



UNIVERSITÀ
DEGLI STUDI
DI PADOVA

UNIVERSITÀ DEGLI STUDI DI PADOVA

Dipartimento di *BIOMEDICINA COMPARATA E ALIMENTAZIONE*

SCUOLA DI DOTTORATO DI RICERCA IN SCIENZE VETERINARIE

INDIRIZZO COMUNE

CICLO XXVI

NOVEL APPROACHES IN ANDROLOGY EXAMINATION AND FOLLICULAR FLUID BIOCHEMICAL CHARACTERIZATION IN THE OPTIMIZATION OF REPRODUCTIVE TECHNOLOGIES IN FARM ANIMALS

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ABSTRACT

The purpose of the studies reported in this Thesis was to increase our understanding of two aspects of the reproductive system in farm animals: the andrological evaluation and the follicular fluid composition. The final aim was to give some tools that can be helpful in optimizing the application of assisted reproductive technologies. Studies were conducted in bulls, rams, alpacas, lamas and dairy buffalo cows.

The first study was designed to investigate the efficacy of scrotal thermographic monitoring in the evaluation of young bulls with low semen production after GnRH administration. Yearling bulls with low semen quality were selected. Scrotal surface temperature (SST) and serum testosterone (T) were evaluated before and after administration of 10.5 µg Buserelin Acetate IV. Bulls were divided in two groups: LowTemp bulls had a decreased SST at 60 minutes; HighTemp bulls had an increased SST. After 60 minutes from Buserelin administration, LowTemp bulls had higher T values compared to HighTemp bulls: 14.32 ± 0.53 ng/ml vs. 10.30 ± 1.37 ng/ml respectively (mean \pm SEM) ($P < 0.05$). Reproductive performances in both groups improved after Buserelin administration, but the improvement was more marked in LowTemp bulls. In conclusion a decreased SST after GnRH administration was associated with better testicles functionality.

The second study was done to assess the predicting value of trans-scrotal ultrasonography (TSUS) and testicular fine-needle aspiration cytology (TFNAC) on sperm production in rams. TSUS and TFNAC were performed on eighteen adult rams at the beginning of the breeding season. After evaluation, semen was collected twice with

electroejaculation from each ram, and then epididymal spermatozoa were collected after slaughtering. Ultrasonography appearance of testicular parenchyma was analyzed to create a four classes division of the animals. Relative percentages of spermatogenic cells and Spermatic Index (SI = percentage of spermatozoa on the total of spermatogenic cells) were calculated after observation of TFNAC slides. Rams having a better echogenicity had a higher SI and a higher total sperm and number of straws per ejaculate either with electroejaculation or epididymal spermatozoa than the worst groups. Several cytological parameters were correlated with sperm production: the most interesting correlation was between SI and number of straws obtained by epididymal collection (0.667; P=0.003. In conclusion, TSUS and TFNAC can be usefully integrated in breeding soundness evaluation of rams, as these techniques had a potential as a marker of semen production.

The third study documents the variability of follicular fluid (FF) composition of South American camelids, in different stages of ovarian activity. In the first experiment, ovaries of alpacas were collected at slaughterhouse and FF was aspirated dividing sample on the base of ovarian cycle stage and follicular dimension. Follicle size affected FF composition, either by biochemical analysis or electrophoresis. Conversely, the influence of corpus luteum was minimal. In the second study, ovaries of thirty lammas were scanned ultrasonographically to identify wave emergence. FF was aspirated trans-vaginally at three different stage of development (growing, static and regressing phase). The electrophoresis analysis revealed differences between groups in 4 classes of weight (250 KDa, 75 KDa, 25 KDa, 15 KDa). This study offers new information concerning the biochemical composition of follicular fluid in South American camelids, suggesting that

oocytes grow and mature in an environment that changes in function of the follicle stage, while is minimally influenced by the presence of the corpus luteum.

The aim of the fourth study was to characterize the biochemical and fatty acids composition of the follicular fluid (FF) in water buffalo. Ovaries of buffalo were collected after slaughtering during breeding season. Fluid was aspirated from follicles of small and large follicles (< 6 mm and > 6 mm respectively) and dividing females in those in luteal or follicular phase of estrous cycle. Biochemical analysis and gas chromatography were performed. Biochemical and fatty acids compositions were influenced by both follicular dimension and phase of ovarian cycle. Biochemical composition and its variation was in agreement with previously study conducted in buffalo and other species. Twenty-two fatty acids were identified in FF; nine were saturated fatty acids, six monounsaturated fatty acids and seven polyunsaturated fatty acids. The most dominant fatty acids were linoleic acid, oleic acid, palmitic acid, stearic acid and arachidonic acid. All the identified fatty acids concentrations varies at least because of follicle dimension or phase, with the exception of γ -linoleic acid and arachidonic acid which concentrations remain stable in all classes.

The fifth study was designed to investigate the effect of different diets on the testicular fine-needle aspiration cytology (TFNAC) in rams. Eighteen rams were fed with three different diets since 8 months of age up to 1 year. The diets were: 1- pasture (N=6); 2- hay and commercial concentrate (N=6); 3- hay, commercial concentrate and a supplementation of conjugated linoleic acid (CLA) containing equal parts of cis-9, trans-11 and trans-10, cis-12 isoforms (N=6). After slaughtering, testes were collected to perform TFNAC. No statistical differences were observed in live weight and testicular

weight between groups. CLA supplemented rams had higher relative percentage of early spermatogenic cells (primary spermatocytes and AB spermatids) and lower percentage of CD spermatids and SI compared to other groups. Total CLA concentration in diets was similar in pasture and CLA supplemented groups, but rams graze at pasture receive almost all cis-9, trans-11 isomer. Rams fed with hay and concentrate received a lower quantity of CLA in the diet. In conclusion, CLA supplementation and in particular the trans-10, cis-12 isomer affected negatively the TFNAC results in peripubertal rams probably because it retarded the onset of puberty.

RIASSUNTO

Lo scopo degli studi riportati in questa Tesi è di aumentare le nostre conoscenze su due aspetti della biotecnologia applicata alla riproduzione negli animali da reddito: la valutazione andrologica e la composizione del fluido follicolare. Il fine è stato quello di fornire degli strumenti che possono essere utili nell'ottimizzazione della gestione riproduttiva. Gli studi sono stati condotti su tori, arieti, alpaca, lama e bufale.

Il primo studio è stato creato per investigare l'efficacia del monitoraggio termografico scrotale nella valutazione di giovani torelli con scarsa produzione di seme, dopo somministrazione di GnRH. Sono stati selezionati torelli di circa un anno di età con scarsa qualità seminale. Sono stati valutati, prima e dopo la somministrazione di 10.5 µg di Buserelina, la temperatura superficiale dello scroto (SST) e il testosterone sierico (T). I tori sono stati divisi in due gruppi in funzione della risposta termica alla somministrazione di Buserelina: tori LowTemp con una diminuzione di SST a 60 minuti dal trattamento; tori HighTemp con un aumento della SST. Dopo 60 minuti dalla somministrazione di Buserelina, i tori LowTemp esibivano livelli di T maggiori rispetto a quelli dei tori HighTemp: 14.32 ± 0.53 ng/ml vs. 10.30 ± 1.37 ng/ml rispettivamente (media \pm SEM) ($P < 0.05$). Le performance riproduttive di entrambi i gruppi sono migliorate in seguito alla somministrazione di Buserelina, tuttavia il miglioramento è stato più marcato nei tori LowTemp. In conclusione, una diminuzione della SST dopo la somministrazione di GnRH è associata a una migliore funzionalità testicolare.

Il secondo studio è stato eseguito per accertare il valore predittivo dell'ecografia trans-scrotale (TSUS) e della citologia testicolare per aspirazione con ago sottile (TFNAC) sulla produzione spermatica negli arieti. TSUS e TFNAC sono state eseguite su diciotto arieti

adulti all'inizio della stagione riproduttiva. Dopo la valutazione, il seme è stato raccolto mediante elettroeiaculazione per due volte da ciascun ariete, e gli spermatozoi epididimali sono stati raccolti dopo la macellazione. L'aspetto ecografico del parenchima testicolare è stato analizzato per creare una classificazione in quattro classi degli animali. Le percentuali relative delle cellule spermatogenetiche e l'indice spermatico (SI = percentuale di spermatozoi sul totale delle cellule spermatogeniche) sono stati calcolati dopo la lettura dei vetrini di TFNAC. Arieti con una migliore ecogenicità testicolare sono risultati avere un più alto SI e un maggior valore di spermatozoi totali e numero di dosi inseminanti prodotte per eiaculato sia per elettroeiaculazione che spermatozoi epididimali rispetto ai gruppi peggiori. Alcuni parametri citologici sono stati correlati con la produzione spermatica: la più interessante correlazione trovata è quella tra lo SI e il numero di dosi ottenute tramite raccolta epididimale (0.667; $P=0.003$). In conclusione, TSUS e TFNAC possono essere utilmente integrati nella valutazione della breeding soundness degli arieti, poiché queste tecniche sono potenzialmente degli indici di produzione spermatica.

Il terzo studio documenta la variabilità della composizione del fluido follicolare (FF) nei camelidi Sud Americani, in diversi stadi di attività ovarica. Nel primo esperimento, ovaie di alpaca sono state raccolte al macello e il FF è stato aspirato dividendo i campioni sulla base dello stadio dell'attività ovarica e alla dimensione follicolare. Le dimensioni follicolari influenzano la composizione del FF, sia la composizione biochimica sia quella elettroforetica. Diversamente, l'influenza della presenza del corpo luteo è minima. Nel secondo studio, le ovaie di trenta lama sono state valutate per via ecografica per individuare l'emergenza follicolare. Il FF è stato aspirato per via transvaginale a tre diversi stadi di sviluppo (fase di crescita, statica e di regressione). L'analisi

elettroforetica ha rivelato delle differenze tra i gruppi in quattro classi di peso molecolare (250 kDa, 75 kDa, 25 kDa, 15 kDa). Questo studio fornisce nuove informazioni sulla composizione biochimica del FF nei camelidi Sud Americani, suggerendo che gli oociti crescono e maturano in un ambiente che varia in funzione dello stadio follicolare, mentre è solo minimamente influenzato dalla presenza del corpo luteo.

Lo scopo del quarto studio era di caratterizzare la composizione biochimica degli acidi grassi del fluido follicolare (FF) di bufala. Ovaia di bufala sono state raccolte dopo la macellazione avvenuta all'interno della stagione riproduttiva. Il fluido è stato aspirato da follicoli di piccole e grandi dimensioni (< 6 mm e > 6 mm rispettivamente) e dividendo gli animali in quelli in fase follicolare e fase luteale del ciclo. Sono state eseguite un'analisi biochimica e una gas cromatografia. La composizione biochimica e degli acidi grassi sono influenzate sia dalle dimensioni follicolari che dalla fase del ciclo ovarico. La composizione biochimica e le sue variazioni sono in accordo con gli studi precedenti condotti su bufale e altre specie. Sono stati identificati ventidue acidi grassi nel FF; nove sono acidi grassi saturi, sei sono acidi grassi monoinsaturi e sette poliinsaturi. Gli acidi grassi più rappresentati sono l'acido linoleico, acido oleico, acido palmitico, acido stearico e acido arachidonico. Le concentrazioni di tutti gli acidi grassi identificati variano in funzione di almeno una dimensioni follicolari o fase del ciclo, ad eccezione dell'acido γ -linoleico e dell'acido arachidonico le cui concentrazioni rimangono stabili.

Il quinto studio è stato creato per investigare l'effetto di diverse diete sulla citologia testicolare per aspirazione con ago sottile (TFNAC) negli arieti. Diciotto arieti sono stati alimentati con tre diete differenti dagli 8 mesi all'anno di età. Le diete sono state: 1-

pascolo (N=6); 2- fieno e un mangime commerciale (N=6); 3- fieno, mangime commerciale e integrazione di acido linoleico coniugato (CLA) contenente parti uguali d'isomero cis-9, trans-11 e isomero trans-10, cis-12 (N=6). Dopo la macellazione, i testicoli sono stati raccolti per eseguire una TFNAC. Non sono state osservate differenze statistiche nel peso vivo e nel peso dei testicoli tra i gruppi. Gli arieti che hanno ricevuto l'integrazione di CLA, avevano percentuali relative di cellule precoci della spermatogenesi (spermatociti primari e spermatidi AB) più elevate e percentuali inferiori di spermatidi CD e indice spermatico rispetto agli altri gruppi. La concentrazione totale di CLA nelle diete era simile nei gruppi al pascolo e integrato con CLA, ma gli arieti al pascolo hanno ricevuto quasi unicamente l'isomero cis-9, trans-11. Gli arieti alimentati con fieno e mangime hanno ricevuto una minore quantità nella dieta. In conclusione, l'integrazione di CLA e in particolare dell'isomero trans-10, cis-12 influenza negativamente i risultati della TFNAC in arieti peripuberi probabilmente perché ritardano l'inizio della pubertà.

1 - PREFACE

In the recent past, great progress has been made in the reproductive management of farm animals. In the context of reproductive technologies, the 1940s and 1950s saw the establishment of artificial insemination and the introduction of cryopreserved semen in cattle breeding. In the early 1960s, first steps in manipulation of estrous cycle have been made, since progestogens showed to be highly effective through when administered by the intravaginal route. In the 1970s embryo transfer and the freezing of embryos in cattle became commercial realities, and they started to be incorporate in breeding improvement programs. In the same years, the availability of prostaglandins and gonadotrophin-releasing hormone gave new possibilities for estrous cycle control in many species. The first steps in mammalian cloning were taken in the mid-1980s and few years later came the ability to produce embryos in the laboratory. The 1990s saw the emergence of somatic cells cloning with the birth of the first cloned animals, Dolly the sheep, opening the way for the production of transgenic farm animals that became a reality only few years later.

The introduction of reproductive technologies has given a tremendous boost to the farm system, giving possibilities that was previously unthinkable. Their applications allow to improve reproductive performance of a single animal; accelerate the genetic selection; give the possibility to move genetic materials all over the world moving entire breeds or species; through transgenic technologies it is possible to erase or create new genes depending of the demand.

For all the reasons mentioned, reproductive technologies have become a reality and most of them are commonly applied in many farms all over the world.

Despite the amazing advancing in the reproductive technologies field, more work has to be done in order to improve the efficiency of reproductive technologies in all species and make their application even more effective.

This thesis is focused on two aspects connected to the reproductive technologies: the andrological examination considering two species (bovine and ovine); the characterization of follicular fluid composition in two species (buffalo and South American camelids). These aspects are extremely important for the optimization of the reproductive biotechnologies. On one hand, the possibility to exclude infertility/subfertility males reduces cost and improves the efficiency of all techniques. On the other hand, knowledge of follicular environment can give a lot of information about the oocyte quality, the requirements for the development and follicular fluid itself can be used directly during in vitro process.

The aim is to provide information that can be used to optimize the application of reproductive technologies.

2 – INTRODUCTION

2.1 Assisted reproductive technologies in farm animals

Assisted reproductive technologies (ART) have made tremendous advances, especially in the last 20 years. ART include: artificial insemination (AI), estrus synchronization, induction of parturition, multiple ovulation and embryo transfer (MOET), cryopreservation of oocytes and embryos, sperm sexing, in-vitro production (IVP), embryo bisection, nuclear transfer and microinjection of DNA constructs (Niemann and Rath, 2001). ART offer a powerful technology for genetic improvement (Amiridis and Cseh, 2012), but have also an enormous potential for conserving rare breeds or species (Long, 2008). Estrus detection, AI and estrous cycle control are basic methods in ART that are commonly applied in farm animals (Drost, 2007). AI was the first ART to be applied commercially for genetic improvement in the mid-1900s, and the advantages of AI in terms of disease control, the import/export of frozen semen, elimination of bulls on farms are well established (Farin et al, 2007). AI represents also the base for the improvement of other ART such as estrus synchronization: fixed-time artificial insemination (TAI), embryo transfer and the use of sexed semen (Farin et al, 2007). The use of AI, especially in dairy cattle, has become so common that most of it is practiced directly by the producer or herd managers.

Although, Walter Heape firstly performed an embryo transfer (ET) in rabbit in the April 1890 (Betteridge, 2003), its commercial use had become feasible in the late 1970s in bovine and ovine, while the first successful ET in buffalo was performed in 1983 (Drost et al, 1983). ET application enables the acceleration of the proliferation of genetic material from the dam as well as of the sire (Farin et al, 2007). In addition, the later developed ability in freezing and transport embryos made ET an extremely powerful

technology for disease control and biosecurity programs, genetic salvage of valuable individuals and development of new lines or breeds (Farin et al, 2007).

IVP of embryos refers to the use of laboratory procedures to generate embryos for transfer, freezing or other ART including cloning and transgenesis (Farin et al, 2007). IVP offers enhance the genetic improvement, enhancing the offspring from a single female, compare to ET production. The first calf resulting from IVP was reported in 1982 (Brackett et al, 1982), the first lamb in 1984 (Betteridge, 2003), the first live buffalo calves in 1991 (Drost, 2007). Comparing the efficacy of conventional ET to that of IVP in cattle, Bousquet reported that success rates for IVP (4.7 embryos per ovum pick-up session, 48% blastocysts from oocytes recovered) greatly exceeded the published results of ET (Bousquet et al, 1999). A confirmation came from Mapletoft that reported about 3.4 times more embryos and 3.2 more pregnancies in a 60-days period when applying IVP in comparison to ET (Mapletoft and Hasler, 2005). IVP has a further advantage as it allows to obtain conceptions using oocyte from prepubertal donors reducing the generation interval and enhancing genetic selection (Amiridis and Cseh, 2012).

While the developed AI technology has provided a possibility to exploit a genetically superior male, the reproductive potential of the female has been unutilized. With the introduction of ET techniques, MOET and above all IVP, there was a greatly increase of the number of offspring that a cow can produce (Wu and Zan, 2012).

Other ART have been used for the production of identical offspring called clones (Mapletoft and Hasler, 2005). The main application is for expanding superior genetics (Farin et al, 2007). A technique of cloning is the embryo splitting, that means to divide the embryos in two half, as it naturally happens in homozygotes twins' pregnancies (Farin et al, 2007). Pregnancy rates of 50%or more per demi-embryo have been

reported, resulting in a net pregnancy rate of more than 100% per original bovine embryos (Gray et al, 1991). The other possibility is the nuclear transfer that allows to product an unlimited numbers of genetically identical offspring (Farin et al, 2007). Willadsen performed his first nuclear transplantation in 1986 (Willadsen, 1986), the birth of lambs cloned from cultured embryonic cells was reported in 1996 (Campbell et al, 1996) while the birth of the first clone from a mammary cell taken from an adult ewe was reported one year later (Wilmut et al, 1997). Somatic cell cloning has now been successful for cloning cattle, sheep, horses, mules, goats, pigs, dogs, cats and many of laboratory animals, anyway the efficiency is very low, less than 1% (Farin et al, 2007). Despite the great advances made in the ART development in the last years, the efficiency is still far from optimal (Drost, 2007).

2.1.1 Application of assisted reproductive technologies in farm animals

The economic implications of reproductive management are critical because they affect farm profitability directly through the different productions (milk, meat, fiber, etc.), genetic improvement and culling rates, but also indirectly through breeding and veterinary costs (Olynk and Wolf, 2008). In particular the sustainability of ART has been studied in diary cattle (Giordano et al, 2011; Lima et al, 2010; Overton, 2005). All these studies concluded that the use of AI instead of natural service in dairy cattle reproductive management is more convenient (Giordano et al, 2011; Lima et al, 2010; Overton, 2005, Valergakis et al, 2007). The use of TAI has been reported to be more profitable than AI at estrus detection and an increased marginal feed cost and greater genetic advantages make it also more profitable (Lima et al, 2010). The application of reproductive management strategies becomes more important in herds with problems in heat detection (Olynk and Wolf, 2008).

With the introduction of MOET and embryo freeze-thaw methods the breeding industry has the tools to increase the number of calves from donors of high genetic merit (Wu and Zan, 2012). It is reported that 300 embryos may be produced from a single cow using MOET with a pregnancy rate after ET of around 50% (Thibier, 2005; Wu and Zan, 2012). The development of ovum pick-up (OPU) and IVP techniques enhanced the possibility of exploiting donor genetic resources. OPU is a trans-vaginal ultrasonically guided oocyte collection method that can be performed 2-3 times a week to maximize oocyte recovery (Galli et al, 2014). Approximately 1000 oocytes can be collected from a single donor using OPU (Presicce et al, 2011; Van Wagtendonk-de Leeuw, 2006). Despite the great advances in IVP, the efficiency is still low, and the production of embryos is usually not greater than 20 – 25 % (Galli et al, 2014). The pregnancy rate of in vitro produced embryos is around 50 %, either using fresh or frozen embryos (Thibier, 2005). As a consequence of the new discoveries, the trend in cattle embryo production in the last 10 years has changed. While in vivo production of embryos remained stable at about 500,000 embryos per year, in vitro production of embryos is steadily growing and passed from about 50,000 embryos of 2000, to 400,000 embryos in 2011 (Galli et al, 2014). In some countries, such as Brazil, IVP is now the first source of embryos. More advanced techniques, like somatic cell nuclear transfer, at the moment are still too inefficient to have a practical relevance among breeders (Galli et al, 2014).

2.2 Breeding Soundness Evaluation

Breeding soundness evaluation (BSE) is an overall assessment of the ability of a male animal to mate and to initiate reproduction. It is recommended to carry out BSE before breeding in order to eliminate sub-fertile animals from breeding programs (Gouletsou and Fthenakis, 2010). In bulls it is estimated that in an unselected population, sub-fertile bulls represent the 20% of the total bulls population (Barth, 2007), while in

Australia veterinary practitioners report that between 10 and 20% of bulls fail a routine BSE (Fordyce et al, 2006). The greatest economic loss that is attributable to sub-fertile animals is delayed conceptions (Barth, 2007). BSE includes: libido assessment, physical examination, examination of the reproductive organs and semen evaluation either in bulls (Barth, 2007; Ellis et al, 2005; Irons et al, 2007), or in rams (Kimberling and Parsons, 2007; Ley et al, 1990; Ruttle and Southward, 1988).

A breeding soundness certificate does not guarantee fertility or the absence of risk of transmitting ill effects to the herd, but it suggests a reduced risk (Irons et al, 2007). The benefits of examining bulls are well documented as satisfactory bulls reached a 9% higher pregnancy rates than questionable bulls (Farin et al, 1989). In the young, BSE can be used to measure the pubertal advancement of the physical and reproductive systems (Chenoweth et al, 1994). BSE gives temporal limited information of the breeding potential of an animal, therefore, a satisfactory classification no implied guarantee of future reproductive performance (Ellis et al, 2005, Kennedy et al, 2002). After a BSE has been concluded, environmental and physiological stressors can unfavorably influence some physical and seminal characteristics (Ellis et al, 2005).

BSE is an important tool for selecting males; anyway most of breeders are still selected from the farmers primarily on the basis of the phenotype and the genotype (Ridler et al, 2012).

2.2.1 Bull Breeding Soundness Evaluation

Guidelines for bull BSE have been described by Barth (Barth, 2007).

A bull requires four attributes to be fertile:

- 1- Freedom from disease
- 2- Good libido
- 3- Physical soundness

4- Good semen quality

These attributes must be held in the forefront of all decisions regarding herd sire selection and BSE.

Libido and serving capacity: In spite of the importance of libido and serving capacity to fertility, the use of standardized tests has not become a part of routine BSE. In the years, various testing methods have been investigated but a twenty-minute exposition to restrained cows test seems to be the most practical and is sufficient for most purposes (Blockey, 1981). Bulls to be tested must first receive 10 minute of sexual stimulation by allowing them to watch another bull serving the cows. Then, bulls are exposed to restrained cows in a small paddock or pen for 20 minute and observed for expression of sex drive, ability to serve cows and the number of services completed in the period. The results of the test are expressed as “Serving Capacity” or the number of mating that a bull performs in a test situation. In a 20-minute test, a serving capacity of 0 to 1 is considered low, 2 to 3 in medium and 4 or more services are considered to indicate high serving capacity. Bulls with higher serving capacity score were shown to achieve higher conceptions rate in females at first estrus than lower scores bulls (Blockey, 1989).

Physical soundness: Body condition is important, as body weight is positively related to the semen concentration in the ejaculate (Devkota et al, 2008). Whereas any systemic illness can affect reproductive performance, a great deal of emphasis must be place on the musculoskeletal system. Bulls must to be able to walk in order to detect females in heat and breed them, with the full weight of the bulls that has to be borne on the hind legs and feet during the mating act.

