^{TP*}	52.93	196-1855
Tot. number sperm collected (10^6spz)	**756.51±568.15**	75.10	121.39-1963.69	**1124.83±783.01**^{NB*}	69.61	110.91-2523.68	**473.28±432.37**^{TP*}	91.36	74.41-1046.01

Table 3. Descriptive statistics of sperm concentration and total number of sperm recovered. *p<0.05

	Group BS (N=17)			Group TP (N=16)			Group NB (N=14)		
	Mean±SD	**CV (%)**	**Min-Max**	**Mean±SD**	**CV (%)**	**Min-Max**	**Mean ± SD**	**CV (%)**	**Min-Max**
Motile sperm (%)	**85.88±8.70**^{TP*,NB*}	10.13	70.00-95.00	**65.94±23.47**^{BS*}	35.59	5.00-90.00	**64.29±23.52**^{BS*}	36.58	15.00-90.00
QM (0-5)	**3.78±0.60**^{NB*}	15.87	2.75-4.50	**3.19±0.92**	28.84	1.50-4.50	**3.02±0.73**^{BS*}	24.17	2.00-4.00
SMI (%)	**80.74±9.18**^{TP*,NB**}	11.37	65.00-90.00	**64.84±19.74**^{BS*}	30.44	17.50-85.00	**62.32±17.93**^{BS**}	28.77	22.50-85.00

Table 4. Descriptive statistics of subjective motility evaluation. *p<0.05; **p<0.01

	Group BS (N=17)			Group TP (N=16)			Group NB (N=14)		
	Mean±SD	**CV(%)**	**Min-Max**	**Mean±SD**	**CV(%)**	**Min-Max**	**Mean±SD**	**CV(%)**	**Min-Max**
MT (%)	**97.29±2.71**	2.79	88.17-99.65	**90.23±16.81**	18.63	34.82-99.92	**85.34±20.55**	24.08	31.03-99.46
MP (%)	**13.56±7.19**	53.02	8.26-38.30	**12.76±4.26**	33.39	1.02-17.69	**15.38±6.77**	44.02	4.83-28.78
VAP(µm/s)	**103.02±13.21**^{TP***,NB***}	12.82	76.14-124.43	**67.12±23.39**^{BS***}	34.85	9.88-96.61	**68.41±23.00**^{BS***}	33.62	18.67-98.28
VSL(µm/s)	**48.48±6.29**^{TP**,NB**}	12.97	35.30-61.52	**35.63±11.67**^{BS**}	32.75	5.77-49.21	**37.06±12.36**^{BS**}	33.35	11.91-57.74
VCL(µm/s)	**142.18±17.54**^{TP**,NB***}	12.34	105.76-164.12	**104.05±38.58**^{BS**}	37.08	19.80-142.41	**98.92±31.93**^{BS***}	32.28	29.17-139.85
ALH (µm)	**5.14±0.90**^{NB*}	17.51	3.23-6.23	**4.20±1.53**	36.43	1.30-5.92	**3.81±1.16**^{BS*}	30.45	1.39-5.29
STR (%)	**49.66±5.69**^{TP**,NB***}	11.46	41.74-67.07	**54.76±2.69**^{BS**}	4.91	51.53-60.48	**56.18±3.32**^{BS***}	5.91	52.32-62.67
LIN (%)	**35.43±5.93**	16.74	30.16-54.18	**34.83±3.78**	10.85	28.62-41.37	**36.51±4.83**	13.23	28.86-47.92
BCF (Hz)	**5.69±0.91**	15.99	3.72-7.29	**6.00±1.38**	23.00	2.39-8.17	**5.72±1.12**	19.58	3.32-7.26
WOB (%)	**71.14±5.91**^{TP**,NB*}	8.31	61.00-81.51	**62.88±6.35**^{BS**}	10.10	50.10-74.66	**64.16±7.42**^{BS*}	11.56	50.24-77.38

Table 5. Descriptive statistics of kinetics parameters assessed by CASA. *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$

	Group BS (N=17)			Group TP (N=16)			Group NB (N=14)		
	Mean±SD	**CV (%)**	**Min-Max**	**Mean±SD**	**CV (%)**	**Min-Max**	**Mean±SD**	**CV (%)**	**Min-Max**
YOPRO$^-$/IP$^-$(%)	**85.65±8.33^{TP*,NB***}**	9.73	63.20-94.79	**72.94±15.33^{BS*}**	21.02	44.51-89.48	**73.55±9.34^{BS***}**	12.48	57.89-86.35
YOPRO$^+$/IP$^-$ (%)	**4.05±2.26^{TP**,NB**}**	55.80	1.12-9.14	**12.63±9.37^{BS**}**	74.19	4.08-38.82	**9.03±4.49^{BS**}**	60.49	2.23-17.75
YOPRO$^+$/IP$^+$ (%)	**9.67±7.78**	80.45	2.46-28.19	**13.19±10.01**	75.89	3.44-43.19	**15.49±7.86**	53.98	3.91-34.42

Table 6. Descriptive statistics of sperm viability assessed by flow cytometry. *: p≤0.05; **: p≤0.01; ***: p≤0.001

