UNIVERSITÀ DEGLI STUDI DI NAPOLI "FEDERICO II"

Ph.D. Thesis

"Genetic, cytogenetic and morphometric analyses in the study of reproductive problems that affect animal productions in the main livestock and pets species"

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Non prendere la vita troppo sul serio: tanto, per quanto tu possa faticare,
alla fine non ne uscirai vivo.
(Elbert Hubbard)

Introductive Abstract

Zootechnics has the mission to improve animal production, according to the human needs, that will influence market demand and animal and human health. Animal reproduction gives an important contribution giving rise to an "extreme Tango" in which genetics is the crazy score with a solid melody but which can change depending on the context in which this music is played. This thesis reports the research studies carried out during the doctorate.

The Disorders of Sexual Development are the main topic of the first chapter. These are genetic diseases involving the reproductive system that cause sterility to the carriers and economic losses to the breeders. The animals involved in this study are dogs and horses, they were examined clinically, cytogenetically and genetically in order to identify DSD etiopatogenesis of each subject.

The main topic of the second chapter is the improvement of stallions' sperm quality using phytotherapic drugs. This study has a dual purpose, to improve the natural breeding and the techniques of artificial insemination. Morphometric analyzes and chromatin fragmentation tests were performed on the ejaculates in addition to the qualitative and quantitative evaluations routinely carried out.

The Third Chapter describes a work born by a collaboration among different research groups. Exploiting the training acquired on the morphometric evaluation of the spermatozoa of different mammals' species we have applied the same techniques on the spermatozoa of Apis mellifera Ligustica, in order to characterize its semen.

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List of abbreviations

CHAPTER 1

(*AMH*) Anti-Mullerian Hormon
(AMHR2) Anti-Mullerian Hormone Receptor Type 2
(*AR*) Androgen Receptors
(*BAC*) Bacterial Artificial Chromosome
(*CAG*) Cytosine-adenine-guanine triplet repeats
(*CAIS*) Complete androgen insensitivity syndrome
(*CFA*) Canis Familiaris
(*CGRP*) Calcitonin gene-related peptide
(*CYP17A*)*1* Cytochrome P450 Family 17 Subfamily A Member 1
(*DAX1*) Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1
(*DHT*) Dihydrotestosterone
(*DSD*) Disorders of Sexual Development
(*ECA*) Equus Caballus
(*EIF3CY*) Eukaryotic translation initiation factor 3
(*Emx2*) Empty Spiracles Homeobox 2
(*EOG*) General objective examination
(*EOP*) Particular objective examination
(*FGF9*) Fibroblast growth factor 9
(*FOXL2*) Forkhead box L2
(*Fst*) Follistatin gene
(*GnRH*) Gonadotropin Releasing Hormone
(*hCG*) Human Chorionic Gonadotropin
(*HES3*) Hes Family BHLH Transcription Factor 3
(*HMG*) Family (high mobility group) -box DNA-binding protein
(*HPRT*) Hypoxanthine Phosphoribosyltransferase 1
(*INSL3*) Insulin-like factor 3
(*LH*) Luteinising hormone
(*Lhx1*) LIM Homeobox 1
(*Lhx9*) LIM Homeobox 9
(LOF) Lost of function
(*MAMLD1*) Mastermind Like Domain Containing 1
(MURCS) Cloacal aplasia of the ducts of Müller, renal aplasia and dysplasia of the somites thoracic cervico

(MVPA)	Department of Veterinary Medicine and Animals Production
(n.d.)	No Data
(OVUD)	University Veterinary Didactic Hospital
(PAIS)	Partial androgen insensitivity syndrome
(PCR)	Polymerase Chain Reaction
(PGD2)	Prostaglandine D2
(*PISRT1*)	Polled intersex syndrome regulated transcript 1
(PMDS)	Persistent Muller's Duct Syndrome
(LGR)	Leucine-rich repeat-containing G-protein coupled receptor
(PSA)	Arabian thoroughbred
(*R-spo1*)	R-Spondin 1
(*SDR5A2*)	5-alpha reductase gene
(*SF1*)	Splicing Factor 1
(*SOX*)	Sry-related HMG box
(SOX9)	SRY-Box 9
(*SRY*)	Sex-determining Region Y
(T)	Testosterone
(*TDF)*	Testis-determining factor
(*TES)*	Testin LIM Domain Protein
(TESCO)	Testis-specific enhancer of *SOX*9 Core
(*TGF-ß)*	Transforming growth factor
(*WNT)*	Wingless-type MMTV integration site family
(*Wnt4)*	Wnt family member 4
(*WT1)*	Wilms tumor 1
(*ZFY)*	Zinc Finger Protein Y-Linked
(*ZNF33bY)*	Zinc Finger protein 33b on Y

CHAPTER 2

(A)	Area
(MD)	Major diameters
(mD)	Minor diameters
(P)	Perimeter
(R)	Roughness
(sd)	Standard deviation
(SDF)	Sperm DNA Fragmentation
(SF)	Shape factor
(TSC)	Total Sperm Count