Examination of the reproductive organs: Examination should start only after the bull has been adequately restrained. The shape of the scrotum, although influenced by

the environmental temperature, can give some information about the thermoregulatory abilities. Presence of a scrotal neck above the testes is of critical importance because this region contains the pampiniform plexus. Short scrotum or excess of fat in the scrotal neck will result in abnormal heat exchange that affects seminal quality (Brito et al, 2003; Coulter et al, 1997; Fernandes et al, 2008; Rahman et al, 2011). Testicular shape also influences daily sperm production: bulls with long ovoid-shaped testicles produce more spermatozoa than those having normal ovoid-shape testicles or spheroid-shaped testicles (Bailey et al, 1996). Palpation must be carry on the testicular cord to detect abscess, varicoceles or a scrotal hernia, on the epididymis to exclude inflammation, granuloma or obstruction, and on the testes that must move freely within the scrotum and almost equal in size. Palpation of the testes can detect problems such as: abscesses, tumors, hematoceles or calcifications. Scrotal circumference measures are highly correlated with the daily sperm production so they are the most important part of the examination. Particular attention should be given in the evaluation of young bulls; in fact breed, body weight and age at the onset of puberty affect scrotal circumference measurements. To measures scrotal circumference, testes must be positioned firmly into the lower part of the scrotum, then a looped tape measure is places the greater diameter of the testes. Different studies reported the minimal requirements in scrotal circumference in bull depending on breed and age (Eriksson et al, 2012; Garcia Guerra et al, 2013; Hingdon III et al, 1999). Inadequate scrotal circumference has been reported as the principle cause of unsatisfactory classification of bulls (Kennedy et al, 2002). A transrectal internal examination is required to evaluate accessory sex glands and inguinal rings. Clinicians should detect every alteration from the normal conditions.

Semen quality: Any methods have been used to collect semen (electroejaculation, artificial vagina, massage, etc.); semen must be analyzed for some parameters and classified:

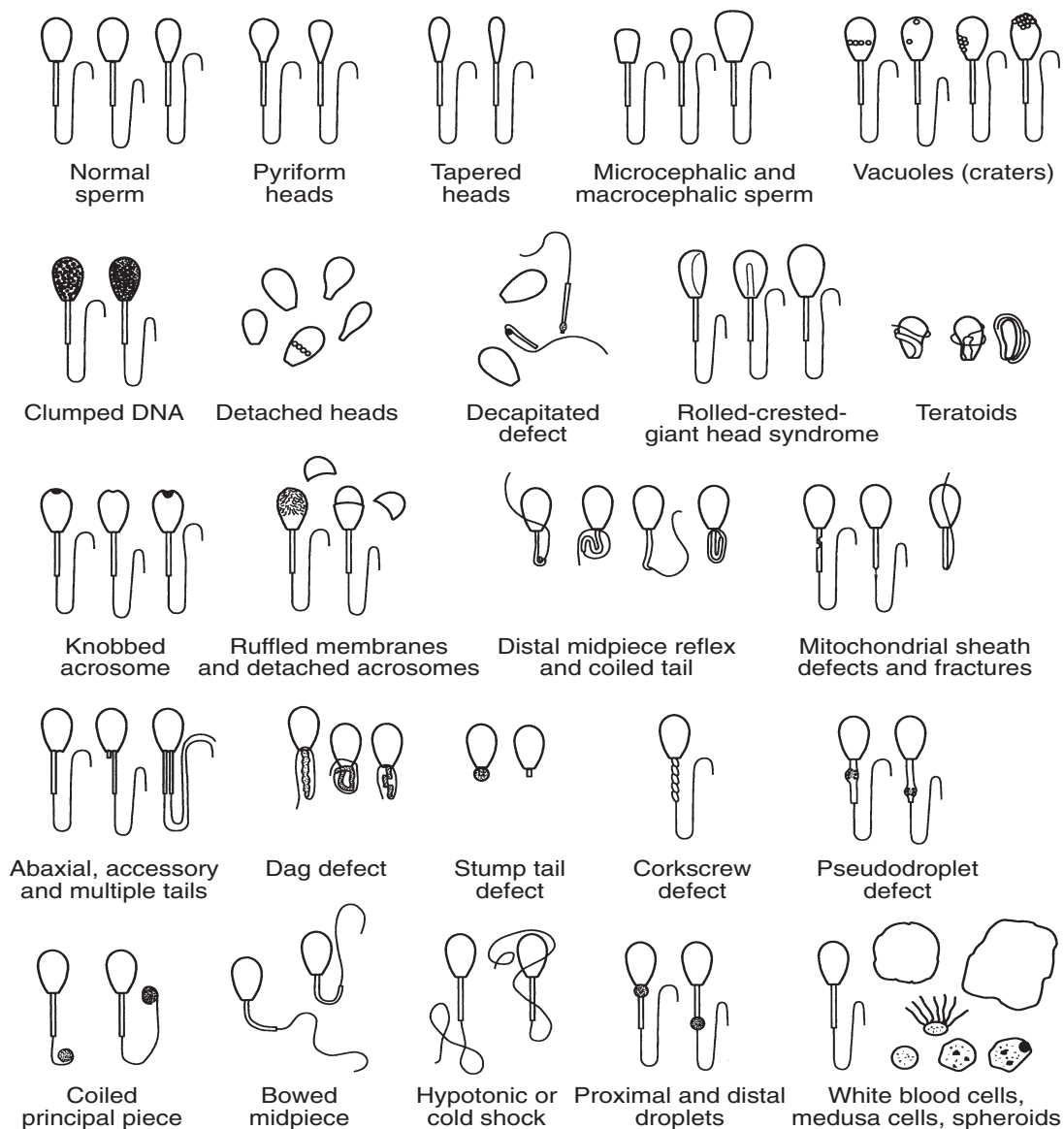
- Semen density
 - Very Good (VG) = creamy, grainy semen with > 750 million sperm/mL
 - Good (G) = milk-like semen with 400-750 million sperm/mL
 - Fair (F) = skim milk-like semen with 250-400 million sperm/mL
 - Poor (P) = translucent semen with < 250 million sperm/mL
- Gross motility or mass motility: is determined from a drop of semen placed on a warm slide under microscopy at 40x magnification. It depends of three factors: concentration, percentage of progressively motile spermatozoa and the speed of progression. The classification is as follow:
 - VG = rapid dark swirls
 - G = slower swirls and eddies
 - F = no swirls, but prominent individual cell motion
 - P = little or no individual cell motion

Mass motility was also assessed on a 0 (no activity) to 5 (rapid swirling motion) scale (Fitzpatrick et al, 2002).

- Individual motility: is the percentage of spermatozoa that are moving in the ejaculate:
 - VG = 80% to 100% motile
 - G = 60% to 79% motile
 - F = 40% to 59% motile
 - P = < 40% motile

- Sperm morphology: because spermatozoa are translucent and virtually invisible on bright-field microscopy, to evaluate sperm morphology is necessary to stain them. The common abnormal morphology forms of spermatozoa are reported in figure 2.1. Sperm abnormalities can be classified in different way. A classification is based on the site of origin of the defect, so a *primary defect* is one that originates during spermatogenesis within the testes, while a *secondary defect* is a defect of maturation that originates in the epididymis. Another classification is based on the effect of the abnormality on the fertility: *major defects* are those that have been associated with infertility; *minor defects* on the contrary do not affect fertility. From the practical point of view is of huge importance to identify if the defects are *compensable* (increasing the insemination dose would increase fertilization rate) or *non-compensable* (no effect induces by increasing the insemination dose). The tolerance levels of sperm abnormalities are usually defined as: < 20% of head defects; < 25% acrosome-tail defects; < 30% total defects. Percentage of normal spermatozoa is the semen trait with the highest repeatability ($r = 0.64$) (Fitzpatrick et al, 2002).

Figure 2.1 Common abnormal morphologic forms of spermatozoa (Barth, 2007)



After examination bulls are placed in one of the follow categories:

- **Satisfactory:** bulls that met all the minimum requirements
- **Questionable:** bulls that are able to mount but have a below-normal fertility or bulls with an undesirable trait that can be transmitted to the offspring
- **Decision deferred:** pubertal bulls or mature bulls that have suffered injuries that could affect temporarily the fertility
- **Unsatisfactory:** bulls whose use is expected to result in poor fertility

2.2.2 Ram Breeding Soundness Evaluation

Examination for breeding soundness in ram is recommended to take place at least 6 – 8 weeks before the onset of the breeding season, to allow time for the resolution of temporary problems or purchase of new animals when necessary (Ridler et al, 2012). BSE in ram follow what has been reported in bulls. Libido and serving capacity observation are very important, but is frequently neglected, although approximately 10% of rams have no interest in breeding ewes. Physical examination has to be carrying on with particular attention to the musculoskeletal system and the body condition. It is recommended a body condition score of 2.5 to 3.5 for the rams entering the breeding season (Kimberling and Parsons, 2007). In the examination of the reproductive organs, scrotal circumference is a widely used parameter in assessing breeding soundness in rams (Ridler et al, 2012). Scrotal circumference in rams is highly correlated with testicular weight, number of sperm in the testes and number of sperm in the epididymis, moreover it has been described that an increased scrotal circumference is associated with increased ejaculate volume and sperm motility (Ridler et al, 2012). Because of the seasonality of reproduction in the ovine specie, testicular volume in rams suffers a difference of up to 30% between spring and autumn (Rosa and Bryant, 2003). For this reason is difficult to establish cut-off values to classify animals on the base of scrotal circumference. Normally, in yearling rams, it has been considered a minimal requirement of scrotal circumference of 33 cm (Kimberling and Parsons, 2007; Van Metre et al, 2012). In the evaluation of ram semen it is common to observe some white blood cells (WBCs) that can indicate a sample contamination by preputial fluids, but also inflammations or infections. A ram with more than 10 WBCs per 20x field should be considered for treatment and re-evaluated (Kimberling and Parsons, 2007). The classification of rams after BSE is not unique as it is in bulls. Kimberling and Parsons

recommended a classification of rams in: excellent, satisfactory, questionable and unsatisfactory (Table 2.1). Other sources described a classification in three categories: “sound/satisfactory”, “temporarily unsound/questionable” or “unsound” (Ridler et al, 2012).

Table 2.1: BSE classification in rams (Kimberling and Parsons, 2007)

Recommendations for Classifying the Potential Breeding Capacity of Rams

	Excellent	Satisfactory	Questionable	Unsatisfactory
Ram Lambs, 6–12 mos	SC > 33 cm Mot > 50% >90% Normal sperm	SC > 30 cm Mot > 30% >70% Normal sperm	SC < 30 cm Mot < 30% <70% Normal sperm	SC < 30 cm Mot < 30% <50% Normal sperm
Yearlings, 12–18 mos	SC > 35 cm Mot > 50% >90% Normal sperm	SC > 33 cm Mot > 30% >70% Normal sperm	SC < 33 cm Mot < 30% <70% Normal sperm	SC < 33 cm Mot < 30% <50% Normal sperm

Mot, motility; SC, scrotal circumference.

2.2.3 Breeding soundness evaluation in extensive system

Little is known of the individual reproductive performance on extensive systems where, usually, multiple sires served a group of females (Holroyd et al, 2002). In these herds a low conception rate is attributed to a high proportion of bulls with low breeding soundness scores (McCosker et al, 1989). A possibility for the producers is to record the calf output and to determine paternity of progeny. This allows a monitor on genetic progress within a herd for replacement sires or to evaluate existing sires (Holroyd et al, 2002). Determining paternity is a procedure that requires the identification of DNA markers for each sire and then a DNA typing and gives a resolution of 86.5 % (range 74.4 – 97.3 %) (Holroyd et al, 2002). In this study the calf output of a single bull is related to the group dimension: in groups of 8-24 it ranges from 11 to 36 %, while in smaller groups (2-7) it was 24 – 94 % with a moderate repeatability (Holroyd et al, 2002).

2.2.4 New possibilities in Breeding Soundness Evaluation

The development of new diagnostic techniques has introduced new tools that can be used to integrate the classical BSE.

Ultrasonography: Diagnostic ultrasonography, typically with the equipment similar to that employed for trans-rectal imaging in cows, has been used for examining the reproductive tract of bulls (Kastelic and Brito, 2012) and rams (Scott, 2012). Although ultrasonography is widely used in many fields, it has been used more sparingly in male evaluation (Kastelic and Brito, 2012). Trans-scrotal ultrasonography is a convenient and non-invasive technique that is ideal for on-farm situations (Chapwanya et al, 2008). It allows visualization of tissue interfaces within the scrotum, as well as a measurement of scrotal circumference (Love, 1992). Moreover, ultrasonography allows the diagnosis of pathological conditions within the testes even if they are non-palpable (Ahmad and Noakes, 1995). Applicability of trans-scrotal ultrasonography in the BSE is still controversial (Kastelic and Britos, 2012). In bulls, testicular pixel intensity was positively correlated with daily sperm production in studies conducted in both extensively and intensively managed bulls (Kastelic et al, 2001; Brito et al, 2012), but was not associated with ejaculate volume, sperm concentration or total sperm number in the ejaculate in studies involving AI bulls (Gabor et al, 1998b; Brito et al, 2002). In rams, trans-scrotal ultrasonography has been proposed as an effective tool in the examination of testes and epididymis (Gouletsou et al, 2003), but up to now no information on its predictive value of sperm production is available.

Fine-needle aspiration cytology: Has been proposed as a tool in the diagnosis of infertility and other pathological conditions affecting testes (Mehrotra and Chaurasia, 2007; Valeri et al, 2010). Testicular fine-needle aspiration cytology (TFNAC) reports are available in many species such as dogs (Dhalbom et al, 1997; Romagnoli et al, 2009),

cats (Gouletosou et al, 2012), stallions (Pereira-Leme and Ozanam-Papa, 2010), alpacas (Stelletta et al, 2011) ad boars (Stelletta et al, 2012). TFNAC has been described to enhance BSE accuracy in bulls (Chapwanya et al, 2008), while no report are available in ram. In our preliminary work, we assess the applicability of TFNAC in ram describing the normal cytological composition in healthy rams, but without investigating his relation with fertility or semen quality (Vencato et al, 2013).

GnRH challenge test: Different studies suggest that a GnRH stimulation test can be used to evaluate a bull reproductive potential (Gabor et al, 1998; Lunstra and Coulter, 1997). An exogenous administration of GnRH or analogues has the effect to increase serum levels of LH and testosterone (Abdel Malak and Thibier, 1982; Gabor et al, 1995; Tannen and Convey, 1977). The increment of the testosterone levels has been suggested to be a good indicator of semen quality (Gabor et al, 1995). Frequently, the GnRH challenge test has been associated with the thermography evaluation of the scrotum (Gabor et al, 1998; Kastelic et al, 1996a), as it was described that the percentage of motile spermatozoa is related to the temperature gradient before and after GnRH administration (Gabor et al, 1998). First studies were conducted monitoring LH and testosterone for at least 9 hours (Abdel Malak and Thibier, 1982). LH levels increase from 15 minutes after GnRH administration, reaching the maximum levels at 105 minutes. Thereafter, the LH levels decrease continuously and return to pretreatment levels at about 6.5 hours after GnRH administration. Testosterone levels after GnRH administration increase slower than those of LH, reaching maximum values between 60 and 210 minutes, then it decrease but do not return to basal levels within 6.5 hours (Abdel Malak and Thibier, 1982). In more recent studies, period of monitoring was reduced to 90 minutes reaching satisfactory results (Gabor et al, 1995; Gabort et al, 1998).

Seminal plasma composition: Seminal plasma in normal physiology is associated with the ejaculation of spermatozoa and their survival in the female reproductive tracts (Juyena and Stelletta, 2012). Researches in different species highlight other roles for seminal plasma, including: 1) activation of the motility of spermatozoa; 2) buffering to provide the optimal osmotic and nutrient medium; 3) prevention of premature activation and stabilization of the membrane; 4) protection of spermatozoa from phagocytosis in the horse; 5) regulation of sperm transport and elimination; 6) hastening of ovulation in cows and induction of ovulation in pigs and camelids; 7) assistance in sperm-ovum interactions; 8) activation of the expression of embryotrophic cytokines; 9) influence over fertility (Juyena and Stelletta, 2012). Because of all these reasons, the composition of seminal plasma can influence the fertility of breeder and could be use as a parameter to predict fertility (Argov-Argaman et al, 2013). Although this aspect is not yet well developed, some studies showed the relation among seminal plasma components (proteins, fatty acids, etc.) with sperm function, cryopreservation success of spermatozoa, resistance of spermatozoa to stress factors (Caballero et al, 2012; Martinez-Soto et al, 2012; Muiño-Blanco et al, 2008; Towhidi and Parks, 2012).

In-vitro sperm adhesion tests: These test have been proposed to predict to fertilization capability of spermatozoa. The sperm zone pellucida binding test is based on the idea that in order to successfully fertilize and oocyte, a spermatozoa has to be able to pass the zone pellucida (Florman and Ducibella, 2006). This capability can be investigated in three methods: the sperm adhesion to the zone pellucida test; the sperm penetration trough the zone pellucida test; the in-vitro fertilization (IVF) test (Apichela and Stelletta, 2012). These tests are very useful in predicting the fertilization capability in-vitro (Oehninger et al, 2000), but give no indication about the ability of spermatozoa to move from the site of deposition of semen (that varies among species) and the site of

fertilization (the oviduct). For this reason its value as predictor of in-vivo fertility is contradictory (Braudmeier et al, 2002). The second test is the sperm oviductal cell test, which is based on the evidence that, in-vivo the adhesion of spermatozoa to the oviductal cells is necessary to develop the ability of fertilization (Apichela and Stelletta, 2012). This test consists in the incubation of spermatozoa together with explanted of oviductal tissue and the count of the number of spermatozoa adhering to the oviductal cells after incubation (Apichela and Stelletta, 2012). Although this test gave some good indication, it still needs further confirmation.

2.3 Follicular fluid

Follicles are structure enclosed in the ovary, consisting of a cumulus-oocyte complex (COC) and the granulosa cells surrounded by the follicular fluid (FF) (Avery et al, 2003). FF is in part a transudate of serum and is also partially composed of locally produced substances, which are related to the metabolic and secretory activities of granulosa and theca cells (Albohomsen et al, 2011; Avery et al, 2003; Bender et al, 2010; Revelli et al, 2009; Rodgers and Irving-Rodgers, 2010; Tabatabaei et al, 2011).

FF plays a major role in autocrine and paracrine regulation and also in physiological, biochemical and metabolic aspects of oocyte maturation and the process of ovulation (Shabankareh et al, 2013). FF has different specific functions (Orsi et al, 2005; Shabakareh et al, 2013):

- Protects oocytes from factors that induce premature resumption of meiosis;
- Protects oocyte from proteolytic attacks;
- Facilitate the extrusion of oocytes during ovulation;
- Enhance sperm attraction, motility and acrosome reaction;
- Provides a suitable microenvironment for the development, growth and maturation of oocytes and is fundamental for maintenance of fertility in female.

2.3.1 Ovarian Follicular Fluid Formation

The formation of FF commences when the theca cells become vascularized (Rodgers and Irving-Rodgers et al, 2010). Capillaries are rare in the part of ovarian parenchyma containing primordial follicles (Hermann and Spanel-Borowski et al, 1998; van Wezel and Rodgers, 1996), and only later developed as a network surrounding the early antral follicles (Jiang et al, 2002). In large species, such as bovine, ovine, buffalo, human, camelids, the thecal capillaries form a multilayers network (Yamada et al, 1995) that is not uniform around the follicle, with fewer capillaries in the apex region (Jiang et al, 2002). The capillaries networks continue to develop as follicles growth in a manner that varies between dominant and subordinate follicles (Berisha and Scharms, 2005), differs among follicles with oocytes of different quality (Huey et al, 1999) or decline upon follicular atresia (Huey et al, 1999; Jiang et al, 2003). Alteration in capillary blood pressure or flow could affect formation of FF during specific times of follicles development (such as ovulation). For fluid to pass from capillaries to follicular antrum, it needs to pass through endothelium, subendothelial basal lamina, thecal interstitium, follicular basal lamina and the membrana granulosa. These structures form a “blood-follicles barrier” (Zhou et al, 2007). Although the barrier permits the diffusion of proteins of up to 500 kDa (Albohomsen et al, 2011), the composition of FF is similar to serum with respect to components only up to 100 kDa (Gosden et al, 1988). On the other hand, for increasing sizes above 100kDa plasma proteins are found at progressively lower concentration in FF than plasma (Rodgers and Irving-Rodgers, 2010). For the same reason, large molecules produced by oocytes and granulosa cells cannot pass the barrier establishing a potential osmotic gradient that could be responsible for recruiting fluid into the follicle (Rodgers and Irving-Rodgers, 2010). Molecules that contribute to create the osmotic gradient are: hyaluronan, proteoglycans and glycosaminoglycans

(Clarke et al, 2006; Saito et al, 2000). Evidence suggests that granulosa cells can facilitate water transport via aquaporins that have been detected in the granulosa cells of different species (Rodgers and Irving-Rodgers, 2010).

The regulation of FF depends on two mechanisms:

- Gonadotropins, PGE1, PGE2 and testosterone upregulates mRNA levels of versican, a molecule that stimulates the synthesis of proteoglycans and glycosaminoglycans increasing the osmotic gradient (Russel et al, 2003)
- The permeability of the barrier varies during the follicular development for alteration of capillaries blood flow but also because the basal lamina composition changes at around the pre-antral and primary stages (Rodgers et al, 1998)

The metabolic activity of follicular cells together with the changes of the permeability of the blood-follicle barrier during the follicular growth lead to modification of FF composition (Albohomsen et al, 2011). Different studies reported that a different composition of the FF in different-sized follicles can be expected (Albohomsen et al, 2011; Andersen et al, 1976; Franchimont et al, 1990; Grimec et al, 1984; Hammon et al, 2000; Homa and Brown, 1992; Khan et al, 2011a; Leroy et al, 2004; Nandi et al, 2007; Schweigert and Zucker, 1988; Shabankareh et al, 2013; Tabatabaei et al, 2011). It has been reported that FF composition also fluctuates with ovarian cycles stages in buffalo (Abd Ellah et al, 2010; Eissa, 1996; Khan et al, 2011a; Khan et al, 2012) and in bovine (Shabankareh et al, 2013). Moreover, FF composition changes between dominant follicle (in particular during the pre-ovulatory growth) and subordinate follicle (Ainsworth et al, 1980; Bender et al, 2010; Fortune et al, 2004). Because FF is partially originated from serum, its composition is influenced by the nutritional status of the animal (Comin et al, 2002; Hammon et al, 2000). In particular, dietary restriction limits FF concentration of

growth factors and gonadotropins leading to a reduction in growth of the follicles and a greater percentage of not ovulating follicles (Comin et al, 2002).

2.3.2 Prediction of oocyte quality

The assessment of oocyte quality is becoming one of the major objectives both in human and animal *in-vitro* production (IVP) (Revelli et al, 2009). Multiple methods of oocyte selection have been proposed.

The study of oocyte morphology is the most common, it is quick and simple but is not fully satisfactory (Balaban and Urman, 2006). Other studies have focused on the expression of genes in granulosa cells and oocyte looking for specific molecular markers (Patrizio et al, 2007) or chromosomal defects (Dawson et al, 2006). Anyway, because of the complexity of the procedures and their expense, these techniques are not applicable to the clinical practice.

FF provides the microenvironment for the development of the oocytes and it is reasonable to think that its composition may play a role in the determination of oocytes quality and the potential to achieve embryo development (Albohomsen et al, 2011; Ali et al, 2004; Bender et al, 2010; Eissa et al, 1996; Revelli et al, 2009; Wallace et al, 2012). Moreover, knowledge on composition of FF can also provide information about the requirements for cell and oocyte maturation (Shabankareh et al, 2013), and may be used as a guide for the formulation of specific media for IVP (Duarte et al, 2012; Gérard et al, 2002). Different substances have been studied as oocyte quality markers:

Hormones: it has been shown that steroidal content of FF changes markedly during the preovulatory growth (Ainsworth et al, 1980; Tabatabaei et al, 2011). High FF concentration of FSH, hCG and LH have been reported to promote oocyte maturation and are associate with oocytes having and higher probability to be fertilized (Revelli et al, 2009). Growth hormone (GH) seems to play a similar role as it enhances the

estrogens (E) production by the granulosa cells (Lanzone et al, 1996) as well as the exposition of FSH and LH receptors (Revelli et al, 2009). Anyway, the use of GH as a predictor of oocyte quality is contradictory as Mendoza described a high correlation between GH levels in FF and IVP successful rate (Mendoza et al, 2002), while Tarlatzis found the opposite (Tarlatzis et al, 1993). Also, prolactin, E, progesterone, androgens and corticoids indication have been contradictory as they were correlated with embryo development in some studies but not in others (Revelli et al, 2009).