	Group BS (N=17)			Group TP (N=16)			Group NB (N=14)		
	Mean±SD	**CV (%)**	**Min-Max**	**Mean±SD**	**CV (%)**	**Min-Max**	**Mean±SD**	**CV (%)**	**Min-Max**
Length (µm)	**9.14±0.29^{NB*}**	3.17	8.55-9.69	**9.09±0.21**	2.31	8.65-9.46	**8.93±0.16^{BS*}**	1.90	8.57-9.16
Width(µm)	**5.09±0.17^{NB**}**	3.34	4.81-5.36	**5.09±0.09^{NB***}**	1.77	4.95-5.23	**4.95±0.92^{BS**,TP***}**	2.02	4.82-5.09
Perimeter(µm)	**25.00±0.64^{NB*}**	2.56	23.55-25.78	**24.97±0.42**	1.68	24.17-25.76	**24.57±0.35^{BS*}**	1.38	23.78-25.00
Area(µm^2)	**38.43±1.98^{NB**}**	5.15	34.08-40.91	**38.15±1.05^{NB***}**	2.75	35.80-39.73	**36.33±0.88^{BS**,TP***}**	2.34	34.36-37.40
Fun 1	**1.80±0.07**	3.89	1.70-1.93	**1.79±0.05**	2.79	1.66-1.88	**1.81±0.05**	2.75	1.74-1.89
Fun 2	**0.77±0.02^{NB*}**	2.60	0.74-0.80	**0.77±0.01**	1.30	0.75-0.80	**0.76±0.02^{BS*}**	2.63	0.73-0.79
Fun 3	**0.28±0.02**	7.14	0.26-0.32	**0.28±0.01**	3.57	0.25-0.31	**0.29±0.01**	3.45	0.27-0.31
Fun 4	**0.95±0.01**	1.05	0.94-0.98	**0.95±0.01**	1.05	0.94-0.97	**0.96±0.01**	1.05	0.95-0.97

Table 7. Descriptive statistics of sperm morphometry assessed by ASMA. *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$

	Group BS (N=17)			Group TP (N=16)			Group NB (N=14)		
	Mean±SD	**CV (%)**	**Min-Max**	**Mean±SD**	**CV (%)**	**Min-Max**	**Mean±SD**	**CV (%)**	**Min-Max**
Head abnormalities (%)	**1.80±1.10**	61.11	0.00-4.00	**1.33±0.69**	51.88	0.00-2.67	**1.05±1.13**	106.86	0.00-4.00
Decapitated head (%)	**3.49±5.34**	153.01	0.00-2.00	**5.50±4.54**	82.55	6.67-18.67	**3.10±2.24**	68.67	6.67-8.67
Proximal droplets (%)	**2.82±4.04**	143.26	0.00-16.00	**2.71±2.81**	1.04	0.00-10.00	**6.10±4.56**	74.33	1.00-13.00
Tail abnormalities (%)	**6.67±4.01**	60.12	2.00-16.00	**8.46±4.61**	54.49	2.00-17.33	**10.48±6.91**	64.48	3.33-23.33
Total sperm abnormalities (%)	**14.78±8.10**	54.80	3.33-32.67	**18.00±9.00**	50.00	6.67-34.67	**20.71±7.49**	35.48	10.67-37.33

Table 8. Descriptive statistics of sperm abnormalities. Non significant differences between groups were found.

4.3.7 Correlations between sperm concentration, number of sperm recovered and testicular cells and indexes

Group BS

Non significant differences were found in this group ($p>0.05$).

Group TP

Non significant differences were found in this group ($p>0.05$).

Group NB

Positive relationship was found between MI and sperm concentration ($r=0.557$ $p=0.038$).

4.3.8 Correlation between sperm motility and testicular cells and indexes

Group BS

Positive relationships were found between SI and percentage of motile sperm ($r=0.502$ $p=0.040$), quality of motility ($r=0.547$ $p=0.023$) and Sperm Motility Index ($r=0.595$ $p=0.012$).

Moreover, VAP was positively correlated with Sertoli cells percentage and SEI (r=0.512 p=0.036 and r=0.527 p=0.030, respectively) and between BCF and spermatids ab (r=0.619 p= 0.008);

Group TP

Quality of motility (QM) was negatively related to Sertoli cells percentage and SEI (r=-0.585 p=0.017 and r=-0.578 p=0.019, respectively). Besides, a positive relationship was found between the same parameter and SSEI (r=0.539 p=0.031);

Group NB

Negative relationships were found between percentage of spermatogonia and percentage of motile sperm, quality of motility and SMI (r=-0.657 p=0.011; r=-0.553 p=0.040 and r=-0.656 p=0.011, respectively).

Moreover, high spermatogonia percentage was negatively related to VAP (r=-0.674 p=0.008), VCL (r=-0.623 p=0.017), VSL (r=-0.688 p=0.007), LIN (r=-0.572 p=0.033) and WOB (r=-0.632 p=0.015) (**Figure 2**). Unless Non significant, negative relationships were found with the remaining kinetics parameters: Mot (r=-0.511 p=0.062), MP (r=-0.528 p=0.052), ALH (r=-0.523 p=0.055) and BCF (r=-0.530 p=0.051).

On the contrary, positive relationship were found between percentage of primary spermatocytes and percentage of subjective motility (r=0.609 p=0.021). For sperm kinetics, positive

relationships were found between the same germ cell and MT (r=0.577 p=0.031), VAP (r=0.539 p=0.047), VCL (r=0.630 p=0.016), ALH (r=0.638 p=0.014) and BCF (r=0.570 p=0.033).

Finally, a negative relationship was found between MI and Total motility (r=0.562 p=0.036).