CHAPTER 3

(CCD)	Colony Collapse Disease
(HL)	Head Length
(NA)	Nucleus Area
(NL)	Nucleus Length
(NW)	Nucleus Width
(PL)	Perforator Length
(TaL)	Total length of the Tail
(ToL)	Total length of the Sperm

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CHAPTER I

ABSTRACT

In zootechnical field reproduction, ability of an individual isa parameter of greatest importance because it is the basis for its ability to produce incomes. Phenotypic sex consists of a set of somatic and functional features that distinguish the male gender from the female one. The DSDs are an emerging problem in dog breeding and among the main causes of horses' subfertility and sterility. Up to now the genetic bases of the development of reproductive abilities are only partially known thus the etiopathogenesis of most cases of DSDs is unknown. Aim of this Ph.D. research work is to evaluate clinically and genetically dogs and horses affected by DSDs due to genetic causes to create a collection of Dog and Horse DNA used for genetic and genome analyses to look for DSDs causative mutations. For this purpose, 11 dogs and 5 horses have been evaluated in this study. The first classification was performed by clinical and (when possible) histological examinations. Then karyotype analysis and SRY gene were characterized to establish chromosome and genetic sex. 8 dogs and 3 horses were positive for the SRY gene, in 3 dogs and 2 horses SRY was absent. For equine species ZFY, ZNF33bY, and EIF3CY genes were also analyzed. Furthermore, in one case the EIF2s3Y and HPRT genes were examined. 8 dogs with chromosomal and genetic arrangement 2n=78, XY; Sry-positive were analyzed for some specific genes involved in the development of the reproductive system, SRD5A2 and MAMLD1 and AR and their sequences were compared to that of 11 control dogs. In both clinical and control cases the sequencing of SRD5A2 and MAMLD1 showed the presence of polymorphisms that do not seem to have an impact on sexual development. The same result was for AR, no mutations or polymorphisms associated with the altered phenotype were found. As regard horses, despite the clinical features attributable to specific DSDs, only in one subject it was possible to obtain a certain diagnosis with the diagnostic tools currently available.

Abstract

Introduction

The awareness of living in a world in which globalization and market demand are increasingly thinking of a common and homologated sense of the taste, appearance, lifestyles and eating habits is projecting the Green side of industry, the zootechnical and breeding one to take more and more extreme and unidirectional decisions. This is evident, for example in the choice of reproducers, increasingly similar to each other that respond to a demanding business model, subjects less and less in balance with the nature system and more and more distant from the concept of biodiversity. Biodiversity, the disorder with which nature balances things, is lost with the artificial genetic improvement that enhances extreme productions to the detriment of others not less important, such as rusticity or fertility. Genetic improvement, use of consanguineous breedings, arbitrary breeding choices not supported by technicians, are now leading to the extinction of native species and breeds that once were the wealth of the territories in which they evolved with a synergy that was nothing short of perfect. This leads us to turn our studies on the genetic aspects of fertility, that is the ability of an individual, male or female, to procreate in a specific period of its existence. In mammals the concept of fertility is closely linked to the survival of a population. In humans, the psychological factor has an incisive effect on the fertility potential of a subject and is closely linked to the concept of sexuality -the complex of manifestations related to sex. Sex is made up of a set of somatic and functional characters that distinguish the male gender from the female one. Sometimes, this difference is not well defined, and, in the adult, it can be the cause of infertility. It is the case of the Disorders of Sexual Development (DSD) that in animals of zootechnical and veterinary interest, causes economic losses (Lyle, 2007; Meyers-Wallen, 2009; Poth et al., 2010; Zavadilova and Zink, 2013; Kinsel et al., 1998). To study the mechanisms underlying male and female sexual differentiation, what is still hidden to the eyes of scientists behind a very complex hormonal cascade, is one of the aims of our daily work. To date we know that, for the development of the genetic sex, the crossroads

between male and female are mainly linked to the presence or absence of the Y chromosome and of the SRY gene located on it (Parma and Radi, 2012). The goal is to put together all the pieces of the puzzle that, before or together with SRY, decide the fate of the gonadal and morphological sex of the individual. To calculate DSDs incidence of (DSD) in animals of zootechnical and veterinary interest is very difficult especially for large animals, there is no a case registration system, moreover freelancers and zootechnical companies often do not ask for the support of a laboratory of genetics to confirm the diagnosis. This is where our work starts, deepen and contribute to the research of the genetic bases of pathological conditions that involve animal reproduction without losing sight of the environment we share with the animal world and its respect that should be an innate and not indoctrinated concept.