Growth factors: inhibin B (Chang et al, 2002) and activin A (Lau et al, 1999) have been proposed as predictors of the quality of the embryos, but these results still need to be confirmed. The Anti-mullerian hormone instead gave contradictory results (Revelli et al, 2009). The role of the Insuline-like growth factors (IGF) system has been widely studied (Fortune et al, 2001; Fortune et al, 2004; Revelli et al, 2009). In the past time this system was described to be significant related to the oocyte quality and embryo development (Revelli et al, 2009), but a recent studies showed that it is not associated with the fertilization outcome (Asimakopoulos et al, 2008). Further studies are needed to establish the possible role of IGF system as a marker of oocyte quality. Interleukins (ILs) can be found in FF produced at local level or deriving from the plasma ultrafiltrate. Different studies showed that some ILs are correlated with embryo quality and pregnancy rate in women (Revelli et al, 2009).

Reactive oxygen species (ROS): the impact of oxidative stress on oocyte maturation seems to be deleterious, although an exact role is not yet clarified (Revelli et al, 2009). For this reason an elevated total antioxidant capability of FF was found to be higher in follicles having oocytes that will have and higher fertilization rate (Oyawoye et al, 2003).

Anti-apoptotic factors: studies on the activation of specific cellular apoptosis pathways sentenced that these factors are not reliable marker of the quality of the oocyte (Revelli et al, 2009).

Proteins, peptides and amino-acids: FF contains several proteins that derive from blood plasma or are secreted both by granulosa and thecal cells (Revelli et al, 2009). Different proteins have been investigated as markers of good follicular development, but just few of them have been concluded to have a possible role: endothelin-2 (Plonowski et al, 1999), homocysteine (Boxmeer et al, 2008), lactoferrin (Yanaihara et al, 2007), prorenin (Revelli et al, 2009). Among amino-acids, alanine and glycine have shown a good predictive value on IVF outcome in bovine (Sinclair et al, 2008), while D-aspartic acid were found to be directly related with good morphology and the fertilization rate in human (D'Aniello et al, 2007).

Sugars: hyaluronan was positively related to the level of apoptosis in granulosa cells, and it was higher in FF of follicles containing non-fertilized oocytes (Saito et al, 2000).

Prostanoids: despite the fact that prostaglandin F₂alpha and PGE₂ were found in higher concentration in FF of follicles containing mature oocytes, because of their very short half-life it is improbable that they will be used as oocyte quality predictors in the clinical practice (Revelli et al, 2009).

Metabolomics: is actually the most used approach in studying FF. It is the dynamic quantitative assessment of all low molecular weight substances that are present in FF at given time (Revelli et al, 2009). Studies on metabolomics have undertaken profiling for a selective class of metabolites, such as fatty acids (O'Gorman et al, 2013; Shaaker et al, 2012; Warzych et al, 2013; Zeron et al, 2001), amino-acids (Booth et al, 2005) and sugars (Preis et al, 2005; Warzych et al, 2013). Some results showed that decrease levels of glucose and increase levels of proline, lactate, leucine and isoleucine in FF resulted in

a successful pregnancy (Wallace et al, 2012). Fatty acids seem to have an important role as fertilization outcome. N-3 fatty acids had a positive correlation with pregnancy rate (Shaaker et al, 2012), while the concentration of the C16:0, C16:1, C18:1cis9 and C22:5n3 fatty acids, together with the activity of two enzymes (Δ^9 -desaturase and elongase) allowed prediction of the morphology COC with an accuracy of 72% (Warzych et al, 2013). O’Gorman found that the fatty acids composition of FF from follicles where oocytes fertilized and developed to early cleavage stage embryos and oocytes that fertilized but failed to cleave, differed for the concentration of nine different fatty acids (O’Gorman et al, 2013).

3 – OBJECTIVES

Aim 1: two studies were conducted to evaluate new approaches in the andrological evaluation. The first study (chapter 4) was done to evaluate the possible use of thermography during GnRH challenge test in the examination of young bulls (around one year,) with low semen production. The second study (chapter 5) was designed to determine the prediction value of trans-scrotal ultrasonography and testicular fine-needle aspiration cytology on sperm production in adult rams.

Aim 2: two studies were conducted to determine the composition of follicular fluid in South American camelids and buffalo. The study in chapter 6 was designed to determine the effects of ovarian cycle and follicle developmental stage in the follicular fluid of South American camelids. The study in chapter 7 investigates the biochemical and fatty acids composition of buffalo follicular fluid from different size follicles.

Aim 3: study in chapter 8 was done to investigate the effect of different nutrition of the testicular cytology of peri-pubertal rams, with particular focus on the Conjugated Linoleic acids supplementation.

4 - INTEGRATED EVALUATION OF SCROTAL TEMPERATURE AND TESTOSTERONEMIA DURING GnRH CHALLENGE TEST IN YOUNG BULLS WITH LOW SEMEN PRODUCTION

4.1 Abstract

The aim of this study is to assess the suitability of thermography monitoring of scrotal surface temperature as a method to monitor testicular function. Yearling bulls (n=23) with low semen quality were selected. Scrotal surface temperature and serum testosterone (T) were evaluated before and after administration of 10.5 µg Buserelin Acetate IV. Thermographic images of scrotum were recorded at 0, 15, 30, 45 and 60 minutes post-GnRH, while blood sampling was only performed at 60 minutes post-GnRH. Bulls were divided in two groups: LowTemp bulls (n=10) had a decreased scrotal surface temperature at 60 minutes; HighTemp bulls (n=13) had an increased scrotal temperature. After 60 minutes LowTemp bulls had higher T values compared to HighTemp bulls: 14.32 ng/ml ± 0.53 vs. 10.30 ± 1.37 ng/ml (mean ± SEM) (P<0.05) for LowTemp and HighTemp bulls respectively. Reproductive performances in both groups improved after GnRH administration, resulting in an increased number of inseminating doses from each collection, which was higher in LowTemp bulls. Pearson correlation test indicates a negative relationship between T and scrotal surface temperature (r=-0.554). In conclusion, a decreased scrotal surface temperature 60 minute after GnRH treatment was associated with improvements of semen quality after treatment.

4.2 Introduction

In the bull, puberty is defined as an ejaculate with at least 50 millions sperm with more than 10% progressive motility (Bagu et al, 2006; Rawlings et al, 2008; Wolf et al, 1965). Dairy bulls attain their mature rate of spermatogenesis at 1 year of age and

gonadotropins and steroid hormones are involved in the process of initiation and maintenance of spermatogenesis (Thibier, 1975). Serum testosterone (T) increases up to 11 months of age, followed by a decrease at 12 months of age (Rawlings et al, 1972; Thibier, 1975). Bull calves can be divided in those attaining puberty early or late (Aravindakshan et al, 2000; Bagu et al, 2006; Evans et al, 1995). Bulls attaining late puberty are a problem for producers because of their increased cost of maintenance. Thermal and functional regulation of the testis are based on systemic and local information (Sharpe, 1984; Tahka, 1989). Testicular function is controlled peripherally via anterior pituitary gonadotropins, while the response of the testis to these stimuli happens locally (Sharpe, 1984). LH stimulates testosterone secretion by the Leydig cells and also modulate blood flow and capillary permeability in testes (Tahka, 1989). Testosterone and its metabolites regulate the activity of androgens and its receptors, which are essential for proper sexual differentiation and maintenance of normal spermatogenesis (Holdcraft and Braun, 2004). Elevated scrotal surface temperature (SST) has been related to poor semen quality in humans (Zorgniotti, 1982) and bulls (Arteaga et al, 2005; Barth and Bowman, 1994; Brito et al, 2003; Coulter et al, 1997; Kastelic et al, 1996c; Kastelic et al, 2001; Setchell, 1998; Vogler et al, 1993), as well as to low *in-vitro* fertility (Fernandes et al, 2008). Therefore, thermoregulation of the testes is essential for sperm production (Kastelic et al, 1996b; Lunstra and Coulter, 1997). Exogenous administration of GnRH increases T secretion from Leydig cells (Abdel Malak and Thibier, 1982; Gabor et al, 1995; Tannen and Convey, 1977). An overall increase in SST was observed following GnRH administration, however the temperature increment detected in the lower aspect of the testicle (defined as “bottom of the scrotum” by Kastelic et al, 1996a), were consistently higher than those recorded from the “top of the scrotum”. Different studies suggest that a GnRH stimulation test can be used to evaluate

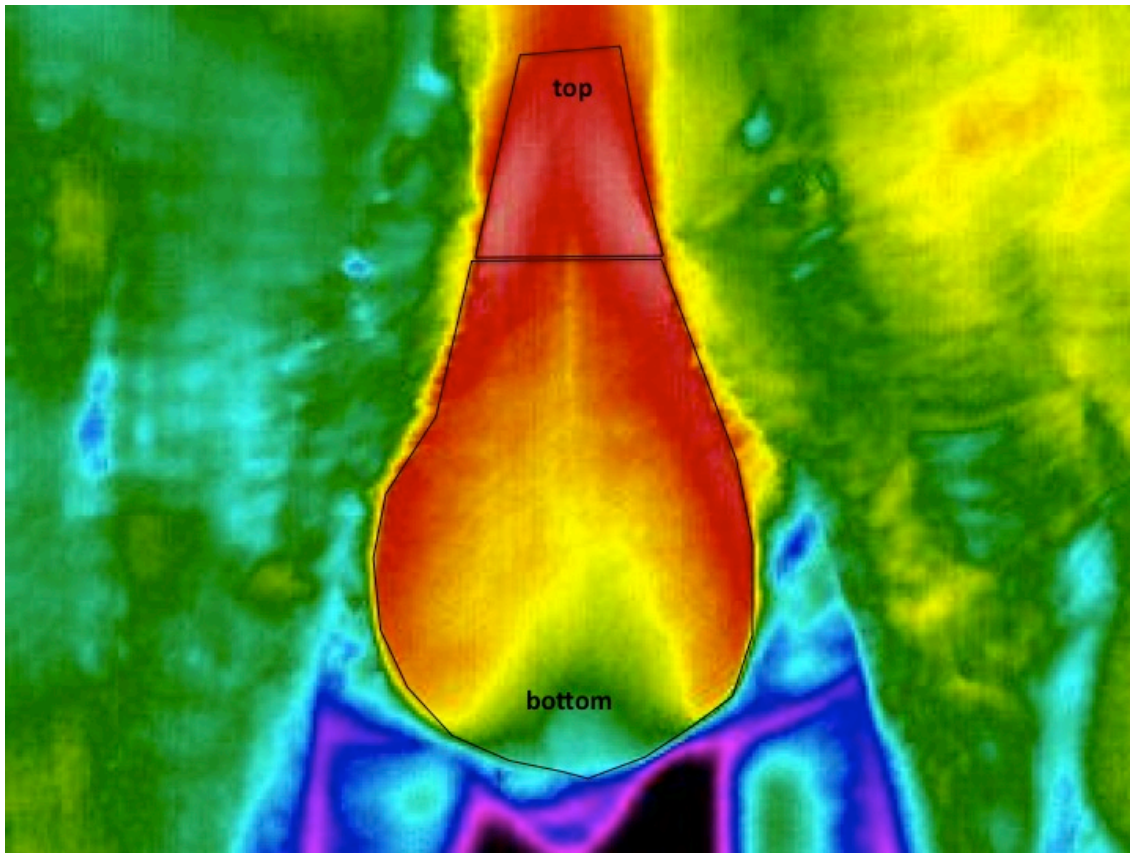
a bull reproductive potential (Gabor et al, 1998, Lunstra and Coulter, 1997): T is measured before and after the administration of GnRH, and the increase in T is an indicator of semen quality (Gabor et al, 1995). It has been proposed that high interstitial T might activate a mechanism of secretion based on a neural local regulation of the scrotal blood flow and muscular contraction, resulting in a change of SST. Therefore, thermographic monitoring may be a suitable method to study testicular function considering the increase of SST related to an increase of T (Gabor et al, 1998). Moreover the percentage of motile spermatozoa is inversely related to the temperature gradient before and 45 minutes after GnRH administration (Gabor et al, 1998). The aims of this study were a) to assess the relationship between thermal response and semen production following GnRH treatment, and b) to develop an andrological evaluation test applicable to young bulls with low semen production, through the integrated information collected with infrared-thermography and testosterone concentration following GnRH challenge test.

4.3 Materials and methods

The present study was started in March 2011 at the bovine semen center (Intermizoo S.P.A., Brussa, Venice, Italy), latitude 45°38'20"N 12°56'51". Semen was collected from young Holstein bulls (n=120) approximately 1 year old as a part of routine progeny test, using an artificial vagina and a teaser male 3 times per week for 3 weeks. From this herd, 23 bulls (n=23) with low semen quality (producing less than 150 straws/collection) were selected. Experimental animals were of 12.5 to 13.5 months of age, 318-370 Kg BW and in good health conditions. Bulls were housed in the same barn in individual pens and fed a balanced diet. The study was performed indoor in order to avoid environmental interferences on the thermographic images and maintain constant temperature and humidity conditions. Bulls were restrained in standing position until

the end of the examination. GnRH stimulation test was performed administering 10.5 µg IV of the GnRH analogue Buserelin (Receptal®, INTERVET). Thermographic monitoring of the scrotum was performed using an infrared camera (Flir Systems™ model P25). At the beginning of each thermographic session environmental temperature and humidity data were entered into the infrared thermo-camera (thermocamera) using a standard thermometer and hygrometer (WMR80A Full Wireless Meteo Station, Oregon Scientific, USA), while the reflected temperature were automatically calculated by the thermocamera using the following formula: [environmental temperature - environmental temperature x (1-emissivity)]. Temperature ranged from 14 to 18°C, humidity from 53 to 65 % and reflected temperature from 12.7 to 15.3 °C. Camera was held at the level of the scrotum, approximately 1.0 meter behind the standing bull (Brito et al, 2012), the tail was raised to view the scrotum and no manipulation of scrotum was made before thermography. Thermographic images were taken immediately prior to administration of Buserelin [Time0] and at 15 minutes intervals up to 60 minutes post-Buserelin [Time15; Time30; Time45; Time60]. SST was evaluated using the program ThermaCam Researcher Basic™ provided from Flir Systems™. A “bottom area” corresponding to the skin covering the testes and a “top area” corresponding to the vascular cone (Figure 4.1) were identified as described previously (Kastelic et al, 1996a, Kastelic et al, 1997, Kastelic et al, 1996b).

Figure 4.1 Scrotal thermography in bull; top: indicate the skin covering the vascular cone; bottom: indicate the skin covering the testes



Differences of mean SST of bottom and top areas before and after GnRH administration (Δ SST) were considered. Blood samples for T evaluation were taken from the jugular vein in order to avoid manipulation of the tail region during the examination that could modify perineal surface temperature. Blood samples were collected at Time0 and at Time60, stored at 4 °C and centrifuged within 60 minutes. Serum samples were maintained at -80°C until analysis. T was determined using an IMMULITE® 1000 immunoassay analyzer from SIEMENS. Differences in mean T before and after GnRH administration (Δ T) were considered. After the first (and only) GnRH administration semen collection was performed 3 times weekly throughout the study. Ejaculates were analyzed using a Computer-Assisted Sperm Analysis (CASA) (Hamilton Thorne

Bioscience). The following parameters were considered: concentration, sperm motility (MOT), sperm progressive motility (PROG), average path velocity (VAP), straight-line velocity (VSL), amplitude of lateral head displacement (ALH), straightness (STR) and linearity (LIN). Number of semen doses obtained from each bull was also determined. Bulls were divided depending on the thermal response to Buserelin administration at Time60. To assess the possible effect of a single injection of GnRH on semen production, semen quality data obtained from our experimental animals were compared to semen quality data from a group of 30 bulls of the same age (12 – 13 months) and with similar low semen production (less than 150 doses produced per ejaculate) collected in the same bull center during the years 2009-2011 (Control bulls).

All data were analyzed using the General Linear Model procedures of SAS (1990). Two-way repeated measures ANOVA was performed to investigate the main effects of group and time and group by time interaction. Effect on semen production was studied using Two-way repeated measure ANOVA, group and time were considered as independent variables and semen parameters as dependent variables. The study lasted from 3 weeks before GnRH administration until the collection of 3000 doses of semen necessary for the progeny test. Pearson correlation indices were calculated among all parameters considered.

4.4 Results

Contrary to our expectations, not all bull showed the expected increase in SST: scrotal temperature increased in 13/23 (indicated as HighTemp) and decreased in 10/13 (indicated as LowTemp) bulls. LowTemp bulls had a significant decrease in SST at Time60 (Δ SST= -0.72 ± 0.22 °C) compared to SST at Time0, while HighTemp bulls had a significant increase in SST (Δ SST= 0.93 ± 0.25 °C) ($P < 0.05$). All SST data are reported in table 4.1.

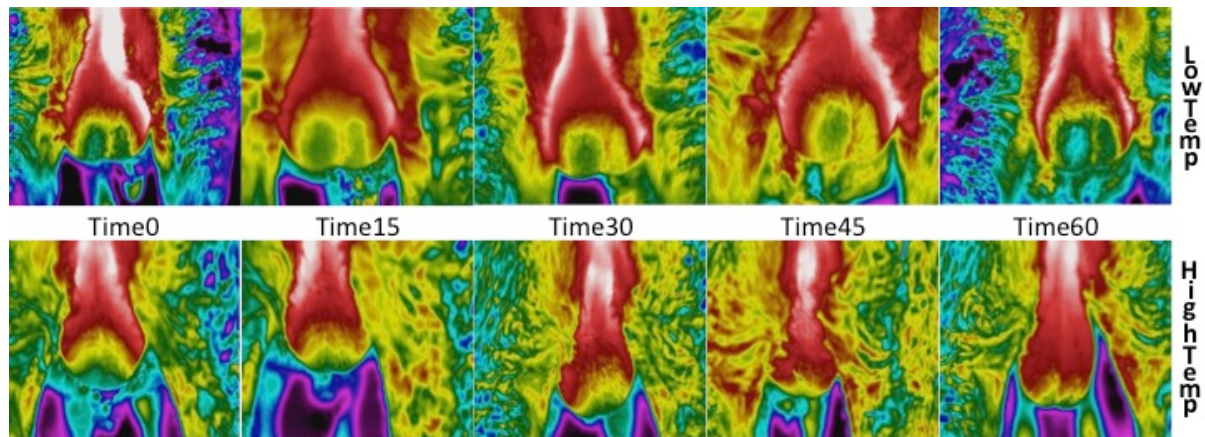
Table 4.1 Mean and Δ scrotal surface temperature (\pm SEM) ($^{\circ}$ C) measured by infrared thermography in the bottom and the top areas of the scrotum

		LowT(n=10)		HighT(n=13)	
	<i>Time (min)</i>	<i>Mean \pm (SEM)</i>	Δ <i>Temperature \pm SEM</i>	<i>Mean \pm SEM</i>	Δ <i>Temperature \pm SEM</i>
Bottom	0	26.87 \pm 0.36 ^a	0	26.61 \pm 0.55 ^a	0
	15	26.00 \pm 0.39 ^b	-0.87 \pm 0.20	26.75 \pm 0.56 ^a	0.14 \pm 0.20
	30	25.78 \pm 0.40 ^{b*}	-1.09 \pm 0.18	26.76 \pm 0.54 ^{a*}	0.15 \pm 0.26
	45	26.21 \pm 0.56 ^{b*}	-0.67 \pm 0.34	27.78 \pm 0.48 ^{b*}	1.17 \pm 0.31
	60	25.98 \pm 0.49 ^{b*}	-0.88 \pm 0.26	27.89 \pm 0.42 ^{b*}	1.21 \pm 0.30
Top	0	30.14 \pm 0.37 ^a	0	30.86 \pm 0.38 ^a	0
	15	29.34 \pm 0.35 ^{b*}	-0.80 \pm 0.18	30.53 \pm 0.42 ^{a*}	-0.32 \pm 0.26
	30	29.27 \pm 0.28 ^{b*}	-0.87 \pm 0.22	30.97 \pm 0.34 ^{a*}	0.11 \pm 0.21
	45	29.60 \pm 0.50 ^{b*}	-0.54 \pm 0.28	31.40 \pm 0.42 ^{b*}	0.54 \pm 0.26
	60	29.58 \pm 0.44 ^{b*}	-0.56 \pm 0.26	31.51 \pm 0.32 ^{b*}	0.65 \pm 0.19

*indicates statistical difference (P<0.05) between groups within time; ^{a,b} indicate statistical differences between time within group and testicular area

Thermometric responses at Time15 and Time30 were variable individually and there was no significant correlation with T, while responses at Time45 were similar to Time60 (Figure 4.2). There was no significant difference in basal T between HighTemp and LowTemp bulls: 6.87 \pm 1.79 vs. 6.84 \pm 1.54 ng/ml (mean \pm SEM). LowTemp bulls had a significant higher T at 60 minutes after GnRH administration: 14.32 \pm 0.52 vs. 10.30 \pm 1.37 ng/ml (mean \pm SEM) for LowTemp and HighTemp bulls respectively) (P<0.05).

Figure 4.2 Scrotal thermography monitoring during GnRH challenge in a Lowtemp and a Hightemp bull.



Semen quality parameters were compared before and after GnRH test. Both groups improved their reproductive performance (Table 4.2), and LowTemp bulls displayed a significant higher improvement compared to HighTemp ($P < 0.05$). Data from Control bulls were divided in a first period corresponding to the first three weeks of collection and a second period corresponding to the successive collections until the achievement of 3000 semen doses. In the first period semen production in Control bulls did not differ from both experimental groups before GnRH administration (Table 4.2). Following treatment, both LowTemp and HighTemp bulls had a higher semen production than Control bulls during the second period ($P < 0.05$) (Table 4.2). The Pearson correlation semen was calculated considering all parameters (T, SST and semen quality). There was a negative correlation between T and SST in young bulls with low semen quality -0.554 ($P < 0.05$); between LowTemp and HighTemp for ΔT (-0.43 ; $P < 0.05$) and T at Time60 (-0.48 ; $P < 0.05$), while there was no correlation between groups and basal T concentration.

Table 4.2 Semen analyses before and after GnRH administration in Lowtemp, Hightemp and control groups

	LowTemp		HighTemp		Control	
	<i>BEFORE GnRH</i>	<i>AFTER GnRH</i>	<i>BEFORE GnRH</i>	<i>AFTER GnRH</i>	<i>1st period</i>	<i>2nd period</i>
<i>Volume (ml)</i>	3.99 ± 0.55	4.43 ± 0.47 ^{a*}	3.51 ± 0.37	3.87 ± 0.33 ^b	3.35 ± 0.26	3.62 ± 0.45b
<i>Concentration (x10⁶/ml)</i>	715.11 ± 167.21	885.79 ± 182.86*	716.88 ± 155.92	900.35± 148.05*	726.31 ± 76.32	877.11 ± 139.63*
<i>Motility (%)</i>	71.74 ± 9.09	84.43 ± 5.03a*	71.66 ± 7.71	84.32 ± 3.56a*	68.56 ± 4.41	68.70 ± 4.54b
<i>Progressive (%)</i>	48.14 ± 6.55	59.07 ± 4.40a*	49.69 ± 5.58	57.83 ± 3.38a*	46.49 ± 2.57	48.30 ± 2.74b
<i>VAP μm/s</i>	89.34 ± 14.84	108.69 ± 9.34a*	91.34 ± 14.58	113.46 ± 6.20a*	77.51 ± 5.81	77.70 ± 6.32b
<i>VSL μm/s</i>	51.46 ± 10.52	66.72 ± 7.39*	45.77 ± 10.44	69.14 ± 6.36*	64.76 ± 5.15	65.02 ± 5.40
<i>VCL μm/s</i>	121.17 ± 26.82	146.07 ± 16.44	102.95 ± 33.61	154.13 ± 40.51	112.43 ± 19.95	137.08 ± 18.70
<i>STR %</i>	54.6 ± 11.10	70.93 ± 7.74*	46.75 ± 10.62	70.72 ± 6.44*	67.61 ± 5.94	68.78 ± 5.92
<i>LIN %</i>	31.77 ± 6.47	43.02 ± 4.78*	28.05 ± 6.40	42.48 ± 3.93*	41.71 ± 3.74	42.43 ± 3.71
<i>ALH μm/s</i>	4.99 ± 1.02	5.91 ± 0.66a*	4.18 ± 0.95	6.19 ± 0.57a*	4.77 ± 0.40	4.31 ± 0.57b
<i>DOSIS n°</i>	138.89 ± 36.49	228.40 ± 33.11 ^{a*}	126.24 ± 37.30	182.50 ± 33.40 ^{b*}	113.58 ± 10.87	157.49 ± 16.33c*

*indicates statistical differences between BEFORE GnRH and AFTER GnRH (or 1st and 2nd period) within group; a,b,c indicate differences among groups within period

4.5 Discussion

This is apparently the first report in which testicular function of young bulls with low semen production was evaluated by monitoring thermic response and T before and after GnRH administration. In our study, the combined evaluation of SST and T following a GnRH challenge test was useful and easily applicable in practice and we believe that it

could be considered as a way to improve the classical andrological evaluation for bulls. Basal SST did not differ between groups at Time0 both at the bottom as well as at the top of the scrotum. Two different thermic responses were identified at Time60: a significant decrease in SST for LowTemp bulls and a significant increase in SST for HighTemp bulls. Our results are in partial disagreement with those of Gabor who reported an increase in SST in all bulls after GnRH administration, although in Gabor's study all treated bulls were adults (average age of 24.3 months) (Gabor et al, 1998).