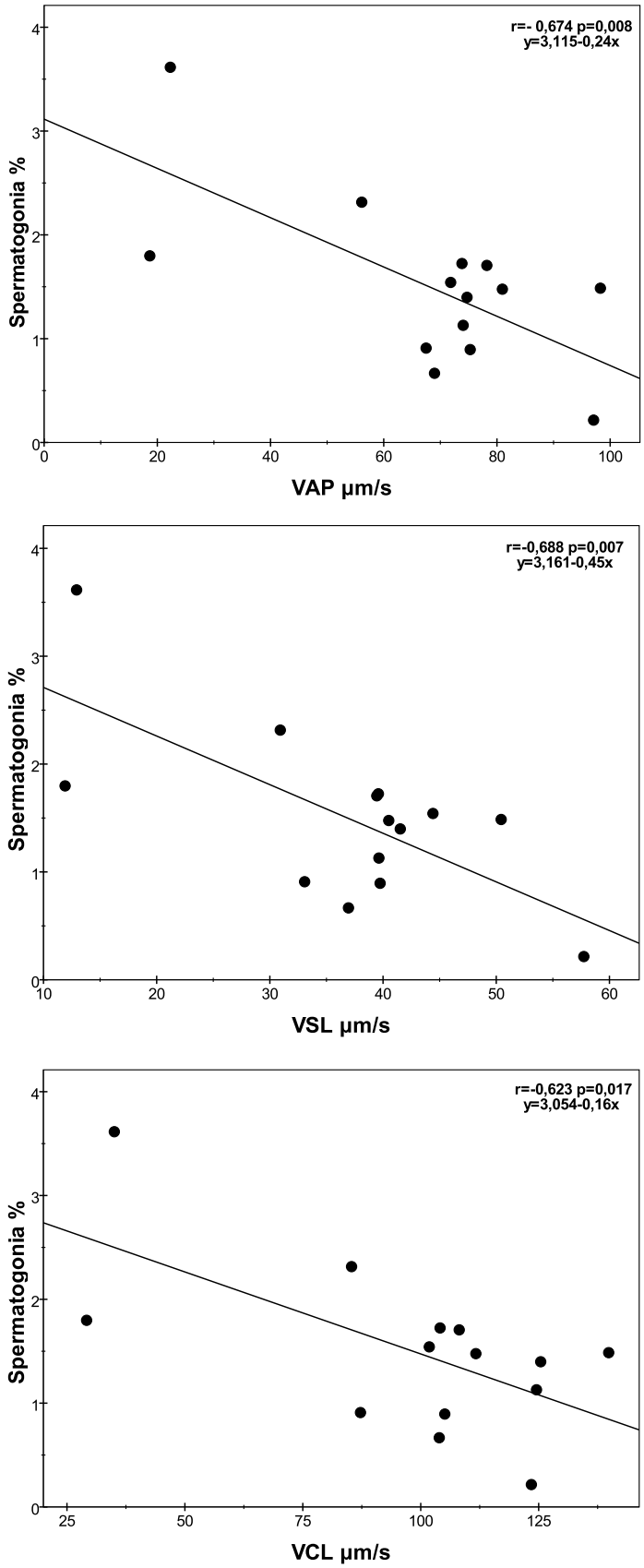

Figure 2. Negative relationships between percentage of spermatogonia and VAP, VSL and VCL out of the breeding season (Group NB).

4.3.9 Correlations between flow-cytometry and testicular cells

Group BS

Non significant correlations were found in this group.

Group TP

Positive relationships were found between viable spermatozoa (yopro$^-$-ip$^-$) and percentage of spermatozoa and SI (r=0.578 p=0.019 and r=0.556 p=0.025, respectively).

Negative relationship was found between the same parameter and testicular weight (r=-.558 p=0.025).

Negative relationships were found between apoptotic cells (yopro$^+$-ip$^-$) and percentage of spermatogonia and spermatozoa (r=-0.530 p=0.035 and r=-0.532 p=0.034, respectively).

Group NB

A positive relationship was found between viable spermatozoa (yopro$^-$-ip$^-$) and primary spermatocytes (r=0.568 p=0.034) but negative with round spermatids / primary spermatocyte ratio (r=-0.573 p=0.032).

Positive relationships were found between dead spermatozoa (yopro$^+$-ip$^+$) and spermatids ab (r=0.545 p=0.044) and round spermatids / primary spermatocyte ratio (r=0.601 p=0.023) but negative with primary spermatocytes (r=-0.591 p=0.026).

4.3.10 Correlations between sperm morphometry and testicular cells and indexes

Group BS

Positive relationships were found between spermatozoa percentage and sperm head ellipticity (Fun1) (r=0.536 p=0.027) and sperm head elongation (Fun3) (r=0.541 p=0.025).

Moreover, positive relationships were found between SI and sperm head ellipticity (Fun1) (r=0.588 p=0.013) and sperm head elongation (Fun 3) (r=0.592 p=0.012).

Finally, a positive relationship was found between sperm head regularity (Fun 4) and round spermatids / primary spermatocyte ratio (r=0.543 p=0.024)

Group TP

Negative relationships were found between sperm head area and percentage of spermatozoa (r=-0.590 p=0.016) and SI (r=-0.561 p=0.024).

Besides, sperm head regularity (Fun 4) showed a positive relationships with percentage of spermatozoa (r=0.580 p=0.019) and SI (r=0.590 p=0.016).

Group NB

Negative relationships was found between sperm head circularity (Fun 2) and percentage of spermatogonia (r=-0.649 p=0.012).

4.3.11 Correlation between sperm abnormalities and testicular cells and indexes

Group BS

Percentage of spermatids ab showed a positive relationships with percentage of head abnormalities and decapitated heads (r=0.530 p=0.029) and decapitated heads (r=0.522 p=0.032). Besides, a negative relations was found between percentage of spermatids cd and sperm head abnormalities (r=-0.564 p=0.018).

Group TP

Percentage of head abnormalities showed a negative relationship with weight (r=-0.510 p=0.043) but positive with percentage of spermatozoa and SI (r=0.505 p=0.046 and r=0.524 p=0.037, respectively).

SI showed a positive relationships with tail abnormalities (r=0.544 p=0.029) and total sperm abnormalities (r=0.539 p=0.031)

Even percentage of spermatozoa showed a positive relationships with tail abnormalities and sperm total abnormalities but both were not statistically significant (r=0.495 p=0.051 and r=0.493 p=0.052, respectively).

Group NB

Positive relationships were found between Sertoli cells percentage and SEI and sperm tail abnormalities (r=0.678 p=0.008 and r=0.682 p=0.007, respectively). Finally, a negative relationship was found between SSEI and sperm tail defects (r=-0.674 p=0.008).

4.4 Discussion

Our results are in agreement with previous reports about epididymal sperm in Iberian red deer (Martinez-Pastor et al., 2005) showing that sperm quality is higher during the breeding season and this occurrence is associated with fully active spermatogenesis.

Sperm motility and kinetics, both assessed subjectively and by CASA system, show that highest values are reached during the reproductive season and the same pattern is described even by flow citometry. As previously shown in roe deer, (Goeritz *et al.*, 2003; Blottner *et al.*, 1996), red deer (Gizejewski, 2004), fallow deer (Gosch and Fisher, 1989) and spotted deer (Umapathy *et al.*, 2007), even in Iberian red deer both total sperm number, percentage of motile and morphologically normal spermatozoa correspond with higher sperm production and quality during the breeding season.