As reported in table 4.1, there was a decrease in SST in LowTemp bulls starting from Time15 at the bottom as well as in the top of the scrotum, while there was an increase in SST in HighTemp bulls starting from Time45 ($P < 0.05$). In both groups the variation of SST at the bottom of the scrotum exceeded the variation SST measured at the top of the scrotum, therefore it is confirmed that bottom SST seems to be more labile (Gabor et al, 1998; Kastelic et al, 1995). Basal T did not differ between HighTemp and LowTemp bulls. The values of T obtained seemed similar to values reported by Thibier (Thibier, 1975) [4.43 ± 3.01 ng/ml]. An increase of T was observed in all bulls at Time60 after GnRH treatment, however LowTemp bull values were statistically higher than those in HighTemp bulls. The ΔT values in our study were of 6.71 ± 1.79 ng/ml for LowTemp bulls and 2.41 ± 0.92 ng/ml for HighTemp bulls. ΔT were higher in LowTemp bulls and lower in HighTemp bulls if compared to Gabor et al. (Gabor et al, 1995). The difference in ΔSST may be due to a different scrotal thermoregulatory capability between testicles of bulls with high vs low fertility. The decrease of SST after GnRH administration may be related to the production of testicular factors causing contraction of on scrotal vessels leading to a decrease in blood flow and therefore a decrease in scrotal temperature (Sharpe, 1984). These testicular factors may be activated as an answer to the increase of interstitial testosterone concentration leading to an overflow at the vascular level.

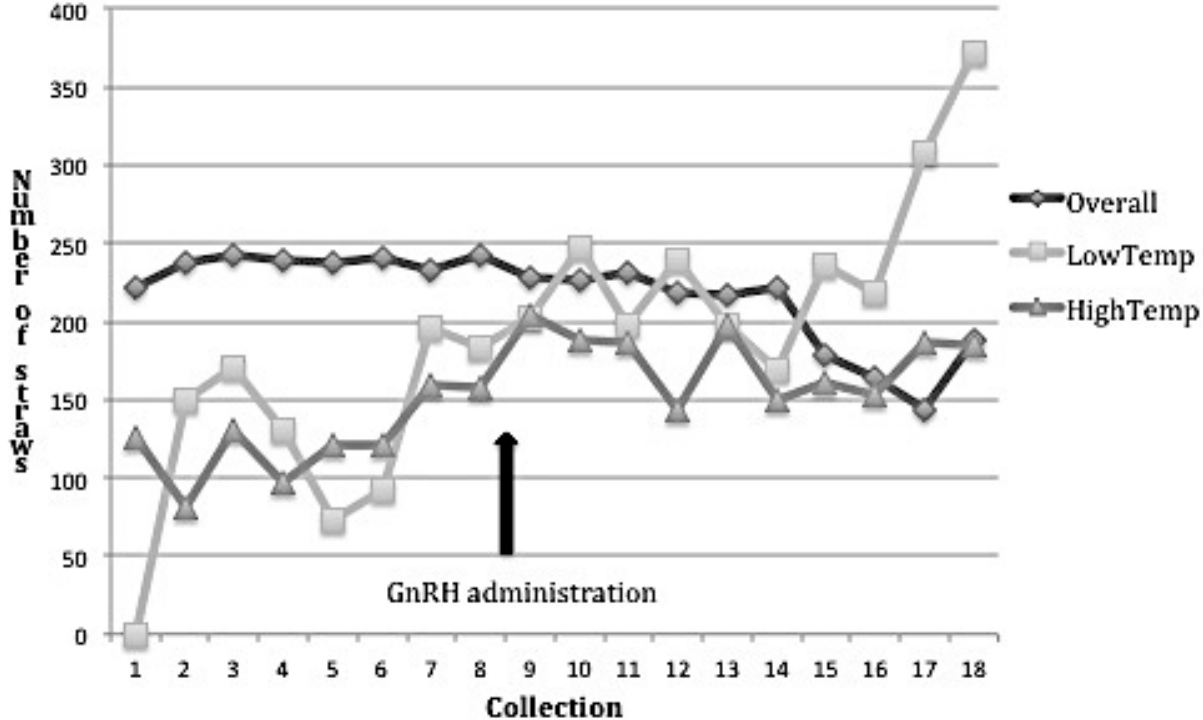
Therefore, the SST decreasing at Time60 in LowTemp bulls might be a result of vascular mechanisms linked to the level of interstitial testosterone concentration. Semen quality parameters did not differ between groups before the GnRH challenge test. Although at the beginning of the study all bulls satisfied the endpoint for puberty at 50 million sperm and 10% motility (Bagu et al, 2006, Rawlings et al, 2008), semen production of these animals was lower compared to other males of the same age. An increase in all semen quality parameters was noticed after GnRH treatment. LowTemp bulls had a higher improvement than HighTemp bulls in terms of volume and number of inseminating doses of 20 million progressively motile spermatozoa. This could be explained by a better thermoregulation expressed in LowTemp bulls allowing to avoid the negative effect of high temperature on sperm production. Semen production improvement was maintained until the end of the collection period (approximately 4 weeks). This result is in accordance with those of Brito that described a relationship among semen quality, decreased scrotal surface and testicular temperature (Brito et al, 2004).

Because of the young age of our experimental bulls (1 year), it is possible that low semen production was caused by a late onset of puberty. The exogenous administration of GnRH caused a peak of T that might have contributed to testicular maturation in late pubertal bulls resulting in the increase of semen production. Semen quality data from experimental groups were compared with semen quality from a Control group of bulls of the same age and low semen production from previous years. During the initial three weeks of collection there was no significant difference in semen production among groups. During the subsequent weeks semen production increased in all groups, but groups treated with GnRH had a higher production in term of number of doses produced per ejaculate than Control bulls (Table 4.2).

Therefore, even if an improvement in semen quality over time was observed in Control bulls, a single GnRH administration may enhance sperm production in late maturing bulls. This finding could be important for bull centers as it may help to reduce the time that usually bulls remain at the center for progeny testing, thereby reducing costs. Considering the production of semen doses before and after GnRH administration, the estimated time to produce 3000 doses in our study was reduced from 7.33 to 4.00 weeks in LowTemp bulls and from 8.00 to 5.33 weeks for HighTemp bulls. Further studies are required to prove the effects of GnRH administration on semen quality in late maturing bulls.

In figure 4.3 the production of straws per collection of the bulls used in this study was compared to the number of straws per collection obtained from the totality of bulls during their progeny test semen collection.

Figure 4.3 Straws production per collection prior and after GnRH administration in experimental groups and overall production of bulls during progeny test



In conclusion, it is confirmed that SST after GnRH treatment can be used to evaluate testicular function. Low maturing bulls with potentially normal fertility may benefit from a GnRH challenge thus reaching puberty within a normal range of time, while late maturing bulls with potentially subnormal fertility could be identified by measuring their SST. The use of a GnRH challenge test can be made also by collecting the post-GnRH blood sample 60 minutes later instead of 90 as reported by Gabor et al (Gabor et al, 1998).

5 – TRANS-SCROTAL ULTRASONOGRAPHY AND TESTICULAR FINE-NEEDLE ASPIRATION CYTOLOGY IN THE EVALUATION OF RAM SPERM PRODUCTION

5.1 Abstract

The aim of this study was to assess the predicting value of trans-scrotal ultrasonography and testicular fine-needle aspiration cytology on sperm production in rams. Ultrasonography examination and testicular fine-needle aspiration cytology were performed on eighteen adult rams of proved fertility. Semen was collected twice using electroejaculation at interval of 1 week, then rams were slaughtered and epididymal spermatozoa were collected. Kinetic analysis of post-thawed semen was performed using a Computer-Assisted Sperm Analysis. Data were analyzed dividing rams in groups on the base of an echogenicity score of testicular appearance, while Pearson correlation indexes were calculated to identify correlation among the considered parameters. The group with better echogenicity score had a higher Spermatic Index (SI) and a higher semen straws production than the worst group ($P=0.047$ for electroejaculation; $P=0.033$ for epididymal spermatozoa). Several cytological parameters were correlated with sperm production: secondary spermatocytes with electroejaculation collection (0.703; $P=0.007$), spermatogonia (-0.746; $P<0.001$) and SI (0.667; $P=0.003$) with epididymal collection. In conclusion trans-scrotal ultrasonography and testicular fine-needle aspiration can add useful information during the BSE in ram.

5.2 Introduction

The BSE of rams should be carried out in flocks before the beginning of the breeding season to eliminate sub-fertile rams (Gouletsou and Fthenakis, 2010; Gouletsou et al, 2003). In the ram, routinely examination includes: libido assessment, physical

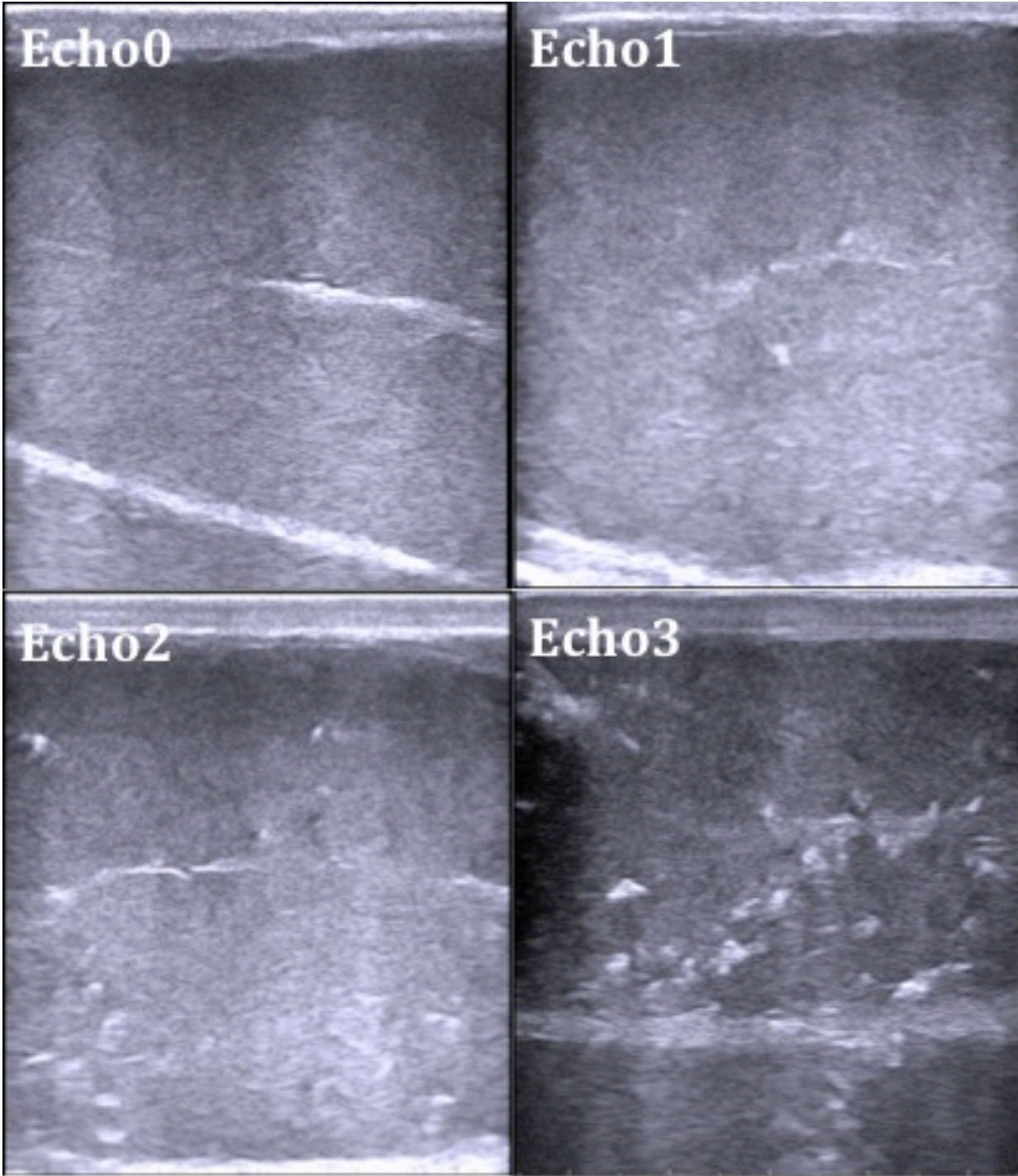
examination, examination of the reproductive organs (external exam, palpation and testicular dimensions) and semen evaluation (Kimberling and Parson, 2007; Ley et al., 1990; Ruttle and Southward, 1988). We are aware of few reports (Ahmad et al, 1991; Gouletsou and Fthenakins, 2010; Gouletsou et al, 2003) that reported the importance of ultrasound imaging in ram BSE. In particular Gouletsou proposed scanning techniques for ultrasound of ram genitalia and described the normal ultrasonographic appearance of these organs (Gouletsou et al, 2003). Testicular fine needle aspiration cytology (TFNAC) has been proposed in the BSE of bulls (Chapwanya et al, 2008), while no report are available on its use in ram, except for a work reporting the normal testicular cytology composition in healthy rams (Vencato et al, 2013) with no mention to its relation with fertility or semen quality. The aims of this study were to study the correlation of TFNAC and ultrasonography examination with semen quality collected either by electroejaculation or from epididymis and to assess if these techniques can be useful in BSE of rams.

5.3 Materials and methods

Eighteen healthy rams of four different breeds (N=5 Alpagota; N=5 Brogna; N=4 Foza; N=4 Lamon) of age ranging from 5 to 7 years and with proven fertility were considered in this study. Animals were housed in a group pen and fed with ad libitum hay and supplementation of commercial concentrate (Compli Sheep®, TECNOZOO). The study was conducted during the 2012-breeding season. A general health examination and clinical examination of the genitalia were performed in the rams, as previously described (Fthenakis et al, 2001), restraining rams in standing position. Ultrasonographic imaging of the testicles was carried out using an ultrasound scanner (MyLabVet™ One, ESAOTE S.P.A., Genoa, Italy) fitted with a multiple frequency (6.0, 8.0, 10.0 MHz) linear transducer. Different frequencies were tested in order to define the

one providing higher quality images, and 10 MHz frequency was used for the entire work. Animals were restrained using a neck lock system, with an operator lifting the tail; no sedation was required. The testicles were pulled downwards within the scrotum and the operator hold each testicle at the opposite site of that where the transducer was applied on. Sagittal and transverse sections of the testicles were taken as previously described (Gouletsou et al, 2003). Testicular parenchyma appearance was classified using a 0 to 3 score scale (Figure 5.1). Score 0 (Echo0): testes with a homogeneous parenchyma with a coarse medium echo-pattern except for the mediastinum testis; score 1 (Echo1): testes with a less homogeneous parenchyma; score 2 (Echo2): testes with highly echogenic structure in relation to the surrounding tissues occupying less than 10% of the parenchyma; score 3 (Echo3): diffuse highly echogenic structure in the parenchyma.

Figure 5.1 Testis ultrasonography appearance classes in rams.



Testicular Fine Needle Aspiration Cytology (TFNAC) was performed in both testicles of all rams as described in previous works in ram and other species (Romagnoli et al, 2009; Stelletta et al, 2011; Vencato et al, 2013) using a 21G butterfly needle connected to a 20 cc empty syringe. Each testicle was held between two fingers and the needle was

inserted in central position avoiding the superficial vessels. During aspiration, the needle was redirected through different plans in order to explore more areas of the parenchyma. The aspirated materials was then distributed in three slides and smeared. Slides were stained using a modified May-Grünwald-Giemsa staining (Sigma-Aldrich) as described by Vencato (Vencato et al, 2013). Slides were immersed for 4 minutes in May-Grünwald, then rinse in distilled water, then were immersed for 20 minutes in a Giemsa solution containin Giemsa and distilled water in ratio 1:20, and finally slides were rinse in distilled water. TFNAC slides were evaluated under an optical microscope, Nikon ECLIPSE E600, with a 1000x magnification, counting at least 200 cells per slide. Firstly a descriptive analysis of cell morphology was made, dividing Spermatogonia, Primary Spermatocyte or Spermatocyte I, Secondary spermatocyte or Spermatocyte II, AB spermatids or “early” spermatids, CD spermatids or “late” spermatids, Spermatozoa and Sertoli cells. Then relative percentages of each cells type were determined. Also, the following indexes were calculated: SI or the percentage of spermatozoa on the total of spermatogenic cells and Sertoli Index (SEI) or the percentage of Sertoli cells on the total of spermatogenic cells, considering the total of spermatogenic cells the sum of spermatogonia, spermatocytes and spermatids. Meiotic indexes in the four classes were also calculated as number of spermatids on the number of primary spermatocytes.

Semen was collected using an electroejaculator (Ruakura Ram Probe Plastic Products, Hamilton, New Zealand). To collect semen, rams were restrained as previously described, feces were removed manually from the rectum and 5 ml of lidocaine 2% were introduced in the rectum. Probe was inserted into the rectum until electrodes were localized above the upper portion of ampullary region. The electrical stimulation was applied for 3 seconds and then stopped for 1 seconds. This cycle was repeated until ejaculate was obtained (usually 6-7 electrostimulations). Semen was collected inside a

graduated tube and then was placed in a warm bath (35°C) and evaluated immediately for concentration, wave motion and total sperm. Ejaculates were diluted using Tryladil (Minitube International, Tiefenbach, Germany) supplemented with 20% egg yolk and heated at 35°C, to obtain a final concentration of 40×10^6 spermatozoa/ml. The samples were equilibrated for 2 hours, during which the diluted semen was packaged in 0.25 ml straws. These were kept 5 cm above liquid nitrogen for 10 minutes and then plunged into the nitrogen (-196.8°C). Semen was collected twice with 7 days interval.

Seven days after the second collection animals were slaughtered. Testicles were removed from the carcass, immediately placed in 5°C saline solution, as reported by Lone (Lone et al, 2011) and bring to the lab within 1 hour. The cauda epididymis was excised from the testicle and several longitudinal incisions were made to expose the spermatozoa to the outer environment. Each cauda epididymis was washed with 3 ml of Tryladil supplemented with 20% egg yolk. Concentration and progressive motility were evaluated. Samples showing $\geq 60\%$ motility were extended to reach final concentration of 40×10^6 spermatozoa/mL. Semen was cryopreserved as described previously. Following thawing semen was analyzed using a CASA with a Hamilton Thorne Bioscience device. The parameters considered were: motility, progressive motility, average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), straightness (STR), linearity (LIN), elongation (ELONG) and area. Number of 0.25 mL straws containing 10×10^6 spermatozoa was also calculated. Mean values and standard deviations of all parameters taking into account were calculated.

One-Way ANOVA was performed using the GLM procedure of the software SIGMASTAT 2.03 in order to identify the predicting values of echogenicity score on TFNAC, total sperm, straws production, motility and progressive motility. Pearson correlation

indexes were calculated using the software SIGMASTAT 2.03 to establish correlation between ultrasonography imaging, testicular cytology and semen quality.

5.4 Results

No problems were recorded during the clinical examination of the rams into the study. Furthermore, no abnormalities were detected in their genitalia. Median body condition score recorded was 3.5 with a range of (2.75 to 3.75) considering a score from 0 to 5 (Rankins and Pugh, 2012). Scrotal circumference in all subjects was higher than 33 cm, satisfying the requirement normally considered in BSE (Van Metre et al, 2012).

Ultrasonographic imaging was obtained using 10 Mhz frequency and 60 mm scanning depth. To standardize the exam, the images were acquired when the mediastinum testis was evident. The classification of parenchyma was as follow: 27.8 % (5/18) of animals had echogenicity score of 0 (Echo0), 33.3% (6/18) had score of 1 (Echo1), 27.8 % (5/18) score of 2 (Echo2) and 11.1% (2/18) had a score of 3 (Echo3).

TFNAC was made successfully in all rams and mean results are reported in table 5.1.

Table 5.1 Testicular fine-needle aspiration cytology in rams (Mean ± SD)

	Mean ± SD
Spermatogonia (%)	10.38 ± 12.85
Spermatocytes I (%)	11.30 ± 3.63
Spermatocytes II (%)	1.58 ± 0.62
AB Spermatids (%)	15.24 ± 4.24
CD Spermatids (%)	17.08 ± 2.97
SI	44.40 ± 8.72
SEI	8.68 ± 5.14

SI= Spermatic Index; SEI= Sertoli Index

Sperm collection was achieved both by electroejaculation and by recovery from the epididymis. The post-thaw characteristics of both types of sperm are given in table 5.2.

No statistical differences were observed between classes

Table 5.2 Semen analysis results depending on collection method (mean \pm SD)

	Electroejaculation	Epididymal Spermatozoa
Motility (%)	44.30 \pm 21.95	63.60 \pm 21.01
Progressive motility (%)	15.18 \pm 6.51	20.47 \pm 5.95
VAP ($\mu\text{m}/\text{sec}$)	81.69 \pm 15.45	95.45 \pm 24.34
VSL ($\mu\text{m}/\text{sec}$)	59.11 \pm 8.60	65.38 \pm 13.27
VCL ($\mu\text{m}/\text{sec}$)	128.83 \pm 29.63	159.07 \pm 44.74
ALH (μm)	15.46 \pm 1.63	16.73 \pm 1.90
BCF (Hz)	15.58 \pm 0.64	15.32 \pm 1.03
STR (%)	73.78 \pm 4.69	69.54 \pm 4.73
LIN (%)	50.00 \pm 5.65	45.24 \pm 4.72
ELONG (%)	42.03 \pm 4.24	44.65 \pm 4.61
AREA (μm^2)	71.54 \pm 56.73	211.50 \pm 182.90

VAP: average path velocity; VSL: straight-line velocity; VCL: curvilinear velocity; ALH: amplitude of lateral head displacement; BCF: beat cross frequency; STR: straightness; LIN: linearity; ELONG: elongation

Table 5.3 shows the results for TFNAC, total sperm, semen straw production, sperm motility and progressive motility for each echogenicity score class.

Table 5.3 Testicular fine-needle aspiration cytology, total sperm per ejaculate, semen straws production, motile and progressive motile spermatozoa in rams classified by testicular echogenicity score (mean \pm SD)

Echogenicity score	Echo0	Echo1	Echo2	Echo3
Spermatogonia (%)	6.24 \pm 2.04	6.04 \pm 1.01	10.1 \pm 6.78	7.15 \pm 0.31
Spermatocyte I (%)	10.2 \pm 3.58	12.7 \pm 1.99	13.0 \pm 3.61	13.4 \pm 3.68
Spermatocyte II (%)	1.74 \pm 0.48	1.77 \pm 0.48	1.85 \pm 1.02	1.50 \pm 0.83
AB spermatid (%)	16.4 \pm 3.64	16.0 \pm 2.95	15.9 \pm 6.32	14.6 \pm 3.72
CD spermatid (%)	17.0 \pm 3.21	18.2 \pm 0.78	15.7 \pm 2.98	17.5 \pm 3.60
SI	49.8 \pm 7.45 ^a	45.9 \pm 3.97 ^{ab}	45.3 \pm 5.24 ^{ab}	38.6 \pm 1.20 ^b
SEI	11.9 \pm 4.16	5.08 \pm 1.33	8.30 \pm 6.95	8.62 \pm 4.67
Total sperm EE (x10⁶)	688.2 \pm 252.7 ^a	351.3 \pm 87.1 ^{ab}	364.3 \pm 119.8 ^{ab}	220.5 \pm 223.6 ^b
Straws electroejaculation	65.8 \pm 29.0 ^a	34.4 \pm 9.13 ^{ab}	36.2 \pm 12.1 ^{ab}	21.0 \pm 24.3 ^b
Motility EE	49.0 \pm 15.7	41.6 \pm 15.8	42.5 \pm 20.4	38.4 \pm 13.9
Progressive motility EE	16.4 \pm 6.8	13.0 \pm 6.8	16.8 \pm 11.0	14.8 \pm 7.8
Total Sperm EPI (x10⁶)	1103.2 \pm 47.3 ^a	1072.9 \pm 64.2 ^a	851.7 \pm 212.0 ^a	366.2 \pm 19.8 ^b
Straws epididymal	108.8 \pm 4.97 ^a	105.0 \pm 6.68 ^a	80.3 \pm 22.8 ^a	33.5 \pm 2.12 ^b
Motility EPI	62.5 \pm 21.3	73.9 \pm 11.9	57.0 \pm 18.4	59.0 \pm 43.1
Progressive motility EPI	20.6 \pm 6,28	23.3 \pm 8.86	11.5 \pm 5.42	19.0 \pm 10.6

Within a row, mean values with different postscripts (a,b) are significantly different ($P < 0.05$); EE: Electroejaculation; EPI: epididymal sperm; SI: spermatid index; SEI: Sertoli index

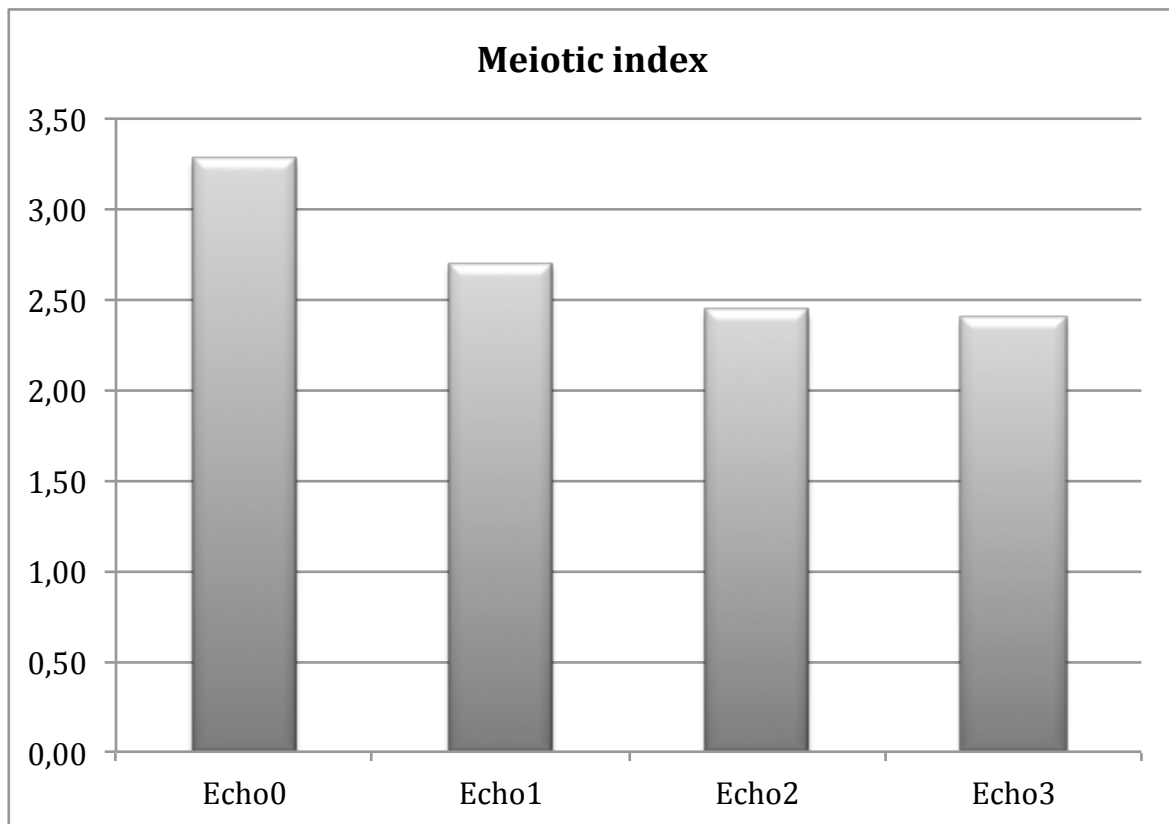
The mean values for the percentages of spermatogonia, spermatocyte I, spermatocyte II, AB spermatids and CD spermatids were not significantly different between the four

echogenicity score classes. The SI declined from Echo0 (49.8 ± 7.45) to Echo3 (38.6 ± 13.9) class with values of 45.9 ± 3.97 for Echo1 and 45.3 ± 5.24 for Echo2. The difference between Echo0 and Echo3 was significant ($P = 0.031$). The SEI showed some differences between the four classes (no significant).

The total sperm and number of straws produced following electroejaculation was higher for Echo0 class rams ($688.2 \pm 252.7 \times 10^6$; 65.8 ± 29.0) than from Echo1 ($351.3 \pm 87.1 \times 10^6$; 34.4 ± 9.13), Echo2 ($364.3 \pm 119.8 \times 10^6$; 36.2 ± 12.1) and Echo3 ($220.5 \pm 223.6 \times 10^6$; 21.0 ± 24.3) class rams. The difference in total sperm and number of straws between Echo0 and Echo3 was significant ($P = 0.042$ and $P = 0.047$ respectively; Table 5.3). Total sperm and straw production from epididymal spermatozoa was higher from rams in class Echo0 ($1103.2 \pm 47.3 \times 10^6$; 108.8 ± 4.97), Echo1 ($1072.9 \pm 64.2 \times 10^6$; 105.0 ± 6.68) and Echo2 ($851.7 \pm 212.0 \times 10^6$; 80.3 ± 22.8) than in class Echo3 ($366.2 \pm 19.8 \times 10^6$; 33.5 ± 2.12 ; $P = 0.037$; $P = 0.033$; $P = 0.045$; $P = 0.041$; $P = 0.045$ and $P = 0.048$ respectively). The differences between the echogenicity classes for sperm motility and progressive sperm motility (Table 5.3) were not significant for both types of collections. Pearson correlation analysis highlighted some significant correlations between different parameters. In TFNAC examination, the percentage of spermatogonia was negatively correlated to percentage of AB spermatids (-0.667 ; $P=0.002$), percentage of CD spermatids (-0.582 ; $P=0.011$) and SI (-0.685 ; $P=0.002$), while the percentage of primary spermatocytes and secondary spermatocytes were positively correlated to the percentage of AB spermatids (0.633 ; $P=0.002$ and 0.507 ; $P=0.03$ respectively). A positive correlation was observed between the percentage of secondary spermatocytes, total sperm (0.721 ; $P = 0.01$) and the number of straws obtained by electroejaculation (0.703 ; $P=0.007$). The relationship between SI and the number of straws produced from an ejaculation almost achieved statistical significance (0.656 ; $P=0.055$). The total sperm

and the number of straws obtained from epididymal spermatozoa were negatively correlated with the percentage of spermatogonia (-0.698 and -0.746, $P < 0.001$) and positively to the SI (0.612 and 0.667; $P < 0.01$) while there was an indication of a relationship between the number of straws and the percentage of primary spermatocytes (0.418; $P = 0.095$). No correlations were found between TFNAC results and spermatozoa kinetic parameters, with the exception of a positive correlation between the percentage of secondary spermatozoa and ALH of epididymal spermatozoa (0.580, $P = 0.048$) and a negative correlation of SI with ALH of epididymal spermatozoa (-0.606; $P = 0.037$). Meiotic indexes were calculated for each class of ultrasonographic appearance. As shown in figure 5.1, meiotic index seems to decrease with the worsening of the testicular appearance, but only Echo0 class differed statistically from Echo3 class ($P < 0.05$). On the base of these results, ultrasonography appearance of the testis seems to be related to spermatogenesis efficiency in rams.

Figure 5.1 Meiotic index in the different classes of ultrasonographic appearance of the testis



5.5 Discussion

The identification of rams with high potential for fertility by clinical examination of genitalia, observing their mating behavior and evaluation of semen is commonly performed. Although such procedures allow the identification of low fertility rams, they do not provide an indication of the different levels of fertility exhibited by those rams that pass this test. In this study we considered animals that were identified as suitable for breeding and already successfully used in breeding campaigns. In our study we found that animals having good fertility, following the common examination scheme, showed considerable differences in terms of semen quality as showed by the high standard deviations reported in table 5.1. Ultrasonographic imaging and TFNAC were

techniques easily applicable, minimally invasive, highly reproducible and economically sustainable. Ultrasound imaging was useful in identifying rams with condition of testicular degeneration (such as calcification) and the use of an echogenicity score, allowed to divide animals in 4 groups on the base of the appearance of the testis. The predicting value of this classification on the sperm production was satisfactory. Rams in the echogenicity score class Echo0 produced significantly more total sperm and straws both following electroejaculation and epididymal spermatozoa collection than those in class Echo3. Similarly, the rams in Echo1 and Echo2 produced more total sperm and straws than those in Echo3 in both methods of semen collection but the differences were significant only for the epididymal sperm collection. Motility and progressive motility were not significantly different between groups. However, motility EE (Table 5.3) of sperm shows a systematic decline from Echo0 to Echo3 suggesting that this aspect should be investigated in future.

This is the second study that reported the use of TFNAC in rams. All spermatogenic cells were identifiable on smears, and in all rams it was possible to recover sufficient tissue for evaluation. Vencato, performing TFNAC in 1 year-old rams, reported results that seem to be partially different from those obtained in this study (Vencato et al, 2013). Although the percentage of primary spermatocytes, secondary spermatocytes, AB spermatids, CD spermatids and SI seem to be similar, we observed a higher percentage of spermatogonia and a lower SEI. These differences may be related to the different age of the rams considered. On the other hand, the values reported in this work seem to be similar to those described for TFNAC in other species, such as dog (Santos et al, 2010) and human (Foresta, 1993). Analyzing the TFNAC results on the base of the echogenicity groups we observed that SI was significantly higher in Echo0 than Echo3. As Echo0 had also the higher production in term of total sperm and straws, it seems to suggest a

predicting value of SI on sperm production. The decreased of meiotic index from Echo0 rams to Echo3 rams seems to suggest that spermatogenesis efficiency is higher in rams having a better testicular appearance.

The determination of Pearson correlation indexes revealed the existence of correlations between some parameters of TFNAC and semen quality, although differences were found between electrojaculation and epididymal spermatozoa collection. SI was positive correlated to total sperm and the number of straws obtained from epididymal spermatozoa collection while a positive tendency was observed with straws obtained from electroejaculation. These findings confirm the importance of SI, which has been described as a measure of the efficiency of testis in producing spermatozoa in human (Foresta, 1993). Other correlations suggest that the increased concentration of early spermatogenic cells, such as the spermatogonia may be related to a reduced spermatogenesis, while the increased concentration of late spermatogenic cells, such as secondary spermatocytes, that are the last $2n$ cell population during spermatogenesis (Hess and Renato de Franca, 2008), may suggest that more cells are undergoing the process of spermatogenesis.

We are aware of only one work that reported results of semen analysis, TFNAC and trans-scrotal ultrasonography (TSUS) from another specie (bulls). In this work, all bulls that were classified as unsuitable for breeding on the basis of TFNAC or TSUS subsequently failed to pass semen analysis, while some of those that were classified suitable subsequently failed to pass semen analysis (Chapwanya et al, 2008). These data seem to suggest the importance of combining the three techniques to achieve an accurate identification of fertile animals. In seasonal breeders, such as the ram, it is even more important to combine different techniques, and in particular TSUS because it

allows to evaluate ram during the non breeding season, in a period where the correct interpretation of semen analysis is difficult.

5.6 Conclusions

TSUS and TFNAC are innovative techniques easily applicable and reproducible in rams and their results can give some indication on the fertility of a ram. This suggests that the integration of testicular ultrasound imaging and TFNAC with the classical analysis can enhance the accuracy of BSE in rams.

6 - INFLUENCE OF OVARIAN ACTIVITY AND FOLLICULAR DEVELOPMENTAL STAGE ON THE BIOCHEMICAL COMPOSITION OF FOLLICULAR FLUID IN SOUTH AMERICAN CAMELIDS

6.1 Abstract

Aim of the study was the characterization of the biochemical composition of follicular fluid in South American camelids at different stages of ovarian activity and follicular development. In the experiment 1 the influence of corpus luteum and follicular dimension on biochemical composition and electrophoretic profiles of alpaca follicular fluid were investigated. Small differences were observed between follicular and luteal phase, while large and small follicles differed for K, P, Glucose, Triglycerides, GPT, CK and proteins of different weights (250 KDa, 200 Kda, 120 Kda, 90 Kda, 75 Kda, 60 Kda, 50 Kda and 37 Kda). In experiment 2 we collected follicular fluid from follicles at three stages of development (growing, static and regressing) in lama. The electrophoresis analysis revealed differences among groups in 4 classes of weight (250 Kda, 75 Kda, 25 Kda, 15 Kda). These results offer new information concerning the biochemical composition of follicular fluid in South American camelids, suggesting that oocytes grow and mature in an environment that changes in function of the follicle stage, while is minimally influenced by the presence of the corpus luteum.

6.2 Introduction

Follicular fluid (FF) is in part a trasudate of serum and is also partially composed of locally produced substances related to the metabolic activity of follicular cells (Albomohsen et al, 2011; Tabatabaei et al, 2011). Metabolic activity and blood-follicle

barrier properties change during the different stages of follicular development; hence, a different composition of the FF in different-sized follicles can be expected (Albomohsen et al, 2011; Khan et al, 2011a; Leroy et al, 2004; Nandi et al, 2007; Tabatabaei et al, 2011). FF has different oocyte-related functions: maintenance of meiotic arrest, protection against proteolysis, extrusion during ovulation, enhancement of spermatozoa attraction, motility and acrosome reaction (Orsi et al, 2005). Changes in FF during estrous cycle influence steroidogenesis, oocyte maturation, ovulation and transport of oocyte to the oviduct as well as the preparation of the follicle for corpus luteum formation (Bender et al, 2010; Eissa, 1996). In vitro studies showed that some metabolites, such as urea (De-Wit et al, 2001), glucose (Hashimoto et al, 2000) and β -hydroxy butyrate (Gomez, 1997) might influence the competence of bovine oocytes.

We are aware of only two works that described the FF composition in South American Camelids considering total proteins, albumin, glucose, total lipids and cholesterol (Pacheco and Coila, 2007; Pacheco and Coila, 2010).

Aim of this study was to study the composition of FF in South American Camelids and in particular the protein electrophoretic profiles of FF collected from follicles at different stages of development.

6.3 Materials and methods

6.3.1 Experiment 1: Biochemical composition and protein electrophoretic profile in different-size follicles in alpaca (*Vicugna pacos*)

Ovaries of twelve adult alpaca females were collected at the slaughterhouse of Huancavelica, Perú, and transported to the local University to be processed. Ovaries from each animal were divided in function of the presence or absence of corpus luteum in at least one of the ovaries, and the FF was collected and divided on the base of the follicles size (small follicles < 3 mm; large follicles > 3 mm) and pooled.

Biochemical analysis was performed using an automatic biochemistry analyzer BT1500, Biotechnica Instruments SpA, Rome, Italy.

One-dimensional Sodium dodecyl sulfate polyacrylamide gel electrophoresis (1D-SDSPAGE) was performed to identify and quantify protein profiles. 1D-SDSPAGE was performed with 7% and 15% polyacrylamide in the gel of separation according to Laemmli (Laemmli, 1970). Samples were diluted 1:20 in sample buffer and a total of 50 µg protein was used for each sample. Samples were boiled in water bath at 100°C for 5 minutes for protein denaturation. Precision plus protein standard (Bio-Rad) was used to identify different protein bands. Electrophoresis was conducted at 75V for 1.5 hours with PowerPac Basic Power Supply (Bio-Rad). Gels were stained with Comassie Brilliant Blue and gel images were processed with Sante Dicom Viewer Image J program. Gel bands density was measured and analyzed to get relative protein concentration. Analysis of data considered the mean of all samples; FF divided in function of presence/absence of corpus luteum (lutealFF and follFF); divided in function of follicular size (smallFF and largeFF).

6.3.2 Experiment 2: Protein electrophoretic profile of FF collected at different stages of follicle development in Lama (Lama glama)

Thirty adult lama females were used in this study. Daily transrectal ultrasonography was performed to determine the wave emergency as previously reported (Adams et al, 1990; Ratto et al, 2003; Ratto et al, 2005). FF aspiration was performed from dominant follicles at three different stages of follicular development in according with Adams (Adams et al, 1990): 1-Early growing phase at day 3-4 after wave emergency (GFF); 2-Early static phase at day 10-11 after wave emergency (StFF); 3-Early regression phase at day 15-16 after wave emergency (RFF). Females have been allocated randomly to the groups

FF was transvaginal aspirated by using a 900 Aloka ultrasound machine and a 5 MHz transvaginal probe equipped with a needle guide. Aspiration has been carried out with an 18G x 1.5 inches needle attached to a 6 cc syringe by a 30 cm silicon tube. After aspiration, FF was deposited in a 5 ml tubes and centrifuged to remove all cells.

1D-SDSPAGE was performed as previously described but processing an unknown quantity of protein and using 14% polyacrylamide in the gel of separation.

6.3.3 Statistical analysis

One-way ANOVA was performed using the GLM procedure of the software SIGMASTAT 2.03 in order to identify differences in protein electrophoretic profile of FF among groups.

6.4 Results

6.4.1 Experiment 1

Biochemical analysis results are reported in Table 6.1. No differences were found between the luteal FF and the follicular FF groups. Potassium, phosphorus, GPT and CK were significantly higher ($P < 0.05$) in smallFF compared to largeFF. Differently, glucose and triglycerides concentrations were significantly lower ($P < 0.05$) in smallFF than largeFF.

Table 6.1 Biochemical analysis of follicular fluids in alpaca (mean \pm SD

	Mean	lutealFF	folIFF	smallFF	largeFF
Creatinine mg/dl	1.48 \pm 0.17	1.41 \pm 0.20	1.56 \pm 0.02	1.59 \pm 0.01	1.32 \pm 0.17
Bilirubin mg/dl	0.75 \pm 0.95	1.22 \pm 1.09	0.19 \pm 0.05	0.22 \pm 0.01	1.56 \pm 1.10
Urea mg/dl	43.91 \pm 2.80	41.60 \pm 1.52	46.62 \pm 0.50	44.79 \pm 2.02	42.18 \pm 2.95
Cl mmol/l	119.91 \pm 4.33	121.13 \pm 5.58	118.62 \pm 0.50	117.34 \pm 1.52	123.73 \pm 4.43
K mmol/l	15.24 \pm 4.52	15.17 \pm 5.73	15.28 \pm 2.58	19.01 \pm 1.61**	10.21 \pm 1.44**
Na mmol/l	147.03 \pm 3.43	151 \pm 0.00	144.14 \pm 1.49	143.00 \pm 0.00	149.18 \pm 2.46
P mg/dl	10.75 \pm 1.95	10.41 \pm 2.49	11.15 \pm 0.85	12.30 \pm 0.46**	8.64 \pm 1.13**
Mg mg/dl	3.39 \pm 0.63	3.79 \pm 0.59	2.91 \pm 0.16	3.15 \pm 0.11	3.79 \pm 0.84
Ca mg/dl	8.26 \pm 0.57	8.30 \pm 0.76	8.21 \pm 0.15	7.82 \pm 0.25	8.85 \pm 0.34
Glucose mg/dl	76.87 \pm 10.74	75.20 \pm 12.18	79.48 \pm 8.46	68.03 \pm 4.56**	88.73 \pm 0.98**
Cholesterol mg/dl	35.30 \pm 15.54	24.72 \pm 5.10	48.60 \pm 14.93	37.94 \pm 20.26	30.00 \pm 0.00
Triglycerides mg/dl	6.93 \pm 2.59	6.33 \pm 2.54	7.90 \pm 2.49	4.90 \pm 1.01**	9.73 \pm 0.98**
Protein g/dl	6.16 \pm 0.63	5.85 \pm 0.71	6.54 \pm 0.05	6.50 \pm 0.00	5.65 \pm 0.74
Albumin g/dl	3.32 \pm 0.43	3.08 \pm 0.46	3.64 \pm 0.05	3.54 \pm 0.05	3.00 \pm 0.54
Globulin g/dl	2.82 \pm 0.21	2.77 \pm 0.25	2.84 \pm 0.05	2.91 \pm 0.10	2.65 \pm 0.20
GGT u/l	28.78 \pm 3.41	31.47 \pm 0.95	25.49 \pm 2.35	29.12 \pm 1.68	28.87 \pm 4.87
GOT-AST u/l	530.60 \pm 103.32	536.87 \pm 134.97	531.62 \pm 42.30	617.52 \pm 49.09	425.55 \pm 41.36
GPT-ALT u/l	20.91 \pm 3.71	21.24 \pm 4.77	20.39 \pm 1.71	23.87 \pm 1.99**	16.97 \pm 1.00**
CK u/l	781.90 \pm 483.56	311 \pm 0.00	1018.09 \pm 410.03	1332 \pm 0.00**	382.64 \pm 96.99**
LDH u/l	3125.23 \pm 1773.02	1631 \pm 0.00	4403.38 \pm 1493.84	5547 \pm 0.00	1963.36 \pm 450.02

** Indicate a statistical difference (P<0.05) between smallFF and largeFF groups.

lutealFF: follicular fluid from animals in luteal phase; folIFF: follicular fluid from animals in follicular phase; smallFF: follicular fluid from follicles <3 mm; largeFF: follicular fluid from follicles >3mm

Using 1D-SDSPAGE fifteen bands of proteins were identified: 250 KDa, 200 KDa, 150 KDa, 130 KDa, 120 KDa, 90 KDa, 75 KDa, 65 KDa, 60 KDa, 50 KDa, 50 KDa, 45 KDa, 37 KDa, 25 KDa, 18 KDa and 15 KDa. The results are reported in Table 6.2.

Table 6.2 Relative percentages of proteins in follicular fluids of alpaca (mean±SD)

Molecular weight	Mean	lutealFF	folFF	smallFF	largeFF
250KDa	2.61±0.53	2.61±0.57	2.61±0.72	3.30±0.40**	2.19±0.08**
200KDa	0.44±0.15	0.43±0.17	0.45±0.19	0.38±0.05**	0.46±0.01**
150KDa	15.26±0.09	15.15±0.98	16.34±0.32	15.51±0.82	15.42±1.07
130KDa	1.68±0.28	1.51±0.17	1.85±0.30	1.59±0.19	1.23±1.09
120KDa	1.36±0.22	1.30±0.23	1.41±0.27	1.17±0.04**	1.52±0.07**
90KDa	0.80±0.17	0.82±0.26	0.79±0.12	0.57±0.17**	0.92±0.07**
75 KDa	10.74±0.38	10.53±0.20*	10.95±0.46*	9.89±1.69**	11.06±0.70**
65KDa	3.16±1.70	2.76±1.60	3.57±2.34	1.62±0.31	3.04±2.71
60KDa	3.65±0.62	3.55±0.73	3.75±0.77	3.30±0.32**	4.35±0.32**
50KDa	3.91±0.29	4.00±0.30	3.82±0.37	3.50±0.33**	4.22±0.13**
45KDa	1.64±0.15	1.61±0.17	1.68±0.19	1.73±0.21	1.39±0.44
37KDa	7.19±1.05	7.85±0.74*	6.54±1.01*	8.66±0.58**	7.64±0.99**
25KDa	5.79±1.53	6.28±0.94	5.31±2.28	7.36±0.75	5.25±1.40
18KDa	0.24±0.11	0.17±0.13	0.30±0.14	0.28±0.11	0.20±0.11
15KDa	1.33±0.45	1.03±0.39	1.64±0.65	1.58±0.45	1.11±0.32

* Indicates a statistical difference (P<0.05) between lutealFF and folFF groups; ** indicates a statistical difference (P<0.05) between smallFF and largeFF groups. lutealFF: follicular fluid from animals in luteal phase; folFF: follicular fluid from animals in follicular phase; smallFF: follicular fluid from follicles <3 mm; largeFF: follicular fluid from follicles >3mm

LutealFF and follFF differ only for the concentration of 75KDa proteins ($P<0.05$) that were significantly higher during the follicular phase. Differences between smallFF and largeFF were observed in 250 KDa, 200KDa, 120 KDa, 90 KDa, 75 KDa, 60 KDa, 50 KDa, 37 KDa ($P<0.05$).

6.4.2 Experiment 2

Among the thirty females considered, two animals have been excluded because of formation of cyst and rupture of the follicle during the collection. The remaining 28 animals were randomly allocated to each class for a total of 9 animals per class. Mean follicular dimensions at the time of FF collection were 7.61 ± 0.74 , 12.89 ± 1.59 , 12.11 ± 2.26 cm in growing, early static and early regressing follicles respectively. Ten molecular weight bands were analyzed in 1D-SDSPAGE images: 250 KDa, 200 KDa, 150 KDa, 100 KDa, 75 KDa, 60 KDa, 45 KDa, 37 KDa, 25 KDa, 15 KDa in addition to the albumin band that is the most present protein. As reported in table 6.3, differences in relative percentage of proteins were observed at 250 KDa, 75 KDa, 25 KDa and 15 KDa molecular weights.