Sperm concentration and total number of epididymal spermatozoa collected during the breeding season were lower than that reported by Martinez *et al.* (2008) but this data could be due to that fact in this case samples were collected during the rutting period when deer show highest sexual activity (mid September–mid October).

During the breeding season percentage of motile sperm, quality of motility and SMI show a positive relationship with spermiogenesis and sperm production (expressed as SI). By contrast, high proportion of spermatogonia out of the breeding season was negatively related with sperm motility suggesting that the involution of spermatogenesis is negatively associated with sperm kinetics.

Furthermore, during the breeding season, both Sertoli cell percentage and SEI were positively correlated with average path velocity (VAP), whereas percentage of spermatozoa and Spermatic index shown a positive relationships with sperm head elongation and ellipticity. Malo *et al.* (2006) found that sperm head elongation and the relative length of principal plus terminal piece seem to influence mainly the straightness of the trajectory.

Moreover, average path velocity (VAP) with straightness (STR) and lateral head displacement (ALH), is associated with cervical mucus penetration and fertility (Aitken, 2006).

Sperm head morphometry show seasonal variations in Iberian red deer which mainly consist of reduction of sperm head length, width, perimeter and area. No previously data have been reported on seasonal variation of sperm head morphometry in this species. Epididymal transit is one of the main factor affecting sperm head morphometry which mainly consist of reduction of sperm head area (Cooper, 2011), width and increasing elongation (Perez-Sanchez *et al.*, 1998). The reasons why, out of the breeding season, with the decline of spermatogenesis, there is a decline of sperm head volume remains to be elucidated. Although we considered spermatozoa collected from the cauda of epididymis it is widely accepted that if spermiogenesis is defective,

the shed spermatozoon may be unable to respond adequately to the epididymal environment (Cooper, 2011). Thus, decline of sperm head volume out of the breeding season could be related to impaired spermatogenesis or epididymal transit.

No relationships were found between sperm head morphometry and sperm concentration and total number of sperm collected (data not shown).

Total sperm abnormalities showed an increasing pattern out of the breeding season although differences were not statistically significant. On the basis of our results, higher Sertoli cells number out of the breeding season is associated with sperm tail defects and suggesting impaired spermiogenesis as confirmed by seasonal pattern by FNAC (see Chapter 3). Furthermore, on the basis of our findings, a higher percentage of spermatids cd out of the breeding season is associated with increased total sperm abnormalities.

The predominant abnormalities found in teratospermic cats, where fewer Sertoli cells are observed in the tubule cross-section are persisting cytoplasmic droplet or a defective midpiece; on the contrary, only few head and acrosomal abnormalities were observed (Neubauer *et al.*, 2004). A high proportion of sperm with a retaining cytoplasmic droplet may be indicative of impaired spermiogenesis (Neubauer *et al.*, 2004).

In conclusion, sperm quality in Iberian red deer is higher during the breeding season when spermatogenesis is fully active. Moreover, during the breeding season Sertoli cell percentage and Spermatic index are positively associated with sperm motility and sperm head elongation. Finally, high percentage of spermatogonia out of the breeding season is negatively related with

sperm kinetics suggesting that involution of spermatogenesis is associated with reduced sperm quality.

5. Preliminary study on Fine Needle Aspiration Cytology (FNAC) and Reproductive Monitoring of Sardinian Red Deer (Cervus elaphus corsicanus, Erxleben 1777) and Fallow Deer (Dama dama, Linnaeus 1758)

5.1 Introduction

The aim of this study was to evaluate *in vivo* the reproductive physiology of Sardinian red deer (*Cervus elaphus corsicanus*, Erxleben 1777) and fallow deer (*Dama dama*, Linnaeus 1758) stags during the breeding season.

Sardinian red deer is an endemic subspecies of Sardinian and Corse, strictly protected under Appendix II of the Bern Convention and Annexes II* and IV of the EU Habitats and Species Directive and included as a Near Threatened on the IUCN Red List (Lovari, 2011).

At the beginning of the XX century, *Cervus elaphus corsicanus* population drastically reduced so that in 1960-1970 it resulted extinct in Corse whereas only 210-230 individuals were estimated in Sardinia (Schenk, 1976). Starting from 1970, due to conservation programs, the population slowly has increased and nowadays nearly 6000 deer have been estimated to in Sardinia. Besides, *Dama dama* was recently reintroduced (1960) and, unless endangered (Lc: least concern category-IUCN), is protected by regional law (L 23/98).

Sardinian red deer is characterised by a smaller size compared to *Cervus elaphus*: body weight is 120-130 kg in stags and 70-80 kg in hinds in the first compared to 220-230 kg in stags and 100-140 in hinds of the latter) (Beccu, 1989; Uccheddu, 2008).

The breeding season (rutting season) starts in early September and lasts until the end of October in Sardinian red deer (Beccu, 1989) and from mid October to mid November in fallow deer (Zomborszky *et al.*, 2005) but it may vary depending on the area.

As other cervids, Sardinian red deer is characterised by a high degree of sexual dimorphism. Malo *et al.* (2005) found that in the polygynic species, sexual characteristic development, such as body mass and antlers growth, are strictly correlated to the endocrine status and reproductive performances of the male (Malo *et al.*, 2005).

Although the Sardinian red deer is an endangered subspecies with peculiar and distinct characteristics, several aspects of its reproductive biology and physiology still remain unknown.

The purpose of this study was to provide the first data about biometrics, semen analysis and testicular cytology of Sardinia red deer and fallow deer and is part of a major project which aims to explore the biology of these species in order to better preserve them. Furthermore, we proposed FNAC as alternative method to evaluate spermatogenesis *in vivo* and to investigate reproductive efficiency in wildlife, even in endangered species.

5.2 Materials and methods

All animal experiments were performed in accordance with DPR 27/1/1992 (Animal Protection Regulations of Italy) which conform to European Community regulation 86/609 and adheres to the guidelines established in the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the Society for the Study of Reproduction.

5.2.1 Electroejaculation and biometrics

Samples were collected *in vivo* from fallow deer (n=6) and, for the first time to our knowledge, from Sardinian red deer stags (n=2) Semen was collected by electroejaculation (EE) during the breeding season (September 2010- November 2010). Electroejaculation (EE) was performed on animals living in captive conditions (Foresta Demaniale Su Filigosu, Oschiri (OT), Italy and Foresta Demaniale Monte Lerno, Pattada (SS), Italy). Stags were immobilised by an intramuscularly injection (Wiener mischung) of xylazine (Rompun, SS, Bayer) and tiletamine-zolazepam (Zoletil 100, Virbac). Doses were: xylazine 1.25 mg/Kg and tiletamine-zolazepam 1.25 mg/kg in Sardinian red deer and xilazine 1.85 mg/kg and tiletamine-zolazepam 1.85 mg/kg in fallow deer. Anesthesia was reverted with an intramuscularly injection of atipamezole

hydrochloride (Antisedan, Pfizer) 1.25 mg/Kg in Sardinian red deer and 1.85 mg/kg in fallow deer.

After immobilisation, the rectum was cleaned from faeces and the prepucial area was washed with physiological saline solution. Electroejaculation was carried out in Sardinian red deer stags using a rectal probe measuring 375 mm and 40 mm width, the length of the electrodes was 80 mm whereas in fallow deer using a rectal probe measuring 295 mm and 25 mm width, the length of the electrodes was 60 mm.

The probe was connected to a power source that allowed controlling of amperage and voltage. Electric impulses progressively increased were applied starting from 0.5 V till maximum 8 V. Each impulse was applied for 5s followed by 5s pause. Semen was collected in fraction in order to avoid the bulbo-urethral fraction and urine contaminations. Samples were quickly diluted 1:1 (v/v) in Tris-citrate- fructose (TCF)-egg yolk (20%) and transported within 1-1.5 h to the laboratory at ≤20°C.

Finally, biometrics were collected: Body weight (kg); Body length (cm); Withers height (cm); Tail length (cm); Ear Length (cm); Tarsus length (cm); Length of forefoot (cm); Width of forefoot (cm); Forefoot pad length (cm); Length of hindfoot (cm); Width of hindfoot (cm) and Hindfoot pad length (cm). Scrotal measurements were recorded: Scrotum Length (cm); Scrotum Width (cm) and Scrotal skin thickness (cm). Finally, antlers measurements were noted: Length of main beam (cm); Circumference of coronet (cm) and Tip to tip (cm).

5.2.2 Sperm analysis

Sperm volume was evaluated with a graduated pipette. Sperm concentration was assessed with haemocytometer (Burker chamber). Sperm viability was evaluated by using a nigrosin-eosin stain (NE). Percentage of viable sperm was obtained evaluating 200 spermatozoa. Finally, sperm motility was assessed subjectively (SMI) and using a CASA system (HTM-IVOS 12.3, Hamilton Thorne Biosciences, Beverly). Semen was analysed at 1:200 dilution in TCF + egg yolk (20%) after 10' incubation at 37°C. CASA sperm analysis set-up was: Frames per second: 30 Hz; Number of frames: 20; Minimum contrast: 10; Minimim cell size: 9; Progressive cells were considered when VAP >25μ/s, STR>80%; Slow cells were considered statics if VAP <10 μ/s, VSL <6.0 μ/s. At least 200 cells were analysed, 15 fields acquired. A total of 10 parameters were evaluated: **MT** (Total Motility), **MP** (Progressive Motility), **VAP** (Average Path Velocity; μm/s) which corresponds to the average velocity of sperm track; **VSL** (Velocity Straight Line; μm/s) which corresponds to the average sperm velocity taking into account the first and the last point of trajectory; **VCL** (Velocity Curvilinear line; μm/s) which corresponds to the average sperm velocity considering point to point the sperm track; **ALH** (Amplitude Lateral Head Movement; μm); **BCF** (Beat Cross Frequency; Hz) which express the beat frequency of the tail; **WOB** (percentage of lateral head displacement; %); **STR** (Straightness; %) which is the VSL/VAP ratio; **LIN** (Linearity; %) which is the VSL/VCL ratio.

Finally, percentages of rapid, medium, slow and static spermatozoa were assessed.

5.2.3 Fine Needle Aspiration Cytology (FNAC)

FNAC *in vivo* was only performed on the right testis, after accurate shaving and disinfecting the area. A 21G butterfly needle, previously connected to a 10 ml syringe, was inserted in the testicular cranial pole, opposite to the epididymis. Constant negative pressure was obtained by clamping with a Klemmer forceps. Finally, the needle was removed and the content was flushed with air on a glass slide and gently smeared.

All smears were left air-dried for 24 h, stained with MGG Quick (Bio-Optica, Milan, Italy) and finally mounted with a coverslip 24 x 60 mm with Eukitt (Bio-Optica, Milan, Italy). The smears were examined under oil at 1000X magnifications using light microscopy (Microscope Nikon Eclipse 80i). At least two hundred consecutive spermatogenic and Sertoli cells were counted in each smears and classified as follows: Sertoli cells, Spermatogonia, Primary Spermatocytes, Secondary Spermatocytes, Early Spermatids, Late Spermatids and Spermatozoa. Proportion of each cell type was determined. Moreover, testicular cytological indexes were determined as follows (Papić *et al.*, 1986; Han *et al.*, 2006).

Sertoli cell index (SEI), expressed as percentage, was assessed by the ratio of Sertoli cells to all spermatogenic cells as index of spermatogenic activity;

Spermatic index (SI), expressed as percentage, was calculated as ratio of spermatozoa to all spermatogenic cells as index of spermiogenic activity;

Sperm-Sertoli index (SSEI) was calculated from the ratio of spermatozoa to Sertoli cells;

Finally, the ratio of the number of spermatids ab/ primary spermatocyte was assessed as index of germ cell loss or mitotic activity (MI).