Table 6.3 Relative percentages of proteins in follicular fluids of llama in different follicular developmental stages (mean \pm SD)

Molecular weight	GFF	StFF	RFF
250 KDa	3.21 \pm 0.58 ^a	4.97 \pm 0.48 ^b	5.68 \pm 0.90 ^b
200 KDa	4.43 \pm 1.45	5.58 \pm 1.85	5.85 \pm 2.13
150 KDa	6.80 \pm 1.75	5.91 \pm 3.62	6.48 \pm 0.61
100 KDa	23.37 \pm 10.17	24.06 \pm 10.46	26.64 \pm 6.48
75 KDa	14.96 \pm 1.54 ^b	11.12 \pm 1.47 ^a	14.12 \pm 1.25 ^o
60 KDa	4.34 \pm 1.31	6.41 \pm 4.34	4.17 \pm 1.47
45 KDa	8.11 \pm 3.52	8.15 \pm 4.14	8.10 \pm 5.28
37 KDa	6.57 \pm 1.15	5.89 \pm 4.04	5.52 \pm 2.19
25 KDa	0.62 \pm 0.55 ^{ab}	1.37 \pm 0.64 ^b	0.25 \pm 0.56 ^a
15 KDa	2.25 \pm 0.73 ^b	1.93 \pm 0.34 ^b	0.47 \pm 0.76 ^a

Different letters in different columns (a,b,c) indicate a statistical difference among groups ($P < 0.05$); GFF: follicular fluid of growing follicles; StFF: follicular fluid of early static follicles; RFF: follicular fluid of early regressing follicles

6.5 Discussion

The composition of FF differs in relation to the different stages of follicles development in different species (Albomohsen et al, 2011; Grimec et al, 1984; Homa and Brown, 1992; Khan et al, 2011a; Khan et al, 2011b; Leroy et al, 2004; Nandi et al, 2007; Schweigert and Schams, 1993; Tabatabaei et al, 2011). Such variations have been demonstrated also in FF of alpaca (Pacheco and Coila, 2007; Pacheco and Coila, 2010) but considering only few parameters (total protein, albumin, glucose, total lipids and cholesterol).

In this study we reported for the first time the complete biochemical composition and protein profiles.

Mean results of biochemical composition of alpaca FF are comparable to the results reported in other species (Albohomsen et al, 2011; Nandi et al, 2007; Tabatabaei et al, 2011). Total protein, albumin and cholesterol seems to be slightly lower, while glucose is consistently lower than those reported from Pacheco (Pacheco and Coila, 2010). No differences were observed between FF collected during the follicular or the luteal phase of the cycle. This finding seems to suggest that the presence of the corpus luteum has no influence on the FF biochemical composition in the follicles present at the same time. Our results are in disagreement with Eissa (Eissa, 1996) that reported in buffalo FF a higher concentration of Ca, P, glucose and total protein during the proestrus and oestrus stages compared to metoestrus and dioestrus. The presence of corpus has also limited influence on the proteins composition. 1D-SDSPAGE revealed that only the 75 KDa and the 37 KDa bands differed between lutealFF and follFF.

It has been reported that the concentration of trace elements and biochemical metabolites composition in the FF fluctuate with the follicle size in different species (Albohomsen et al, 2011; Grimec et al, 1984; Kor et al, 2013; Leroy et al, 2004; Nandi et al, 2007, Tabatabaei et al, 2011). In our experiment 1 we considered two groups of size dimensions, the considered small follicles (diameter < 3 mm) included all the emerging follicles that have not passed through the selection phase, while in the large follicles (diameter > 3 mm) are included all follicles that have been selected for dominance but that could be in any of the stage of development (growth, static, regression). In smallFF we observed a higher concentration of K, triglycerides and a lower concentration of glucose in comparison to the largeFF as it has already been reported in other species (Albohomsen et al, 2011; Leroy et al, 2004; Nandi et al. 2007). The higher concentration

of glucose in the large follicle could have different explanation: a less intensive glucose metabolism in large follicles; a relative larger volume of FF in comparison to the amount of granulosa cells that consumes glucose for their metabolism; an increased permeability of the blood-follicle barrier during follicular growth (Leroy et al, 2004). The difference in the concentration of triglycerides may be explain by the fact that triglycerides probably do not pass through the follicular membrane because they are transported by the Very Low-Density Lipoproteins, that are too large to pass the blood-follicle barrier (Grummer and Carrol, 1988). The origin of triglycerides in the FF may be local and a dilution effect could be observed at the increasing of follicle size (Leroy et al, 2004). The decreased concentration of K in FF with follicular development could be related to the use of glucose during the developmental process that leads to the transfer of K from extracellular sites to the intracellular sites (Tabatabaei et al, 2011). The P levels decreased with the increasing of follicles size in agreement to earlier reports in goat (Mishara et al, 2003) and cattle (Tabatabaei et al, 2011). Moreover a reduction of GPT and CK concentrations was observed with the increasing of the size of the follicles. This is the first report of electrophoretic profile of proteins in FF of South American camelids In experiment 1, 1D-SDSPAGE revealed some differences between smallFF and largeFF involving eight of the fifteen classes of weight identified. In particular, the 200 KDa, 120 KDa, 90 KDa, 75 KDa, 60 KDa and 50 KDa weight proteins concentrations are higher in largeFF than in smallFF. We can assume that the increment of their concentration may be related to the function of some of these proteins in the development of the oocytes.

In the experiment 2, FF was collected only from follicle with large dimensions. The scanning of the ovaries allowed us to identify the stage of follicular development. The growing follicles consist of dominant follicles that not have developed the capability to

ovulate, the early static follicles are follicles able to ovulate in case of mount or induction while the regressing follicles have passed through all the static phase and have started to become atretic.

With the 1D-SDSPAGE images analysis it was possible to identify the evolution of protein composition through the follicular development. Passing from the growing phase to the early static phase, there was an increase of 250 kDa proteins and a decrease of 75 kDa proteins in FF, while comparing RFF and StFF a lower concentration of 25 kDa and 15 kDa proteins and a higher concentration of 75 kDa proteins were observed in the regressing follicles. 1D-SDSPAGE allows the obtaining of information about the modification of classes of protein identified by molecular weight. In our study we observed a variation in some classes of follicle in relation to the developmental stage. These may suggest that different proteins may have a role in a specific moment of the follicular development. In the experiment-2 we found a different concentration of proteins of around 15 kDa. In this band are represented proteins such as Bone Morphogenetic protein 15 (BMP15) (16-17 kDa), β -Nerve Growth Factor (β NGF) (14 kDa) and leptin (16 kDa). These proteins have been studied for their effect on fertility: BMP15 was observed to be at higher levels in FF of eggs that fertilized and cleaved in comparison with the unfertilized and it is positively correlated to E levels (Wu et al, 2007); β NGF is in part responsible of the process of maturation of the oocyte (Barboni et al, 2002); leptin was positively correlated with the fertilization rate (De Placido et al, 2006). Because of their relation with fertility, we could hypothesize that these proteins may be responsible for the decreased expression of the 15 kDa band in the RFF. The 25 kDa band, that is more expressed in the StFF, contains protein such as Ig-k chain C region (25 kDa), TGF- β (25 kDa), Apolipoprotein A-1 (28 kDa), IGF2 (20kDa). Ig-k chain C region and apolipoprotein were already proposed as marker of

follicular maturity (Spitzer et al, 1996), while IGF2 was found to be correlated with oocyte fertilization, cleavage and embryo development (Wang et al, 2006). Among the proteins contained in the 37 kDa band there are Prorenin (40 kDa) which high levels were found in follicles containing immature oocyte (Revelli et al, 2009). Prorenin may be responsible for the higher expression of the 37 kDa band in the smallFF.

The 50 kDa band that is more expressed in largeFF in experiment 1, contains alpha1-antitrypsin (57-49 kDa) that has been described to be higher in FF of mature follicles, and its determination has been proposed as a marker for oocyte selection and fertilization time in-vitro (Spitzer et al, 1996). The 75 kDa band was the only that differed also between lutealFF and follFF. One of the proteins in this band is transferrin (78 kDa) that was found in higher concentration in mature follicle and has a role in promoting cell proliferation (Spitzer et al, 1996).

Despite all the hypotheses on proteins variation during the cycle, further studies are mandatory to isolate single proteins and study their variation, as well as studying their relation with fertility.

In conclusion, FF is a complex mixture containing solute derived from plasma and metabolites of follicular cells. Its composition is strongly influenced by the follicle developmental stage and their dimension, while the influence of presence/absence of corpus luteum appears minimal. Knowledge of FF composition could be useful to extrapolate the requirements for oocyte growth in vitro and may be useful for the formulation of optimized cell culture medium.

7 - BIOCHEMICAL AND FATTY ACIDS COMPOSITION OF WATER BUFFALO (BUBALUS BUBALIS) FOLLICULAR FLUID

7.1 Abstract

Aim of this study was to characterize the biochemical and fatty acids composition of follicular fluid collected from follicles of different size and in different phase of ovarian cycle in water buffalo farmed in Italy. Ovaries were collected at slaughterhouse during the breeding season; follicular fluid was collected by aspiration dividing samples in small and large follicles (< 6 mm and > 6 mm respectively) and in luteal and follicular phase. Biochemical analysis and gas-chromatography were performed. Biochemical and fatty acids composition were greatly influenced by both follicular dimension and phase of ovarian cycle. Biochemical composition and its variation was in agreement with previously study conducted in buffalo and other species. This is the first report of the fatty acids composition of buffalo follicular fluid. Twenty-two fatty acids were identified in follicular fluid; nine were saturated fatty acids, six monounsaturated fatty acids and seven polyunsaturated fatty acids. The most dominant fatty acids were linoleic acid, oleic acid, palmitic acid, stearic acid and arachidonic acid. All the identified fatty acids concentrations varies at least because of follicle dimension or phase, with the exception of γ -linoleic acid and arachidonic acid which concentrations remain stable in all classes.

7.2 Introduction

Reproductive technology in water buffalo has not been developed as it has been in cattle (Drost, 2007) and the nutrients concentrations were reported to be non-physiological in mammalian in-vitro maturation systems (Nandi et al, 2008). For this reason, knowledge on composition of follicular fluid (FF) may be useful in the selection of oocytes (Revelli

et al, 2009) and may be used as a guide for the formulation of specific media for in-vitro production (Duarte et al, 2012; Gérard et al, 2002).

FF is in part a transudate of serum and also partially composed of products of the metabolic activity of follicular cells (Albohomsen et al, 2011; Tabatabaei et al, 2011). Metabolic activity and blood-follicle barrier properties change during follicle development, influencing the composition of the FF (Khan et al, 2011a; Leroy et al, 2004; Nandi et al, 2007). FF plays a major role in autocrine and paracrine regulation and also in physiological, biochemical and metabolic aspects of nuclear and cytoplasmic maturation of oocyte and process of ovulation (Shabankareh et al, 2013). Because of his critical role in determining oocyte quality, in the last years, the studies on FF composition has been focused in establish the relation between a single component and the fate of the oocyte coming from a specific follicle (Revelli et al, 2009).

In buffalo, FF composition has been demonstrated to be influenced by the phase of the ovarian cycle (Eissa, 1996), though in another study no differences were found between FF collected in luteal and follicular phase (Baki Amar et al, 2013). Factors affecting FF composition are: reproductive acyclicity (Khan et al, 2011a; Khan et al, 2012) and above all follicular dimension. Nandi et al reported that glucose and lactate concentration in FF are affected by follicles size (Nandi et al, 2008). The influences of follicle dimension on FF composition were also described in another study for glucose, cholesterol (Khan et al, 2011a). FF composition is also affected by the cystic status of the follicles (Khan et al, 2011b). No reports are available on the fatty acids composition of FF in buffalo. Moreover, there is no information on the FF composition of buffalo farmed in intensive system in Italy.

Aim of this work is to investigate the effect of follicular dimension on the biochemical and fatty acids composition in water buffalo

7.3 Materials and methods

Ovaries were collected at a local abattoir during the month of November 2012, during the buffalo reproductive season in Italy. Ovaries were maintained at 4°C in saline solution and transported to the laboratory within 40 min.

Follicles diameter was measured using a divider compass and metric scale, and follicles were classified in small and large size (< 6 mm and > 6 mm respectively). FF was also divided on the base of the presence or absence of the corpus luteum in at least one of the ovaries of each animal. Samples were so divided in four groups: FF from small follicles of an animal in follicular phase (smallFFF); FF from large follicles of an animal in follicular phase (largeFFF); FF from small follicles of an animal in luteal phase (smallCLFF); FF from large follicles of an animal in luteal phase (largeCLFF).

FF was aspirated using a 18G needle connected to a syringe. After collection FF was centrifuged at 1500 x g for 10 min and the supernatant was stored at -80°C.

Biochemical analyses were performed using an automatic biochemistry analyzer BT1500, Biotechnica Instruments SpA, Rome, Italy. Fatty acids were measured by gas chromatography in according to Carnielli (Carnielli et al, 1996). Samples were placed in glass tubes and were treated by direct methylation using a 3N HCl-Methanol solution (Supelco, Sigma-Aldrich Group, Germany) containing 1 mg/ml of nonanoic acid and 1mg/ml of heptadecanoic acid as internal standard. After flushing with nitrogen, tubes were capped and incubated for 45 minutes at 100° C. After methylation, the solution containing the fatty acids methyl esters (FAME) was placed in an ice bath, and was supplemented with a 10 % solution of K₂CO₃ up to reach a pH of 7. A solution containing hexane (Merck) and B-idrossi-toluene was added before to centrifuge at 3000 rpm for 10 minutes. After centrifuge, 1 microl of the upper layer containing hexane and the FAME was injected into a HP 5890 Gas-Chromatography (Hewlett Packard, CA,

USA) equipped with Omegavax 30 m 0.25 mm x 0.25 microm (Supelco, Sigma-Aldrich Group, Germany). The gas chromatograph was operated with the following temperature program: 70° C initially for 3 minutes, thereafter the oven temperature was raised by 20° C/min until 205° C and held this temperature for 15 minutes. The temperature was then increased again at 0.4° C/min to 213° C and held this temperature for 10 minutes, then the temperature was increased at 5° C/min to 240° C and held for 7 minutes. Peak areas were calculated by HP-Chem station software using nonanoic acid and heptadecanoid acid as internal standard.

Data were analyzed using the GLM procedure of the software SIGMASTAT 2.03. Two-way ANOVA was performed to investigate the effects of the ovarian stage, follicles size and their interactions on the parameters considered in this study. Significant differences were considered with $P < 0.05$.

7.4 Results

A total of fifty-six follicular fluid samples were collected and divided in the four classes: smallFFF (N=16), largeFFF (N=16), smallCLFF (N=12) and largeCLFF (N=12).

Table 7.1 reported the results of biochemical analysis of the FF in the different groups.

Table 7.1 Biochemical analysis of follicular fluid collected from small (< 6 mm) and large (> 6 mm) follicles in follicular and luteal phase in water buffalo (mean \pm SD)

	smallFFF	largeFFF	smallCLFF	largeCLFF
Glucose (mg/dl)	51.0 \pm 9.31 ^b	96.1 \pm 3.87 ^d	36.3 \pm 6.33 ^a	81.9 \pm 7.89 ^c
Triglycerides (mg/dl)	27.2 \pm 1.95 ^b	15.3 \pm 1.53 ^a	32.3 \pm 4.49 ^c	17.3 \pm 2.18 ^a
Cholesterol (mg/dl)	63.6 \pm 28.9 ^b	44.0 \pm 16.2 ^a	67.2 \pm 18.0 ^{bc}	83.1 \pm 34.3 ^{cd}
Total protein (mg/l)	70.4 \pm 3.18 ^b	66.8 \pm 4.32 ^a	73.5 \pm 1.07 ^c	75.9 \pm 0.71 ^d
Albumin (mg/l)	29.5 \pm 1.41 ^a	30.9 \pm 1.02 ^b	31.1 \pm 1.14 ^c	32.4 \pm 0.11 ^d
Globulin (mg/l)	39.4 \pm 2.57 ^b	37.2 \pm 2.68 ^a	42.2 \pm 0.13 ^c	43.9 \pm 0.33 ^d
Urea (mg/dl)	45.8 \pm 9.21	48.8 \pm 11.8	47.4 \pm 4.59	48.2 \pm 4.40
Bilirubin (mg/dl)	0.42 \pm 0.08 ^b	0.33 \pm 0.09 ^a	0.38 \pm 0.03 ^{ab}	0.34 \pm 0.04 ^{ab}
Creatinine (mg/dl)	1.52 \pm 0.17 ^a	1.51 \pm 0.10 ^a	1.67 \pm 0.36 ^{ab}	1.76 \pm 0.37 ^b
Calcium (mg/dl)	9.31 \pm 0.43 ^b	8.67 \pm 0.29 ^a	9.71 \pm 0.44 ^c	9.69 \pm 0.63 ^c
Phosphorus (mg/dl)	12.4 \pm 2.14 ^b	8.61 \pm 0.82 ^a	13.3 \pm 0.50 ^b	9.04 \pm 0.02 ^a
Magnesium (mg/dl)	2.93 \pm 0.33 ^b	2.36 \pm 0.34 ^a	2.92 \pm 0.26 ^b	2.54 \pm 0.19 ^a
Chloride (mmol/l)	120.3 \pm 1.43 ^b	91.3 \pm 3.30 ^a	112.2 \pm 0.22 ^b	78.9 \pm 49.8 ^{ab}
Potassium (mmol/l)	15.5 \pm 0.26 ^d	6.8 \pm 0.07 ^a	9.8 \pm 0.09 ^c	7.5 \pm 0.43 ^b
Sodium (mmol/l)	151.8 \pm 2.67 ^{ab}	151.3 \pm 14.2 ^a	156.3 \pm 2.15 ^{ab}	158 \pm 3.07 ^b
SAP (u/l)	154.4 \pm 64.1 ^a	142.1 \pm 29.4 ^a	227.1 \pm 60.4 ^b	134.7 \pm 70.2 ^a
CK (u/l)	198.4 \pm 43.8 ^b	201.1 \pm 57.9 ^b	184.7 \pm 77.4 ^{ab}	144.7 \pm 76.4 ^a
GGT (u/l)	20.9 \pm 1.00 ^a	22.4 \pm 4.68 ^a	45.9 \pm 18.8 ^b	21.9 \pm 0.81 ^a
GOT-AST (u/l)	241.4 \pm 2.27 ^c	185.3 \pm 15.4 ^b	275.2 \pm 8.75 ^d	167.0 \pm 25.1 ^a
GPT-ALT (u/l)	101.2 \pm 7.02 ^b	57.8 \pm 3.72 ^{ab}	127.9 \pm 14.1 ^c	45.0 \pm 19.2 ^a

Different letters (a,b,c,d) between columns indicate a statistical difference (P<0.05). SAP: Alkaline phosphatase; CK: Creatine Kinase; GGT: Gamma glutamil transpeptidase; GOT-AST: Aspartate aminotransferate; GPT-ALT: Alanine transaminase

Urea was the only parameter that did not differ statistically between any of the groups. Bilirubin, magnesium, phosphorus chloride, GOT-AST concentrations were affected by follicular dimensions (P<0.05), but not by the phase of the estrous cycle. On

the other hand, cholesterol, creatinine, globulin, total protein, sodium and CK were affected only by the phase of estrous cycle. Both follicular dimensions and phase of the cycle affected the other parameters.

In table 7.2 are reported the results of the mean fatty acids composition of FF and the composition in the different groups. Twenty-two fatty acids were identified. The most common fatty acid was linoleic acid (C18:2n6), followed by oleic acid (C18:1n9), palmitic acid (C16:0), stearic acid (C18:0) and arachidonic acid (C20:4n6).

Caprylic acid (C8:0), caprinic acid (C10:0), lauric acid (C12:0), linoleic acid, α - linoleic acid (C18:3n6) and trienoic acid (C20:3n9) concentrations were affected both by follicular dimensions and phase of estrous cycle. Concentrations of nine fatty acids were affected only by the phase of the estrous cycle, among them, palmitic acid and oleic acid. Concentrations of four fatty acids were affected only by follicular dimensions, among them, stearic acid.

Table 7.2 Fatty acids composition (mg/dl) of follicular fluid collected from small (< 6 mm) and large (> 6 mm) follicles in follicular and luteal phase in water buffalo (mean \pm SD)

	Mean	FFFsmall	FFFlarge	CLFFsmall	CLFFlarge
C6:0	0.10 \pm 0.01	0.09 \pm 0.01 ^a	0.09 \pm 0.01 ^a	0.10 \pm 0.02 ^{ab}	0.11 \pm 0.01 ^b
C8:0	0.03 \pm 0.02	0.02 \pm 0.02 ^a	0.02 \pm 0.02 ^a	0.03 \pm 0.02 ^a	0.05 \pm 0.00 ^b
C10:0	0.06 \pm 0.02	0.05 \pm 0.01 ^a	0.05 \pm 0.01 ^a	0.08 \pm 0.02 ^b	0.05 \pm 0.03 ^a
C12:0	0.06 \pm 0.02	0.06 \pm 0.01 ^b	0.05 \pm 0.01 ^a	0.08 \pm 0.02 ^c	0.06 \pm 0.01 ^a
C14:0	0.61 \pm 0.13	0.58 \pm 0.13 ^a	0.52 \pm 0.04 ^a	0.67 \pm 0.18 ^b	0.65 \pm 0.08 ^b
C14:1n5	0.10 \pm 0.13	0.04 \pm 0.02 ^a	0.08 \pm 0.02 ^a	0.12 \pm 0.17 ^{ab}	0.20 \pm 0.24 ^b
C16:0	11.85 \pm 1.55	11.50 \pm 2.16 ^a	10.93 \pm 1.85 ^a	12.12 \pm 1.04 ^{ab}	13.20 \pm 0.34 ^b
C16:1n9	0.55 \pm 0.13	0.54 \pm 0.15 ^b	0.42 \pm 0.11 ^a	0.61 \pm 0.11 ^b	0.64 \pm 0.05 ^b
C16:1n7	1.28 \pm 0.43	1.40 \pm 0.49 ^a	1.21 \pm 0.28 ^a	1.46 \pm 0.07 ^a	0.84 \pm 0.91 ^b
C18:0	7.87 \pm 1.80	7.06 \pm 1.80 ^a	8.35 \pm 1.51 ^{ab}	7.42 \pm 0.97 ^a	9.25 \pm 3.79 ^b
C18:1n9	16.96 \pm 3.04	16.13 \pm 3.64 ^a	15.26 \pm 3.85 ^a	17.83 \pm 1.84 ^a	19.00 \pm 3.61 ^b
C18:1n7	0.67 \pm 0.69	0.42 \pm 0.73 ^a	0.34 \pm 0.29 ^a	0.85 \pm 0.99 ^{ab}	1.18 \pm 0.09 ^b
C18:2n6	20.86 \pm 9.77	17.29 \pm 6.11 ^a	15.25 \pm 3.13 ^a	20.28 \pm 11.19 ^a	35.79 \pm 0.67 ^b
C18:3n6	0.65 \pm 0.23	0.55 \pm 0.21 ^a	0.57 \pm 0.16 ^a	0.63 \pm 0.24 ^a	0.96 \pm 0.16 ^b
C18:3n3	1.30 \pm 1.25	0.79 \pm 0.36	0.99 \pm 0.40	1.64 \pm 1.71	1.86 \pm 2.37
C20:3n6	1.03 \pm 0.25	0.91 \pm 0.19 ^a	1.08 \pm 0.23 ^{ab}	0.97 \pm 0.20 ^a	1.23 \pm 0.48 ^b
C20:4n6	2.60 \pm 1.52	2.19 \pm 0.45	1.97 \pm 0.44	3.62 \pm 2.47	2.09 \pm 0.24
C20:3n9	0.31 \pm 0.10	0.27 \pm 0.10 ^a	0.26 \pm 0.08 ^a	0.39 \pm 0.12 ^b	0.28 \pm 0.01 ^a
C22:0	0.11 \pm 0.09	0.12 \pm 0.03 ^b	0.02 \pm 0.04 ^a	0.13 \pm 0.11 ^b	0.18 \pm 0.10 ^b
C22:4n6	0.34 \pm 0.09	0.38 \pm 0.13 ^b	0.28 \pm 0.04 ^a	0.36 \pm 0.11 ^b	0.33 \pm 0.05 ^{ab}
C24:0	0.16 \pm 0.17	0.10 \pm 0.08 ^a	0.05 \pm 0.09 ^a	0.16 \pm 0.15 ^a	0.41 \pm 0.21 ^b
C24:1n9	0.20 \pm 0.15	0.20 \pm 0.11 ^b	0.04 \pm 0.07 ^a	0.24 \pm 0.09 ^b	0.39 \pm 0.19 ^c

Different letters (a,b,c,d) between columns indicate a statistical difference (P<0.05).

7.5 Discussion

Since FF is in contact with oocyte and granulosa cells, changes in concentration of different components may reflect the requirements of follicular structure, and they may

represent a marker of oocyte quality. While other studies described the biochemical composition of FF in buffalo, to our knowledge this is the first report of the fatty acids composition. Moreover, there is no information about the FF composition of Mediterranean water buffalo farmed in Italy. The existing studies on FF composition in buffalo reported variability in relation to the phase of ovarian cycle (Eissa, 1996), reproductive acyclicity (Khan et al, 2011a; Khan et al, 2012) and follicles size (Nandi et al, 2008).

Ovaries of buffalo cows were collected at slaughterhouse during the breeding season in the Northern hemisphere. FF was collected by aspiration and samples were divided in function of follicular dimensions and the phase of ovarian cycle at slaughtering time.