Germ cells: Sertoli cell ratios were evaluated as index of Sertoli cells functionality.

5.2.4 Statistical analysis

All statistical analyses were performed using the SPSS 17.0 statistical software package (SPSS Inc, Chicago, IL, USA). For each variable descriptive statistics are reported.

5.3 Results

5.3.1 Biometrics

During the breeding season, body weight was 63.83±6.64 Kg in fallow deer stags, 81.5±7.78 kg in Sardinian red deer (mean±SD).

Biometrics of fallow deer (n=6) and Sardinian red deer (n=2) are shown in Table 1 and 2, respectively. Table 3 shows data of scrotal length and width and scrotal skin thickness of fallow deer (n=5) and Sardinian red deer (n=2).Antler measurements of fallow deer (n=3) and Sardinian red deer (n=2) stags are reported in Table 4 and 5, respectively.

	Fallow deer (n=6)		
	Mean±SD	**CV (%)**	**Min-Max**
Body weight (kg)	**63.83±6.64**	10.40	57-72
Body length (cm)	**151.80±14.24**	9.38	134-173
Withers height (cm)	**83,73±2,79**	3.33	80-86.2
Tail length (cm)	**17.20±1.27**	7.38	16-19.2
Ear length (cm)	**14.76±1.36**	9.21	13-17
Tarsus length (cm)	**32.80±4.99**	15.21	28.1-40
Length of forefoot (cm)	**5.72±0.55**	9.62	5.1-6.5
Width of forefoot (cm)	**4.51±0.10**	2.22	4.4-4.7
Forefoot pad length (cm)	**3.4±0.68**	2.20	3.0-4.4
Length of hindfoot (cm)	**5.5±0.40**	7.27	4.9-6.0
Width of hindfoot (cm)	**4.18±0.17**	4.07	3.9-4.4
Hindfoot pad length (cm)	**3.22±0.58**	18.02	2.7-4.0

Table 1. Biometrics data of fallow deer stags (n=6).

	Sardinian red deer (n=2)		
	Mean±SD	**CV (%)**	**Min-Max**
Body weight (kg)	**81.5±7.78**	9.55	76-87
Body length (cm)	**170.75±3.89**	2.28	168-173.5
Withers height (cm)	**97.8±3.96**	4.05	95-100.6
Tail length (cm)	**15.75±2.47**	15.68	14-17.5
Ear length (cm)	**17.1±0.14**	0.82	17.0-17.2
Tarsus lenght (cm)	**30.6±0**		30.6
Length of forefoot (cm)	**6.7±0.71**	10.60	6.2-7.2
Width of forefoot (cm)	**6.4±0.28**	4.38	6.2-6.6
Forefoot pad length (cm)	**3.5±0.56**	16	3.1-3.9
Length of hindfoot (cm)	**6.45±0.64**	9.92	6.6-6.9
Width of hindfoot (cm)	**5.55±0.21**	3.78	5.4-5.7
Hindfoot pad length (cm)	**3.1±0.28**	9.03	2.9-3.3

Table 2. Biometrics data of Sardinian red deer stags (n=2).

	Fallow deer (n=5)			Sardinian red deer (n=2)		
	Mean±SD	**CV (%)**	**Min-Max**	**Mean±SD**	**CV (%)**	**Min-Max**
Scrotal length (cm)	**6.97±1.51**	21.66	5.2-9.65	**9.5±0.57**	6	9.1-9.9
Scrotal width (cm)	**5.33±1.14**	21.39	3.5-7.0	**8.05±0.49**	6.09	7.7-8.4
Scrotal skin thickness (cm)	**0.23±0.03**	13.04	0.20-0.25	**0.28±0.04**	14.29	0.25-0.30

Table 3. Biometrics data of scrotal length, scrotal width and scrotal skin thickness in fallow deer (n=5) and Sardinian red deer stags (n=2).

	Fallow deer (n=3)		
	Mean±SD	**CV (%)**	**Min-Max**
Length of main beam (cm)	**47.66±7.96**	16.70	37.20-57.00
Circumference of coronet (cm)	**13.24±1.46**	11.03	11.05-15.00
Tip to tip (cm)	**55.80±3.93**	7.04	52.30-60.05

Table 4. Biometrics data of antlers measurements of fallow deer stags (n=3).

	Sardinian red deer (n=2)		
	Mean±SD	**CV (%)**	**Min-Max**
Length of main beam (cm)	**56.1±10.4**	18.54	49.0-63
Circumference of coronet (cm)	**12.4±0.57**	4.60	12.2-12.8
Tip to tip (cm)	**37.75±2.76**	7.31	35.8-39.7

Table 5. Biometrics data of antler measurements of Sardinian red deer stags (n=2).

5.3.2 Semen analysis

Ejaculation generally occurred between 2V and 4 V in fallow deer and between 4V and 7 V in Sardinian red deer. Due to inadequate anaesthesia, two fallow deer were excluded from electroejaculation procedure.

None sample was discarded because of urine contamination.

Sperm volume was low in fallow deer (0.37±0.26 ml) whereas abundant in Sardinian red deer (1.80±1.70 ml). As a consequence, total number of sperm recovered was high in fallow deer (1225±719.16X10^6spz) and extremely low in Sardinian red deer (139.2±27.15X10^6 spz).

Sperm concentration, sperm motility index (SMI), viability and kinetics are shown in **Table 6** and **Table 7.**

	Fallow deer (N=4)			Sardinian red deer (N=2)		
	Mean±SD	**CV (%)**	**Min-Max**	**Mean±SD**	**CV (%)**	**Min-Max**
Volume (ml)	**0.37±0.26**	70.55	0.30-0.80	**1.80±1.70**	94.28	0.6-3.0
Concentration(10^6spz/ml)	**3400±2641.82**	77.70	840-6840	**152±158.39**	104.20	40-264
Total sperm number (10^6spz)	**1225±719.16**	58.71	576-2052	**139.2±27.15**	19.51	120-158.4
Motile sperm (%)	**80.00±20.00**	25.00	50.00-90.00	**77.50±3.53**	4.56	75.00-80.00
Quality of Motility (0-5)	**2.50±1.47**	58.88	1.00-4.00	**2.00±00**		
SMI (%)	**71.25±24.28**	34.08	35.00-85.00	**58.75±1.77**	3.01	57.50-60.00
Viability (%)	**93.00±4.24**	4.56	90.00-96.00	**80.00±7.07**	8.84	75.00-85.00

Table 6. Sperm volume, concentration, motility and viability of fallow deer and Sardinian red deer during the breeding season.