Comparing the biochemical composition to the reports available, most of the parameters agree with those reported with exception of glucose that seems to be higher in this study (Baki Acar et al, 2013; Eissa, 1996; Khan et al, 2011a) but similar to Nandi (Nandi et al, 2008).

Biochemical composition was greatly influenced either by follicular dimension or by phase of ovarian cycle. Glucose concentration was higher in FF collected from animals in follicular phase than FF from animals in luteal phase, in according to Eissa (Eissa, 1996). Moreover we found a higher concentration in large follicles compared to small follicles as it has already been reported in other species (Albohomsen et al, 2011; Leroy et al, 2004; Nandi et al. 2007). Tryglicerides, K and P were more concentrated in small follicles than in large follicles in agreement with previously studies (Albohomsen et al, 2011; Leroy et al, 2004; Nandi et al. 2007; Tabatabaei et al, 2011). These aspects are in agreement with the results of the work reported in chapter 6 and have been discussed in paragraph 6.5. Overall, the biochemical composition was extremely variable between groups. The only parameters that remained stable is urea.

The most dominant fatty acids present in the buffalo follicular fluid were linoleic acid, oleic acid, palmitic acid, stearic acid and arachidonic acid. The same fatty acids were reported to be the most concentrated also in human follicular fluid (O’Gorman et al, 2013) and dairy cows (Renaville et al, 2010), but in different order. Palmitic acid, stearic acid and arachidonic acid seems to be less concentrated in buffalo FF than human and dairy cows FF, while oleic acid seems to be more concentrated in buffalo FF (O’Gorman et al, 2013; Renaville et al, 2010). Linoleic acid concentration seems to be higher in buffalo FF than human FF (O’Gorman et al, 2013) and lower than dairy cows FF (Renaville et al, 2010).

Overall, the fatty acids composition of FF was different depending on the follicular dimensions and phase of ovarian cycle. Of the five most common fatty acids, stearic acid was affected by the follicular dimensions, two fatty acids by the phase of ovarian cycle (palmitic acid and oleic acid), one was affected by both (linoleic acid) and one did not differ (arachidonic acid) (Figure 7.1 and Figure 7.2).

Figure 7.1 Effect of the phase of ovarian cycle on the concentration of the most representative fatty acids in buffalo follicular fluid

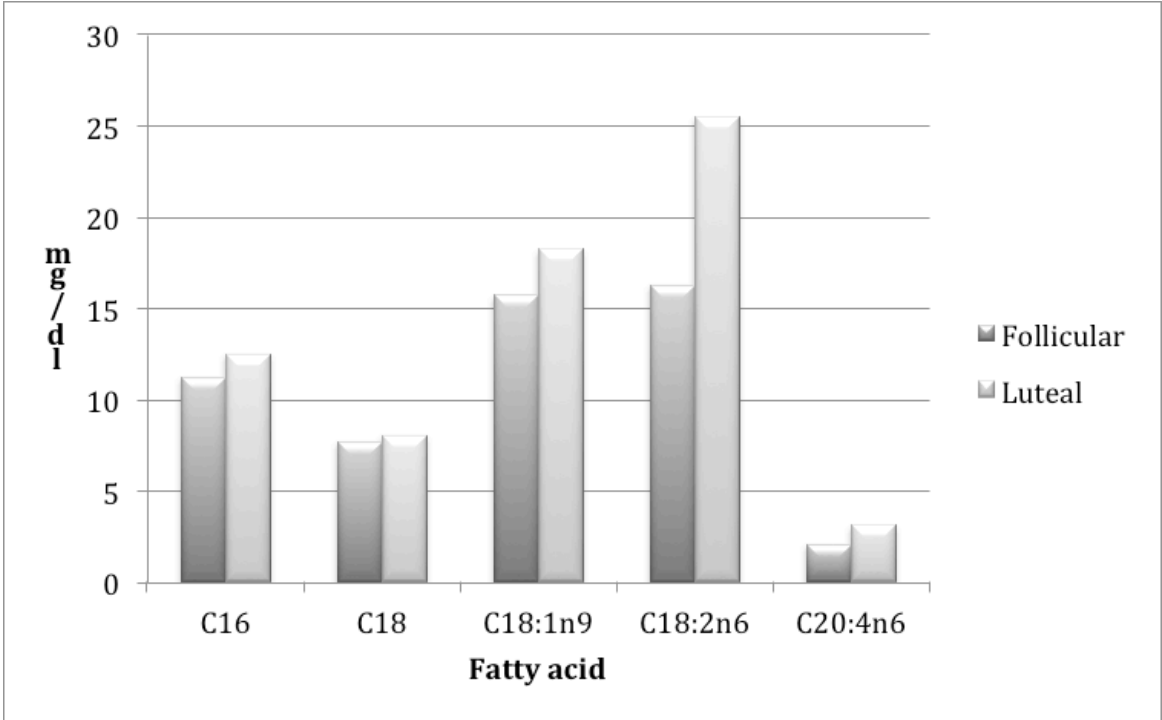
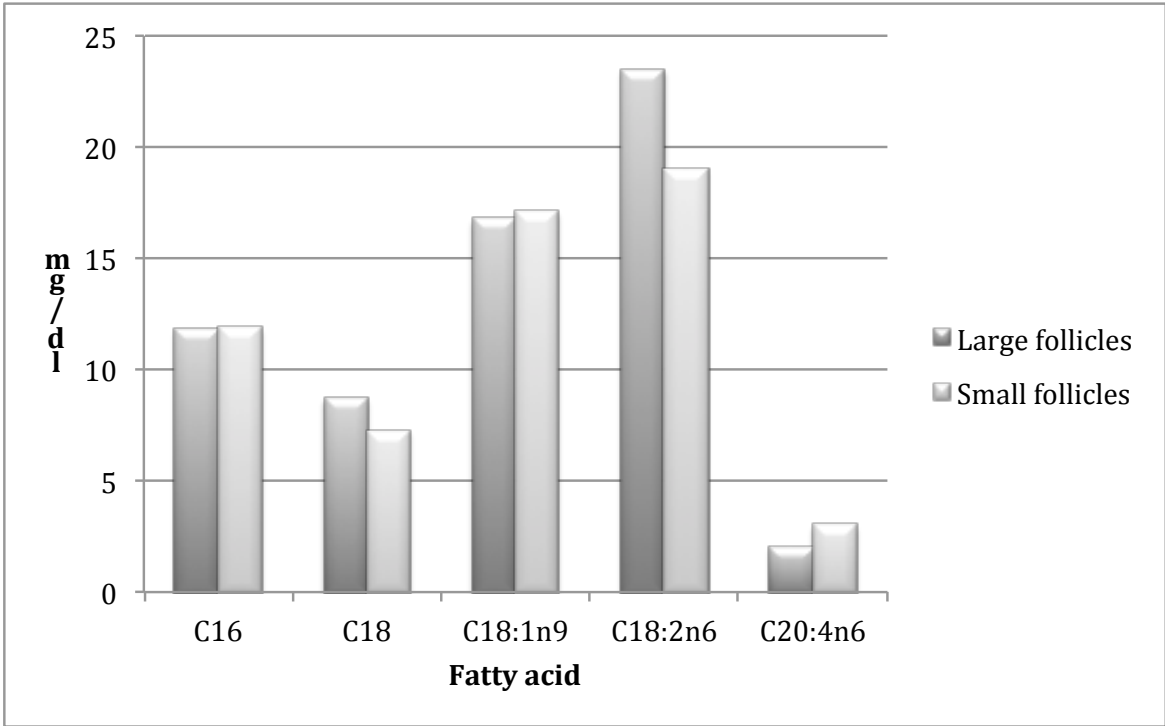


Figure 7.2 Effect of follicular dimension on the concentration of the most representative fatty acids in buffalo follicular fluid



From the twentytwo fatty acids identifies, nine were saturated fatty acids (SFA), six monounsaturated fatty acids (MUFA) and seven polyunsaturated fatty acids (PUFA). Only γ -linoleic acid and arachidonic acid concentrations remain stable in all classes.

Linoleic acid concentrations were extremely variable, though the only significant difference was found in CLFFlarge that shown higher concentration than other groups. Different studies reported that linoleic acid affected negatively the oocyte competence. In particular, his concentration was higher in FF from inactive follicles (Renaville et al, 2010), moreover Homa reported that linoleic acid inhibits resumptions of meiosis in a dose-dependent manner (Homa and Brown, 1992). Palmitic acid is the most abundant fatty acid in human FF and was found to be significantly increased in the FF where the oocytes fertilized but failed to cleave (O’Gorman et al, 2013). Excess of palmitic acid has been shown to induce apoptosis in human and bovine granulosa cells and affected steroidogenesis, which is critical in supporting follicular development and oocyte maturation (Mu et al, 2001; Vanholder et al, 2006). In our study palmitic acid concentration was affected by the presence of the corpus luteum. In particular the CLFFlarge samples had higher concentration of palmitic acid. Linoleic acid and palmitic acid concentrations seem to suggest that the oocytes coming from follicle grown under the influence of a CL may have less possibility to develop. Moreover, elevated levels of palmitic acid and stearic acid were reported to impair post-fertilisation development (Leroy et al, 2005). In contrast, a subsequent study reported that stearic acid was found to be significantly increased in the FF from follicles which oocytes undergo cleavage (O’Gorman et al, 2013), probably because stearic acid can be converted in oleic acid, which is reported to be more concentrated in good-quality oocyte (McKeegan and Sturmey, 2011). In our study stearic acid and oleic acid were higher concentrated in

CLFFlarge samples. These findings suggest that while stearic acid may affect developmental potential of the oocytes, on the other hand oleic acid may have a beneficial effect. Arachidonic acid is the major precursor for prostaglandins and protects granulosa cells from palmitic and stearic acid effects (Mu et al, 2001). Its concentration did not change between inactive and active follicles FF in bovine (Renaville et al, 2010), while it was more concentrated in human FF from follicles with oocytes undergo cleavage (O’Gorman et al, 2013). In our study its concentration did not statistically differ between groups.

Because FF composition can give information on the requirements of oocytes and follicular cells, it may be used as a guide for the formulation of specific IVP medium to enhance in vitro process efficiency.

In conclusion, follicular dimensions and phase of the ovarian cycle influenced FF composition; in turn this could affect oocyte quality. The concentrations of fatty acids that have been proposed as markers of oocyte quality seem to suggest that oocytes collected from large follicles during the luteal phase could be less competent, although this aspect has to be confirmed.

8 - EFFECTS OF CONJUGATED LINOLEIC ACIDS SUPPLEMENTATION IN DIET ON THE TESTICULAR FINE-NEEDLE ASPIRATION CYTOLOGY IN PERIPUBERTAL RAMS

8.1 Abstract

This study was designed to investigate the effects of diets containing different quantity of conjugated linoleic acid on the testicular fine-needle aspiration cytology (TFNAC) in peripubertal rams. Eighteen rams of three breeds (Alpagota, Brogna and Foza) were fed with three different diets: Pasture rams were graze at pasture; Hay rams were fed with hay and a commercial concentrate; CLAsuppl rams were fed with hay, a commercial concentrate ad supplemented with conjugated linoleic acid (CLA) composed by 50% of cis-9, trans-11 isomer and 50% trans-10, cis-12 isomer. Animals were slaughtered at one year of age and testicles were collected to performe TFNAC. No statistical differences were found in live weight and testicular weight at slaughtering time between groups. CLAsuppl rams had higher percentages of early spermatogenic cells (primary spermatocytes and AB spermatids), lower percentages of CD spermatids and lower spermatic index ($P<0.05$) than rams in Pasture and Hay groups. CLA supplementation affected negatively TFNAC results. This effect may be due to negative effect of CLA on serum leptin concentration that causes a delay of the onset of puberty.

8.2 Introduction

Metabolic conditions play an essential role in the modulation of the timing of puberty (Sanchez-Garrido and Tena-Sempere, 2013). In the last two decades, solid evidences have demonstrated a permissive role of leptin in the metabolic gating of pubertal maturation (Barb and Kraeling, 2004; Castellano et al, 2009; Roa et al, 2010). Leptin is an adipocyte-derived hormone secreted by the white adipose tissue in proportion to the

amount of fat stores (Moon et al, 2007; Tena-Sempere 2007). Effects of leptin have been described in hypothalamic, pituitary and testicular function (Spicer, 2001). In particular, leptin indirectly regulates GnRH neuronal function (Quennel et al, 2009) and its action seems to be mediated by kisspeptin system (De Bond and Smith, 2013). Leptin concentrations has been observed to increase during puberty in human males and then decreased after puberty because of the inhibition of leptin secretion induced by testosterone (Spicer, 2001; Tena-Sempere and Barreiro, 2002). Conditions of leptin insufficiency, if persistent in time, are coupled to disturbed function of numerous systems (Sanchez-Garrido and Tena-Sempere, 2013). Among these, also the reproductive axis is affected, as low or null leptin levels are linked to delay or absence of puberty onset and ipofertility (Roa et al, 2010).

Conjugated Linoleic Acid (CLA) supplementation in diet can reduce body-fat mass and serum leptin (Medina et al, 2000; Rahman et al, 2001). In particular, significant loss of adipose tissue was caused by trans-10, cis-12 isomer (t10c12CLA) (LaRosa et al, 2006; Poulson et al, 2004), while cis-9, trans-11 isomer (c9t10CLA) did not cause any effect in the adipocytes (DeClercq, 2010). Because of its role on leptin serum concentrations, it is possible that t10c12 CLA supplementation in diet may affect negatively the onset of puberty in rams.

Testicular fine needle aspiration cytology (TFNAC) is an easily reproducible, poorly invasive and highly diagnostic technique. Its use has been proposed in the BSE of bulls (Chapwanya et al, 2008). In ram, studies of TFNAC described the normal testicular cytology composition (Vencato et al, 2013) and its relation with the sperm production (CHAPTER 5).

Aim of the present study was to investigate, through TFNAC technique, the effect of different diets containing different quantity of CLA and its isomers on the testicular maturation in peripubertal rams.

8.3 Materials and methods

Eighteen rams of three different local breeds (Alpagota, Brogna, Foza), born in a time lapse of 2 months, were divided in three groups of six rams each and fed with three different diets. A group was maintained at pasture (Pasture), a second group was fed with hay and supplemented with a commercial concentrate (Hay) and a third group was fed with hay, commercial concentrate and received a supplementation of a CLAs compound (CLAsuppl).

Before slaughtering animals were weighted and after slaughtering, testes were separated from the carcass and weighted. They were conserved in saline solution at 4°C temperature and transported to the obstetric laboratory of the Department of Animal Medicine, Production and Health of the University of Padua, within 1 hour. TFNAC was performed as previously described (Vencato et al, 2013) using a 21G butterfly needle and a 20 cc empty syringe. The material collected during the aspiration of each testicle was distributed in three slides and smeared. Slides were stained using a modified May-Grünwald Giemsa staining protocol as described in other works (Stelletta et al, 2011; Vencato et al, 2013) and reported in Chapter 5. Slides were evaluated under an optical microscope with a magnification of 1000x, counting at least 200 cells per slide. Relative percentages of spermatogenic cells (spermatogonia, primary spermatocyte, Secondary spermatocyte, AB spermatids, CD spermatids, Sertoli cells) and the Spermatic Index (SI= percentage of spermatozoa on the total of spermatogenic cells) were determined.

Data obtained were analyzed using the General Linear Model procedures of SAS (1990). One-way ANOVA was performed to investigate the effects of diets on TFNAC

parameters. Analysis of the covariates was performed to exclude the effect of age, weight and other factors that can influence the results.

8.4 Results

Rams live weights before slaughtering were respectively 35.65 ± 2.86 Kg, 34.39 ± 5.36 Kg and 35.18 ± 8.96 in Pasture, Hay and CLAsuppl groups. While testicular weights measured immediately after slaughtering were 0.32 ± 0.06 Kg, 0.26 ± 0.09 Kg and 0.25 ± 0.11 Kg in Pasture, Hay and CLAsuppl group respectively.

TFNAC was successfully performed in all testes, and it allowed to collect sufficient quantity of material in order to evaluate the cytological composition. Table 8.1 showed the results of TFNAC in the different groups.

Table 8.1 Relative percentages of spermatogenic cells and Spermatic Index (SI) in the groups of rams fed with different diets

	Pasture	Hay	CLAsuppl
Spermatogonia (%)	0.07 ± 0.17	0.10 ± 0.41	0.039 ± 0.19
I spermatocytes (%)	8.84 ± 8.16^a	6.72 ± 4.41^a	20.81 ± 19.35^b
II spermatocytes (%)	0.10 ± 0.27	0.28 ± 0.74	0.11 ± 0.38
AB spermatids (%)	16.07 ± 9.16^a	15.27 ± 5.74^a	27.77 ± 10.22^b
CD spermatids (%)	27.01 ± 6.30^a	32.40 ± 4.27^a	23.52 ± 9.48^b
SI	47.90 ± 16.21^a	45.24 ± 9.62^a	27.76 ± 18.44^b

Different letters ^(a,b) between diet groups indicate a statistical difference with $P < 0.05$.

Pasture: rams graze on pasture; Hay: rams fed with hay and a commercial concentrate; CLAsuppl: rams fed with hay, commercial concentrate and supplemented with conjugated linoleic acid.

8.5 Discussion

This is the first work that investigates the effect of nutrition on the cytological appearance of testes in ram. Animals were assigned randomly in three groups receiving different diet (pasture; hay and concentrate; hay, concentrate and CLA supplementation) from prepubertal age and up to 1 year of age, and then they were slaughtered.

Concentration and composition of CLA administered to animals differ between diets. Animals raised on pasture received almost the same quantity of CLA than those that were fed with hay, concentrate and CLA supplementation. The analysis of CLA composition of pasture showed that CLA consist almost entirely of c9t10 CLA, while the supply of CLA administered to the CLAsuppl group was composed of 50% c9t10 CLA and 50% t10c12 CLA. Content of CLA received from animals in Hay group was negligible.

Rams were weight just before slaughtering and no significant differences in terms of total weight were observed between groups. After slaughtering, the testicles of each animal were weight and although Pasture group seems to have heavier testes, no significant differences were observed between groups.

TFNAC was performed on all testes recovering sufficient tissue from each one. All spermatogenic cells were identifiable on smears. The observation of the slides showed differences in the cytological composition between groups. CLAsuppl group showed lower percentage of CD spermatids and SI, and a higher percentage of primary spermatocytes and AB spermatids than Pasture and Hay groups.

Values found in Pasture and Hay groups seems to be similar to those reported in rams of the same age (Vencato et al, 2013), while CLAsuppl group showed results that seem to be different, in particular for spermatocyte I, AB spermatids, CD spermatids and SI.

Diet effects, measured through TFNAC examination, appear evident and specifically the supplementation of CLA affected negatively the cytological appearance of testes. In particular, the SI, that is considered a measure of the efficiency of spermatogenesis (Foresta, 1993), is particular affected from CLA supplementation.

Diets high in CLA have been reported to cause a decrease of body-fat mass in rats (Rahman et al, 2001), swine (Tous et al, 2013) and dairy cows (Van Soosten et al, 2012). Some studies reported also a negative effect of CLA on the circulating levels of leptin (Miller et al, 2007; Rahman et al, 2001). Leptin is a 16kDa hormone produced from the white adipose tissue and regulates feed intake, the neuroendocrine-axis and immunological processes (Barb and Kraeling, 2004). Its role in the metabolic control of the onset of the puberty has been widely demonstrated in mice (Ahima et al, 1997; Chehab et al, 1997).

In this study the supplementation of both isomers of CLA, c9t11CLA and t10c12 CLA, had negative effect on TFNAC findings, while animals in Pasture group that received a higher amount of total CLA, but only of the isomer c9t11CLA, were not affected. Hay group received a small quantity of CLA did not differ from Pasture group.

As CLA effects on circulating levels of leptin has already be demonstrated (Rahman et al, 1997), it is possible that the supplementation of CLA had negative effect on the onset of puberty and spermatogenesis and the effect may be mediated by the decreased levels of leptin, although this cannot be demonstrated. In support of this supposition there is also the evidence that the negative effect is reported only in the group receiving also the t10c12 CLA isomer. It has been described that only t10c12 CLA had the effect of reducing the amount of fat stores (LaRosa et al, 2006; Poulson et al, 2004) and because levels of leptin are produced in relation to the quantity of adipose tissue (Moon et al, 2007; Tena-Sempere, 2007), it is credible that only the t10c12 CLA isoform affects leptin

levels. In the future, it would be desirable to study the effects of t10c12 CLA in diet in the circulating levels of leptin in order to prove this supposition.

8.6 Conclusions

TFNAC was a suitable tool in diagnosing diets effects on testicular cells composition. The supplementation of CLA in diet affected negatively the results of TFNAC in comparison to animals fed with forage and concentrate and animals grazed on pasture. Analysis of CLA composition showed that the main difference between diets was the concentration of t10c12 CLA isomer that may be the reason of the differences observed in the cytology.

9 – GENERAL DISCUSSION

Breeding techniques have always been at the center of any livestock enterprise, motivated by curiosity and consolidated by breeder's needs and interests (Galli et al, 2014). The application of ART in livestock requires the integration of the laboratory techniques with the clinical management of animals finalized at obtaining live offspring. The introduction of ART has given a great impulse to the genetic improvement of breeds and species in which they were applied, and also gave a fundamental tool in preservation of biodiversity of endangered breeds and species. Great advances have been made in ART in the last years, but they are still far from efficiency. In this thesis two aspects that influences ART efficiency have been considered in order to obtain knowledge that can be useful in optimizing ART.

The first field to be interested is the andrological evaluation of breeders. Since before the introduction of ART, male had a main role in genetic improvement, as they can give origin to more offspring per year than a single female of any species. Their role has been enhanced by the introduction of AI and maintained its importance in embryo production technologies. For these reasons is mandatory to prove male fertility before to involve animals in breeding programs. The society of theriogenology proposed some guidelines to follow in performing an andrological evaluation (this guidelines are summarized in chapter 2 of this thesis) but some Authors concluded that they are not totally satisfactory (Chapwanya et al, 2008; Gouletsou et al, 2003) and new techniques have been proposed as completion of BSE. In chapter 4 and 5 of this Thesis two of these techniques have been applied.

Chapter 4 reported a study of thermographic and testosterone monitoring in young bulls with low semen production. This study was conducted in collaboration with a bull semen production center. In this center, young bulls with high genetic potential arrived

at about 11 months of age. After a quarantine period, they are evaluated for their breeding potential and then are trained for semen collection using an artificial vagina. Once trained, bulls are collected up to obtain three thousand straws that are distributed, at reduced price or sometimes also for free, in different herds. After reaching the required number of straws, bulls are moved to a resting stable where they have to wait until data of fertility and productivity of the progeny return to the center. This process is the so-called Progeny test. Therefore, bulls are more than four years old when they start producing, and among them only a few percentage become semen producers. During this period bulls are a cost for the center. Furthermore, not all bulls produce three thousand semen straws in the same time and some of them have poor or absent semen production. This because bull calves can reach puberty at different time (Aravindakshan et al, 2000; Bagu et al, 2006; Evans et al, 1995), and bulls attaining late puberty increase the costs for semen producers and decelerate the process of genetic selection.

In the study reported in chapter 4, a novel approach for evaluating young bulls with poor semen production was tested. GnRH stimulation test has been already proposed to evaluate bull reproductive potential (Gabor et al, 1998; Lunstra and Coulter, 1997) in older animals. The administration of exogenous GnRH causes an increase of T that has been related to semen quality (Gabor et al, 1995). Moreover, high interstitial T might activate a mechanism of secretion based on a neural local regulation of the scrotal blood flow and muscular contraction, resulting in a change of SST that can be measures with thermography (Gabor et al, 1998).

In our study, after the administration of an analogue of GnRH, two different thermographic responses have been observed, some bulls presented a decreased SST after GnRH administration and some of them an increased SST. This result is in partial disagreement with a previous study that reported an increase of SST in all bulls (Gabor

et al, 1998), but in animals having an average age of about 2 years. Therefore, we decided to analyze the other results dividing bulls on the basis of thermography findings. Basal T and sperm production before GnRH analogue administration did not differ between groups. As expected, T increased in all bulls after GnRH administration, but ΔT was different between groups. In LowTemp bulls ΔT was significantly greater than HighTemp bulls. Semen production and semen kinetic parameters were not significantly different. After GnRH administration, semen production and almost all parameters of kinetics improved significantly, but improvement of semen production was greater in LowTemp bulls than in HighTemp bulls. On the basis of our results we concluded that a negative variation of SST following the GnRH administration is a marker of testicular functionality. Either thermal or functional regulation of the testis is based on systemic and local information (Sharpe, 1984; Tahka, 1989). Difference in SST variation after GnRH administration may be caused by different production of testicular factors that influences the blood flow (Sharpe, 1984). The administration of exogenous GnRH induces the secretion of T that moved from the cytoplasm of Leydig cells to the interstice and then to the blood. The increase of T in the interstice may activate the testicular factors responsible for the variation of SST. In bulls having a better testicular functionality, the administration of GnRH caused the secretion of higher quantity of T and then of testicular factors that may lead to the decrease of the SST. In bulls having not such a good functionality, this probably not happened, as a consequence the temperature increase because of the testicular stimulation operated from the GnRH that is not balanced by the alteration of the blood flow and muscular contraction. In future studies, the attention should be focused in demonstrating this possibility.