	Fallow deer (N=4)			Sardinian red deer (N=2)		
	Mean±SD	**CV(%)**	**Min-Max**	**Mean±SD**	**CV(%)**	**Min-Max**
MT (%)	**85.08±15.89**	18.68	63.00-97.00	**83.00±15.08**	18.17	72.33-93.67
MP (%)	**47.83±15.49**	32.38	32.00-69.00	**21.00±0.94**	4.49	20.33-21.67
VAP(µm/s)	**101.55±23.58**	23.22	80.5-128.73	**136.9±29.70**	21.69	115.90-157.901
VSL(µm/s)	**86.4±17.35**	20.38	73.47-110.5	**83.15±14.07**	16.92	73.20-93.10
VCL(µm/s)	**150±33.85**	22.57	117.5-182.6	**247.33±63.54**	25.69	202.40-292.27
ALH (µm)	**6.29±2.95**	46.86	4.2-10.63	**8.18±1.01**	12.38	7.47-8.90
BCF (Hz)	**29.13±17.03**	58.44	4.1-42	**29.95±7.94**	26.52	24.33-35.57
LIN (%)	**60.00±6.95**	11.58	50.00-66.00	**36.50±3.06**	8.39	34.33-38.67
STR (%)	**84.92±8.19**	9.64	73.33-92.00	**61.83±3.06**	4.95	59.67-64.00
Rapid (%)	**68.00±29.35**	43.16	34.00-93.67	**64.17±0.23**	0.37	64.00-64.33
Medium (%)	**16.83±14.73**	87.49	2.67-31.00	**18.67±14.61**	78.29	8.33-29.00
Slow (%)	**5.92±8.81**	148.94	0.67-19.00	**6.33±2.83**	44.66	4.33-8.33
Static (%)	**9.00±8.80**	97.76	0.33-18.00	**10.67±12.26**	114.90	2.00-19.33

Table 7. Sperm kinetics parameters assessed by CASA in fallow deer and Sardinian red deer stags.

5.3.3 Fine Needle Aspiration Cytology (FNAC)

FNAC was performed on 4 fallow deer and 2 Sardinian red deer. Only one cytological sample was discarded because of high red blood cells presence. A total of 228, 25± 47,97 cells (mean±SD) were evaluated in fallow deer smears whereas 261 in Sardinian red deer.

Sertoli cells and spermatogenic cells percentages of fallow deer and Sardinian red deer are reported in **Table 8** and **Table 9**, respectively.

	Fallow deer (N=4)		
	Mean ± SD	**CV (%)**	**Min-Max**
Sertoli cells (%)	**6.46±1.57***	24.35	5.00-8.33
Spermatogonia (%)	**1.73±1.51**	87.10	0.96-4.00
Spermatocytes I (%)	**5.34±1.13**	21.16	4.31-6.66
Spermatocytes II (%)	**0.62±0.95**	152.92	0.00-2.00
Spermatids ab (%)	**25.53±9.57**	37.49	14.71-35.50
Spermatids cd (%)	**32.75±6.32**	19.31	27.33-41.18
Spermatozoa (%)	**27.45±4.05**	14.74	22.50-32.06

Table 8. Sertoli cells and spermatogenic cells percentages in fallow deer stags during the breeding season.

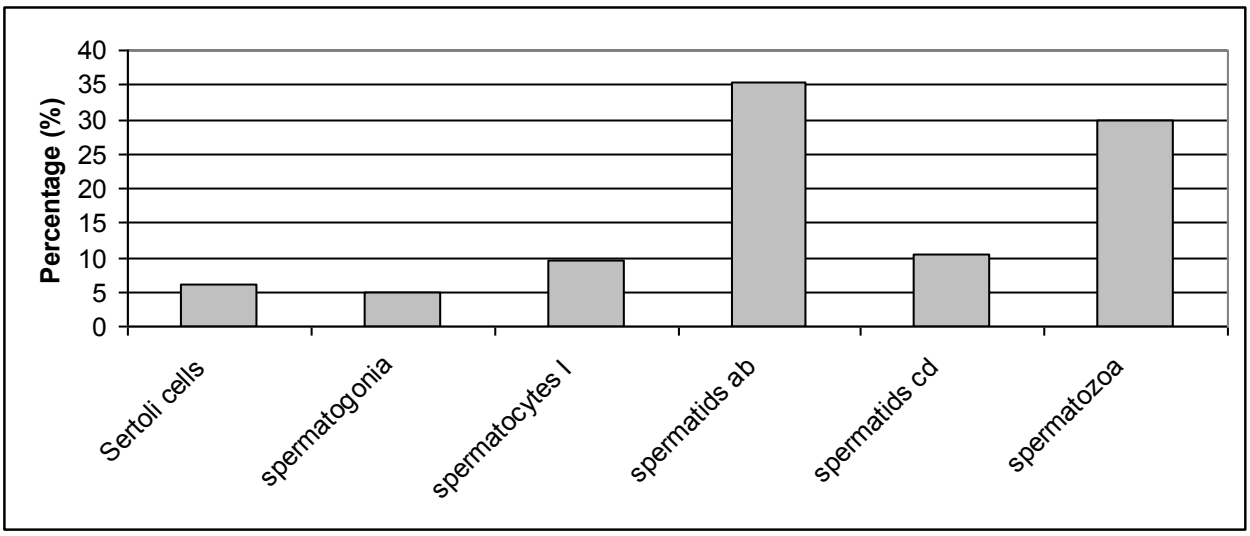

Table 9. Sertoli cells and germ cells percentages in a Sardinian red deer stag during the breeding season.