Semen production increased after GnRH administration starting from the subsequent semen collection and was maintained up to the achievement of three thousand straws.

Although an improvement in semen quality over time could be expected, a single GnRH administration may have been responsible for the increasing of semen production. To investigate this aspect, we analyzed the productivity data of bulls of the same age and with similar semen production, that were present in the same center the year before our experiment. Untreated bulls had an improvement in semen production after the first three weeks of collection, a period similar to the pre-treatment period of experimental groups. We considered the improvement in the control group as a reference of the normal semen production improvement due to the maturation. Control bulls, even in their highest production period, had significantly lower production than both experimental groups. Although these data have to be considered with caution, it may be possible that the difference is attributable to the administration of GnRH. The importance of GnRH in reproduction has been well documented. Episodic administration of GnRH or analogue, in a manner that mimics the pattern of endogenous GnRH secretion, supports gonadotrope function, maintains gonadotropin secretion, and supports and sustains testicular development and function. In contrast, a continuous administration has a temporary stimulation effect but renders the gonadotrope cells refractory to the GnRH inputs, suppressing gonadotropins secretion and testicular function (Adams, 2005). We can speculate, but we need further study to confirm it, that a single GnRH administration has the effects of enhancing sperm ejaculation and promoting spermatogenesis. These effects could explain such a rapid improvement of sperm production, and also because the improvement was maintained over time. Despite these speculations, our study demonstrated the efficacy of the thermography monitoring of SST after GnRH administration as a tool in andrological evaluation. A second advantage of this study was the reduction of the time needed to conclude the

collection of straws necessary for the Progeny test, though it remains without a clear explanation, it represents a reduction of costs for the center.

Chapter 5 reported a study of the application of TSUS and testicular fine-needle aspiration cytology to evaluate sperm production in ram. This study was performed with the collaboration of Veneto Agricoltura.

Ultrasonography allows to investigate the internal architecture of soft tissues, and could provide visualization of testicular and epididymal alterations (Gouletsou et al, 2003). Although its use has already been reported, there are no indications about its role as a marker for sperm production.

Cytology has been proposed at the beginning of 20th century as a diagnostic tool in testicular evaluation. Although it was considered less traumatic than biopsy its use has been controversial for many decades (Vencato et al, 2013). Its application became more common after the introduction of the TFNAC technique, which is described as easily reproducible, poorly invasive and highly diagnostic (Foresta, 1993). TFNAC applications in veterinary medicine are recent. TFNAC has been successfully applied to diagnose infertility and other pathological condition affecting testes in dog (Dhalbom et al, 1997; Romagnoli et al, 2009; Santos et al, 2010), cat (Gouletsou et al, 2012), stallion (Pereira-Leme and Ozanam Papa, 2010), alpaca (Stelletta et al, 2011) and boar (Stelletta et al, 2012). At the time of this study, no data were available on the relation of TFNAC findings and sperm production in ram.

Eighteen adult rams were involved in this study. Rams were of 4 different breeds, were used in previous breeding seasons as breeders with good reported performances. Animals involved were considered for slaughter, this gave us the possibility to recover testis from slaughterhouse and to collect epididymal spermatozoa. TSUS and TFNAC were performed in all animals at the beginning of the breeding season, and then semen

was collected twice using an electroejaculator. After slaughtering, spermatozoa were collected from epididymis. Semen obtained in all collections was treated with specific medium and analyzed after freeze-thaw process.

TSUS was useful in observing testicle parenchyma morphology. It was possible to diagnosed different conditions of testicular degeneration that appear as highly echogenic structures in relation to the surrounding tissues. The grade of degeneration was used to create a classification of parenchyma appearance (Echo0; Echo1; Echo2; Echo3). These classes were used to divide animals and to perform the statistical analysis of TFNAC and semen parameters.

On the other hand, relative percentages of all the spermatogenic cells were measured in TFNAC slides. Both techniques were easily applicable, minimally invasive and economically sustainable.

TFNAC results were compared between classes of ultrasonography appearance. Spermatic index (SI) was the only TFNAC parameter that differed between groups. It was higher in Echo0 rams than Echo3 rams, while no differences were observed between others groups. SI has been described as a measure of the efficiency of testis in producing spermatozoa in human (Foresta, 1993), so it may indicate that Echo0 rams have a higher testicular efficiency. Considerations on the efficiency of spermatogenesis can be formulated on the base of the TFNAC results. Spermatogenesis is the process by which a population of germ cells produces spermatozoa (Holstein et al, 2003). Spermatogonia multiply continuously in successive mitosis, while cells in meiosis are called spermatocytes (Holstein et al, 2003). Usually, spermatogonial cells give rise to spermatocyte after a fixed number of mitotic divisions that is characteristics of each species (six generations in ram) (Franca et al, 2005). From a single primary spermatocyte, through the process of meiosis, four spermatozoa can be originated.

However the efficiency of spermatogenesis is not of 100 %, as a percentage of germ cells are lost during the process by apoptosis or degeneration (Holstein et al, 2003). The efficiency of spermatogenesis can be measured with the meiotic index that is calculated dividing the number of spermatids per the number of primary spermatocytes. In ram the mean meiotic index has been calculated in 3.2 (Hess and Renato de Franca, 2008). Meiotic indexes calculated in our study, suggested that ultrasonography appearance of the testis seems to be related to spermatogenesis efficiency in rams, as it declined from Echo0 rams to Echo3 rams.

The analysis of sperm production highlighted some statistical differences between classes both using electroejaculation and epididymal spermatozoa. Echo0 rams have a higher total sperm and straws production than Echo3 rams, and in general a decrease in sperm production has been observed passing from Echo0 to Echo3. These findings seem to confirm the predicting value of ultrasonography appearance of testicle on sperm production. On the other hand, the differences observed in sperm motility and progressive motility were not statistically significant, so even if there is a possibility that echogenicity classification might be used as a marker of motility parameters, further study are warranted to investigate this possibility.

To study the validity of TFNAC as a marker of sperm production, we calculate the Pearson correlation indexes between TFNAC results and total sperm/number of straws. SI was correlated positively and significantly with sperm production of epididymal spermatozoa, while the correlation was just above significance limit using EE. Other correlations were found between the secondary spermatocytes and the sperm production in EE collection, and a negative correlation between spermatogonia and epididymal spermatozoa production. On the base of these results, TFNAC seems to be a possible indicator of sperm production but it needs further improvement.

Chapter 4 and 5 studies indicated that thermography monitoring of scrotum after GnRH administration, TSUS and TFNAC can be very useful tools in the andrological evaluation. However, further considerations have to be discussed on these techniques. Because of the diffusion of ultrasound machines between veterinarian and also farmers its use seems to be the most practical in the herds. Moreover, it has a great advantage in stagional species such as the ovine. In fact, ultrasonography appearance of the testis does not change in the different stages of the reproductive cycle, so it can be used to evaluate males before the beginning of the breeding season. TFNAC and semen analysis performed in no-breeding season instead, can give some erroneous indications as a reduction of spermatogenesis activity has been described during this period. Although TFNAC is an easily applicable technique, it required more experience in the evaluation of the slides and it require more time to obtain a result than ultrasonography. Its main use could be in the diagnosis of pathological condition of azoospermia or oligospermia has it has been reported in other studies. Thermography is a quiet new technology, because since some years ago it was available only for military purposes and it was very expensive. In the last years, the cost for thermography machine decreased so it became a more sustainable technology, although it is not yet developed. Its applications in veterinary medicine are very different (Stelletta et al, 2012), so it will become an important instrument in the future.

In chapter 6 and 7 are reported two studies of the composition of FF in South American camelids and buffalo. The importance of FF is not a new discovery as its use has already been proposed as a supplementation in the IVP medium (Ducolomb et al, 2013; Nandi and Kumar, 2008; Somfai et al, 2012), and also as a marker of oocyte quality (Albohomsen et al, 2011; Ali et al, 2004; Bender et al, 2010; Eissa et al, 1996; Revelli et al, 2009; Wallace et al, 2012).

South American camelids are a very important economic resources in the countries of origin. The main production is the fiber considering above all the domestic species (alpaca and lama) and the vigogna. This product has raised the interest on these species in other countries of the world, and it represents the reason why many alpaca are now bred in Australia, New Zealand, England, China, etc. Other products are meat and milk, but the consumption is restricted to the countries of origin because most of the other countries do not have a legislation that regulates the production of food from South American camelids. Moreover, some of these animals are used for transport (lama), trekking and pet therapy or kept as a pet.

Reproduction aspects of South American camelids are peculiar. Estrus in camelids may continue for several weeks with ovulation occurring only after the female is mated (Adams et al, 1990). An ovulation-inducing factor has been isolated from seminal plasma and was identified as β -Nerve Growth Factor (β -NGF) (Ratto et al, 2012), a neurotrophin which role in ovarian development and oocyte maturation has already been established (Barboni et al, 2002; Dissen et al, 2009). Because of the different pattern of follicular development compared to the most studied species, it is possible that the modification in FF composition can be different in South American camelids. The study in chapter 6 is focused on the study of composition of FF in alpaca and lama at different stage of ovarian cycle. This study was composed of two different experiments, one in collaboration with the Universidad Nacional de Huancavelica, Perú, the other in collaboration with the University of Saskatchewan, Canada. In experiment 1, ovaries of alpaca were collected from abattoir, FF was aspirated from follicles and samples were divided in four categories depending of presence or absence of corpus luteum and follicular dimension. We analyzed the biochemical composition and protein profiles using a 1D-SDSPAGE technique. Mean biochemical composition of FF in our study was

similar to the results reported in other species (Nandi et al, 2007; Albohomsen et al, 2011; Tabatabaei et al, 2011), but was different from the only available report in alpaca (Pacheco and Coila, 2010) and in particular glucose levels were consistently lower. After some researches in bibliography, we were unable to find an explanation for such a great difference. However, looking also to the references mentionated in that article, we believe that the Authors may have made a mistake in indicating the unit of measurement of glucose, which would explain the difference between our study and their.

No significant differences were observed between FF collected during the follicular or the luteal phase. This finding suggests that the presence of the corpus luteum has no influence on the composition of FF in alpaca, and considering the influences of FF on oocyte quality, it could mean that oocytes collected in both stages have the same developmental potential. FF composition differed a lot between small and large follicles both for biochemical composition and protein profile, confirming previous reports in other species (Albohomsen et al, 2011; Leroy et al, 2004; Nandi et al. 2007), which probably means that oocytes from different size follicles have different developmental potential. In experiment 2 we followed ultrasonographically the ovarian activity of a group of lama females to identify the vawe emergency. Animals were divided in three groups in which FF aspiration was performed at different stages of dominant follicle development.

Because of the small amount of FF collectable from a single follicle we decided to analyze the protein profiles using the 1D-SDSPAGE. Differences in mean relative percentages were significant in four molecular weight classes. It may be possible that some proteins in these classes have a role (either favorable or negative) in establishing oocyte competence. There are no data available at the moment on the protein composition of FF in South American camelids, so it is not possible to compare our

results to other. In the discussion in the paragraph 6.5 we advanced some proposal of proteins that may be included in the molecular weight bands that differed between groups, however, more studies are needed to identify the protein composition of FF in South American camelids and also to discover their role in oocyte development potential.

On the base of our results, we can conclude that FF composition in South American camelids changes in relation to the dimension of the follicles, and in dominant follicles changes in relation to the developmental stage. This study may provide the basis for three types of study in the future: 1-the deepening of the knowledge of the composition of FF; 2-the relation between the FF composition and the oocyte competence; 3-the modification of the composition of IVP medium in relation to environment of origin of the oocytes.

In chapter 7 is reported a study of the biochemical and fatty acids composition of buffalo FF. This study was conducted in collaboration with the Department of Pediatrics of the University of Padua.

Buffalo is a very important animal in the economy of different countries. Because of its adaptation to heat stress it represents a greater animal protein source than cattle in the tropical region (Baruselli et al, 1996). The river buffalo plays a prominent role in rural economy in Asia and it is bred for milk, meat and draft-work. In the Amazon Region of Brazil the buffalo population was estimated in 2.5 million head in 1994 with an increasing trend (Baruselli et al, 1996).

Buffalo breeding in Italy probably started more than one thousand years ago, although no evidences are available on the introduction of this specie in our country since the XII century. Data from the website of the “Associazione Nazionale Allevatori Specie Bufalina” (ANASB) emphasize the role of buffalo in Italy. At the present time, the Italian

buffalo population is about 200,000 animals, the only production of “Mozzarella di Bufalo DOP” employs about 15,000 people and the number of dairies working buffalo milk has been estimated in 400.

Despite the great importance of buffalo, reproductive technologies in this specie have not developed as they have been in cattle (Drost, 2007). Different studies on FF have been published in the past years showing that the composition is influenced by: ovarian cycle (Eissa, 1996), corpus luteum (Baki Amar et al, 2013), acyclicity period (Khan et al, 2011; Khan et al, 2012) and follicular dimension (Khan et al, 2011; Khan et al 2011b; Nandi et al, 2008). No information is available on the fatty acids composition of FF.

Fatty acids composition of FF can be a possible predictor of oocyte developmental competence and embryo vitality (O’Gorman et al, 2013). Different fatty acid composition has been reported between FF from active and inactive follicles in dairy cows (Renaville et al, 2010), while the fatty acids composition of human FF from follicles where oocytes fertilized and developed into multi-cell embryos and from oocyte that failed to cleavage was different in nine different fatty acids (O’Gorman et al, 2013).

In our study, ovaries were collected from slaughterhouse and aspirated once transported to the laboratory dividing the FF in four groups depending on the presence or absence of corpus luteum and on follicular dimension. Firstly a complete biochemical analysis was performed. The results of biochemical analysis seem to agree with previously studies with the only exception of glucose that was higher in this study (Baki Acar et al, 2013; Eissa, 1996; Khan et al, 2011a), but similar to Nandi (Nandi et al, 2008). FF biochemical composition was influenced either by follicular dimensions or phase of the ovarian cycle, differently from what we found in FF from South American camelids that was influenced only by follicular size (chapter 6). Glucose content was higher in FF collected from animals in follicular phase than FF from animals in luteal phase; Eissa

reported the same variation in his study (Eissa, 1996). Glucose was more concentrated in large follicles than small follicles, in accordance to studies conducted in other species (Albohomsen et al, 2011; Leroy et al, 2004; Nandi et al. 2007). Triglycerides, K and P were found to be more concentrated in small follicles than large follicles. Overall, all parameters showed a variability between groups, with the exception of urea that remained stable. Such variability is a consequence of the modification of the metabolism of granulosa cells and of the permeability of the blood-follicle barrier. The determination of the fatty acids composition of buffalo FF is the main innovative aspect of this study, considering that no information is available at the moment. Twenty-two fatty acids were identified; nine were SFA, six MUFA and seven PUFA. The most represented fatty acids were linoleic acid, oleic acid, palmitic acid, stearic acid and arachidonic acid. These five fatty acids were described as the most dominant in FF of human (O’Gorman et al, 2013) and dairy cows (Renaville et al, 2010), although not in the same order. Palmitic, stearic and arachidonic acids seem to be less concentrated, and oleic acid more concentrated in buffalo FF than human and dairy cows FF (O’Gorman et al, 2013; Renaville et al, 2010). Linoleic acid concentration was higher in buffalo than human FF (O’Gorman et al, 2013) and lower than dairy cows FF (Renaville et al, 2010). Fatty acids concentrations were influenced either by follicles size and phase of ovarian cycle. Among the identified fatty acids only two (γ -linoleic acid and arachidonic acid) remained stable in the different groups. Linoleic acid and palmitic acid were significantly higher concentrated in CLFFlarge groups, so in follicles that grown under the influence of a CL. Both fatty acids were described to affect oocyte development: linoleic acid because it inhibits resumptions of meiosis (Homa and Brown, 1992), palmitic acid because it induces apoptosis in granulosa cells affecting steroidogenesis (Mu et al, 2001; Vanholder et al, 2006). Also stearic acid was higher concentrated in CLFFlarge group. The possible role

of this fatty acid as marker of oocyte quality has been discussed: elevated level of stearic acid were described to impair post-fertilisation development (Leroy et al, 2005), in another study its concentration was found to be higher in follicles which oocytes undergo cleavage (O’Gorman et al, 2013). Oleic acid and arachidonic acid were described as positive marker of oocyte quality, oleic acid was more concentrated in good-quality oocyte (McKeegan and Sturmey, 2011), while arachidonic acid, which is the major precursor for prostaglandins, protects granulosa cells from the apoptotic effects of palmitic and stearic acid (Mu et al, 2001). In this study arachidonic acid concentrations did not statistically differ between classes, as it has already been described in dairy cows FF (Renaville et al, 2010). Oleic acid was generally more concentrated in all groups than what is reported in literature, and it was found at increased levels in CLFFlarge. This finding may be a consequence of the elevated stearic acid concentration, as stearic acid is a precursor of oleic acid (Mu et al, 2001).

In conclusion, the FF composition is very variable in buffalo. This aspect must be considered during in vitro processes as oocyte collected from follicles of different size or in different phases of ovarian cycle may have different competence or different requirement during maturation and development.

A study of the effect of three different diets on TFNAC in post-pubertal rams is reported in chapter 8. This study was done in collaboration with the Department of Agronomy, Food, Natural Resources, Animals and Environment (DAFNAE) of the University of Padua.

Eighteen pre-pubertal rams were divided in three groups: 1-grazed at pasture; 2-fed with hay and ovine commercial concentrate; 3-fed with hay, an ovine commercial concentrate and supplemented with CLA.

Many studies have been conducted in the last years on the effect of CLA on health. In animal husbandry great emphasis has been placed on the effect of CLA supplementation in the diet on the lipid composition of milk and meat. It has been reported that CLA supplementation can reduce body fat-mass in human and some animal models (Larsen et al, 2003; Medina et al, 2000; Rahman et al, 2001; Wang and Jones, 2004). In particular, the loss of adipose tissue is caused by t10c12 CLA isomer (LaRosa et al, 2006; Poulson et al, 2004). In animals, diet supplementation with CLA has been studied to reduce fat content in milk in order to meet market demands (Pappritz et al, 2011; Sinclair et al, 2010). No studies were conducted on the effect of CLA supplementation in diet on the spermatogenesis or the onset of puberty.

Rams grazed on pasture and rams supplemented with CLA received the same total quantity of CLA, but with different isoforms concentration. In pasture almost all CLA are in cis-9, trans-11 isoform, while the supplementation was composed by a 50 % of cis-9, trans-11 isoform and 50 % of trans-10, cis-12 isoform. Rams fed with hay and concentrate instead received a little quantity of CLA because they are denaturated during the haymaking. At 1 year of age animals were weighed and sent for slaughtering. No significant differences were observed in terms of live weight and testicular weight between groups. After slaughtering, testes were collected and TFNAC was performed. The analysis of cytology slides point out some differences between groups. Rams receiving a supplementation of CLA had lower percentages of late spermatogenic cells (CD spermatids and SI) and higher percentages of early spermatogenic cells (spermatocytes I and AB spermatids) than both the other groups. So, a negative effect on the onset of puberty or spermatogenesis seems to be induced by CLA supplementation, and specifically by the t10,c12 isoform and not from the c9,t11 isoform. Because of its effect of reducing white adipocytes deposits, t10,c12 CLA induces also a reduction of the

circulating level of leptin, an hormone that is produced from the adipocytes (Miller et al, 2007; Rahman et al, 2001). Leptin role in the onset of puberty has been documented (Ahima et al, 1997; Chehab et al, 2997), and both the deficiency and the excess have negative effects on the onset of puberty (Ahn et al, 2012; Roa et al, 2010). Although the exact mechanism of action of leptin has not been clarify, it has been proposed that it may act through the Kisspeptin-1 system (Sanchez-Garrido and Tena-Sempere, 2013; Tena-Sempere, 2013). Kisspeptin-1 has been described as a key transmitter in the reproductive brain, and kisspeptin-1 system is responsible for the regulation of all aspects of reproductive maturation and function, from brain sex differentiation to the neuroendocrine control of ovulation and sex steroid feedback (Sanchez-Garrido and Tena-Sempere, 2013). Expression studies documented an increase in the hypothalamic expression of the Kiss1 gene during the pubertal maturation, leading to the idea that a rise in Kisspeptin is necessary in inducing puberty (Navarro et al, 2004; Shahab et al, 2005). Supplementation of diet with t10,c12 CLA in prepubertal rams may have induced a reduction of white adipocytes population leading to a lower concentration of leptin than rams grazed on pasture or receiving no supplementation. As a consequence, decreased levels of leptin may be not able to induce a sufficient secretion of Kisspeptin-1 to allow these animals to attend puberty at the same time of the other groups. A delayed puberty may be responsible for the retard of spermatogenesis we observed through TFNAC.

We can conclude that a supplementation of t10,c12 CLA may have negative effects on testicular maturation in peri-pubertal lambs. Even if CLA supplementation has beneficial effect on milk and meat quality, its use is to be avoided in lambs that will be use for breeding. In the future, it would be desirable to study the effects of CLA supplementation also on mature subjects in order to investigate the possible influence on

spermatogenesis. Moreover, further studies are needed to confirm the effects of CLA supplementation of leptin levels and kisspeptin system.

10 – CONCLUSIONS

10.1 Andrological evaluation

1 – Scrotal infrared thermography is a suitable method to monitor responses to GnRH administration;

2 – A decreased scrotal surface temperature after GnRH administration is a sign of a higher testicular functionality compared to an increased temperature response in yearling bulls with low semen production;

3 – A single GnRH administration seems to improve semen production, and the improvement is maintained over time;

4 – Ultrasography testicular appearance is a marker of semen production in rams and a classification can be useful in selecting animals at the beginning of the breeding season because;

5 – Testicular fine-needle aspiration cytology is easily applicable and minimally invasive, several cytological parameters are correlated to sperm production in rams;

10.2 Follicular fluid characterization

1 – Follicular fluid composition is influenced by follicles dimension in some parameters (K, P, Glucose, Triglycerides, GPT, CK) while is not influenced by presence/absence of corpus luteum in alpaca;

2 – Corpus luteum influenced two classes of molecular weight in electrophoresis analysis (75 kDa, 37 kDa), while eight molecular weight bands differed between small and large follicles (250 kDa, 200kDa, 120 kDa, 90 kDa, 75 kDa, 60 kDa, 50 kDa, 37 kDa) in alpaca;

3 – Follicular fluids protein profiles differed in relation to the developmental stage of follicles (growing, static or regressing) in four molecular weight bands (250 kDa, 75 kDa, 25 kDa, 15 kDa);

4 – Biochemical composition of buffalo FF was greatly influenced either by follicular dimension or phase of the ovarian cycle

5 – Fatty acids composition of buffalo FF was different from what is reported in other species, although the five most dominant fatty acids were the same; their role of markers of oocyte quality seems to suggest the poor quality of oocytes collected from follicles in luteal phase, but this aspect has to be confirmed

10.3 Diet effects on testicular fine-needle aspiration cytology in rams

1 – Testicular fine-needle aspiration cytology was easily applicable and reproducible in rams and it allows us in pointing out differences induced by nutrition;

2 – No differences in live weight or testicular weight were observed between rams graze on pasture, fed with hay and concentrate or fed with hay, concentrate and supplemented with conjugated linoleic acid;

3 – Conjugated linoleic acid supplementation affected negatively testicular fine-needle aspiration cytology in peripubertal rams, probably because the trans-10, cis-12 isoform retarded the onset of puberty;

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ACKNOWLEDGMENTS

I would like to express my most deeply appreciation to my supervisor Dr. Calogero Stelletta, who has provided encouragement, spiritual support and beautiful time to share enlightening discussion throughout my research program. I would like also to thanks Prof. Gregg Adams for his ospitality at the University of Saskatchewan, and for providing animals, equipments and ideas for my study on lamas.

I express my gratitude to all persons who have collaborated to the different experiments in included in this thesis: Giancarlo Bertoli, Eleonora Carlo and Giorgio Carrer from Intermizoo spa; Dr. Irene Vazzana from Istituto Zooprofilattico Sperimentale delle Sicilie; Dr. Valerio Bondesan from Veneto Agricoltura; Prof. Jaime Ruiz from Universidad Nacional de Huancavelica; Dr. Martin Arganaraz; Dr. Tamara Badon; Manuela and Sara from the Department of Peditry of the University of Padua; Prof. Giovanni Bittante and Dr. Alessio Cecchinato from the Department of Agronomy, Food, Natural Resources, Animals and Environment of the University of Padua.

I would like also to thank all technicians and students who helped me in these three years.