In Sardinian red deer, testicular cell indexes were: Sertoli index (SEI): 6.53; Spermatic index (SI): 31.84; Sperm-Sertoli index ratio (SSEI): 4.88 and spermatids ab/primary spermatocyte I ratio (MI): 3.68.

	Fallow deer (N=4)		
	Mean±SD	**CV(%)**	**Min-Max**
SEI (%)	**6.93±1.80**	26.04	5.26-9.09
SSEI	**4.34±0.62**	14.28	3.47-4.93
SI (%)	**29.40±4.70**	15.98	23.68-34.54
MI	**4.97±2.22**	44.62	2.5-7.89

Table 10. Testicular cell indexes in fallow deer during the breeding season.

5.4 Discussion

This study provides preliminary data of reproductive physiology of Sardinian red deer and fallow deer stags. Biometrics data are in agreement with previous reports on Sardinian red deer (Beccu, 1989; Uccheddu, 2008) and fallow deer (Asher *et al.*, 1996, 2000; Gosch and Fischer, 1989). Body weight in Sardinian red deer stags was lower than that reported by Beccu (1989) (81.5±7.78 vs 120-130 kg) and this occurrence was likely due to captive conditions.

For the first time to our knowledge, electroejaculation was performed in Sardinian red deer.

Our results are in agreement with previous reports on semen collected by electroejaculation in cervids (Martinez *et al.*, 2008; Gizejewski, 2004; Gosh and Fischer, 1989).

In fallow deer ejaculation occurred at lower voltage (2-4 V) compared to what indicated by Gosch and Fisher (0-23 V) (1989).

In agreement with the observation of Gizejewski (2004) in *Cervus elaphus*, also in Sardinian red deer the ejaculates contained two fractions typical of the mating period, the yellow fraction (YF; accessory gland-derived) and the white fraction (WF; epididymal origin).

By contrast, no samples of fallow deer semen were brown or black as reported by Gosch and Fisher (1989) during the rutting season. Furthermore, both in Sardinian red deer and in fallow deer, the white fraction had milky or creamy consistency.

In Sardinian red deer semen volume showed a wide range, likely due to anesthesia and electroejaculation protocol. In fact, collection of semen using electroejaculation in *Cervus elaphus*

results in ejaculates of high volume due to increased volumes of fluids from accessory glands and epididymis (Gizejewzki, 2004). Total number of spermatozoa recovered in Sardinian red deer were extremely low compared to *Cervus elaphus hispanicus* and *Cervus elaphus* (139.2 X 10^6 spz/ml vs 3.6 X 10^9 and 569.4 X 10^6 to 793.9 X 10^6/ml, respectively) (Martinez *et al.*, 2008; Gizejewski, 2004).

In contrast, in fallow deer semen volume and total number of sperm recovered were in accordance with previous studies (Asher *et al.*, 1996; Gosch and Fisher, 1989).

Testicular cell proportions and indexes indicate an active spermatogenesis both in *Cervus elaphus corsicanus* and in *Dama dama*. Spermatic index in Sardinian red deer (31.84) and fallow deer (29.40±4.70) were higher compared to what observed in Iberian red deer during the post-rutting season (23.03±6.29) and quite similar to that described in human (SI 34.8±13.3) (Foresta *et al.*, 1993), horse (SI: 31.5±8.5), dog (SI: 26.6±3.8) and alpaca (27.28±13.83 in the right testis and 20.63±14.47 in the left testis) (Stelletta *et al.*, 2011). By contrast, differences of SEI are described among species: Sardinian red deer (6.53), fallow deer (6.93±1.80), Iberian red deer (16.06±11.06; range: 3.27-40.99), horse (20.9±17) (Leme and Papa, 2000), dog (4.2±0.8) (Santos *et al.*, 2010), alpaca (39.31±27.17 in the right testis and 45.75±24.79 in the left testis) (Stelletta *et al.*, 2011) and human (30.0±19.5) (Foresta *et al.*, 1993).

In conclusion, FNAC proved to be a reliable and minimally invasive method to evaluate spermatogenesis *in vivo* from wildlife. Further studies are required in order to deepen knowledge

on reproductive biology of Sardinian red deer in order to optimize the strategies to better preserve it.

6. Conclusions

- Fine Needle Aspiration Cytology (FNAC) has proven to be a reliable method to evaluate spermatogenesis and its seasonal variations in wild deer. Both cytological pattern and histological analysis describe the same pattern of seasonal involution.

- Involution of spermatogenesis in Iberian red deer is mainly characterised by a decline of testicular weight, Sertoli cells and spermatozoa percentages and increased elongated spermatids percentage.

- In agreement with previous studies about several seasonal breeders, our results show that Sertoli cells are not a stable population but fluctuate with season in Iberian red. For the first time, this result has been obtained by FNAC technique.

- During the non breeding season, increased percentage of elongated spermatids and constant round spermatids per primary spermatocyte ratio suggest that involution of spermatogenesis involves firstly the spermiogenesis without affecting the meiotic activity. This finding is also supported by the higher spermatids cd: Sertoli cell ratio and the stronger negative correlation between round spermatids and elongated spermatids out of the breeding season.

- Epididymal sperm kinetics, viability and percentage of normal spermatozoa reach the maximum during the breeding season in agreement with previous studies.

- During the breeding season, Sertoli cell percentage and Spermatic index are positively associated with sperm motility and sperm head elongation. Thus, active spermatogenesis is positively related with sperm quality. On the contrary, high percentage of spermatogonia out of the breeding season is negatively related with sperm kinetics suggesting that involution of spermatogenesis is associated with reduced sperm quality.

- Basic data on reproductive biology of Sardinian red deer and fallow deer are in agreement with previous studies. FNAC technique was first applied *in vivo* in these species. High Spermatic index and spermatozoa percentage show fully active spermatogenesis during the rutting and this result is related with high sperm quality and kinetics.

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