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Chapter 1
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1.1 Sexual differentiation

There are a genetic, a gonadal, an anatomical sex and in humans a psychological, a registered and a social sex too. The genetic sex corresponds to the gender identified by the sex chromosomes arrangement of the cells of an individual. By mean anatomical or morphological sex indicate the phenotypic manifestation given by the primary (testicles, spermatic ways, penis, vulva, vagina, uterus, tube and ovaries) and secondary (different skeletal development and muscular mass, different distribution of adipose and piliferous formations) sexual characteristics. Between the time of fertilization and that of gonadal differentiation, the zygotes go through a phase of common sexual development, they are defined sexually undifferentiated because the sexual dimorphism is not such developed to allow a morphological distinction between the two sexes (Christensen and Meyers-Wallen, 2011). Internal and external primary sexual features define gonadal sex. It determines the endocrine sex in physiological conditions, influencing the production of estrogens and androgens. Gonadal sex is important for "brain sexualization", for hormone production and for fertility. Moreover, the regard, the respect and the laws about animals are evolving in a positive way, the individual identity and the social role of the animals are receiving more and more attention; from the veterinary point of view all this would help and encourage to increase the knowledge about all the pathophysiological and behavioral aspects of the animal life.

Normal sexual differentiation shows itself in three sequential phases:
1. establishment of chromosomal sex (genetic)
2. development of gonadal sex
3. development of phenotypic sex

Chromosomal gender (XX or XY) is established at fertilization; this composition is maintained in all cell lines throughout life. In mammals, the sex of the embryo is determined at the time of fertilization, depending on whether the sperm involved is a carrier of an X or Y chromosome. In fact, the female gametes can only contain the X chromosome and are therefore called homogametes, while the spermatozoa can contain the X or Y chromosome and for this they are defined heterogametes and are the real responsible for determining the chromosomal sex. Early studies on the

mechanisms of sexual differentiation in mammals had shown that the presence of the Y chromosome was associated with the development of testicular tissue and a male phenotype, regardless of the number of X chromosomes present. For this reason, it was supposed the presence on the Y of a gene that have a fundamental importance for male sexual differentiation and it started the search for a testicular determining factor connected to the Y chromosome (TDF = testis-determining factor). Further studies allowed the identification, more than 20 years ago, of the *SRY* gene (Parma and Radi, 2012). This gene, a member of the *SOX* family, encodes a transcription factor belonging to the HMG-box family (high mobility group) DNA binding protein (Sekido and Lovell-Badge, 2013), also called *SRY* protein or testicular determination factor. The genetic sex depend on the presence or the absence of a specific gene: *SRY*, that is located on the sex determing region of the Y chromosome, it is necessary both for the activation of the genes that induce the formation of the testis and for the suppression of the genes which induce ovarian formation. In fact, all embryos initially are sexually undifferentiated and only at a precise moment of embryonic growth they are induced to evolve in a specific gender. The arrangement of the sex chromosomes determine the differentiation of the gonads: the presence of a Y chromosome causes the differentiation of the genital ridge in a testis. If the Y-chromosome is absent then the genital crest develops in an ovary. Many genes are involved in the development of gonadal structures and some are already expressed in the undifferentiated gonad.

EMX2 , *LHX9* , *LHX1*, *SF1*, *POD1*, *WT1* are genes responsible for the early proliferation of undifferentiated gonadal tissue prior to the activation of sex-specific genes. In fact, the knocking out of one of them causes regression of the gonad in both XX and XY embryos before its differentiation (Parma and Radi, 2012; Serge and Parada, 2017). These genes are very likely to represent transcription factors implicated in the correct activation of the *SRY* gene (Sekido and Lovell-Badge, 2013). *WT1* and *SF1* are also necessary for the development of kidneys and adrenal glands (Bruening *et al.*, 1992; Luo *et al.*, 1994).

The *SRY* gene located on the Y chromosome encodes for TDF and initiates testicular differentiation. Its expression must respect specific times and must start from the central portion of the gonad, and then extend towards the two poles (Bullejos and Koopman, 2001; Sekido *et al*., 2004); the same direction is then followed during the inactivation phase of the gene. The action of the *SRY* may require the presence of co-factors available only

from a certain moment (Kidokoro *et al.*, 2005). The *SRY* gene is expressed in somatic cells of the gonads for a limited period during which the *SRY* protein exceeds the minimum activation threshold and activates the autosomal *SOX*9 gene. In the gonad, the *SRY* protein binds directly to the *SOX*9 TESCO promoter (TES (testis-specific enhancer of *SOX*9) Core) together with *SF1* (steroid factor, gene transcription regulator also called NR5A1, nuclear receptor subfamily 5, group A, member 1) to up-regulate the expression of *SOX*9 (Kimura *et al.*, 2014). Once expressed, it is the same *SOX*9 protein that, together with *SF1*, activates TESCO, continuing to up-regulate itself through a self-regulating mechanism that goes on even after the expression of the *SRY* gene is terminated (Sekido and Lovell-Badge, 2013). Two *SOX*9 alleles are required for normal testicular development (Foster *et al.*, 1994). The *SOX*9 protein at first is present in low concentration in the undifferentiated gonads of both sexes, thanks to an initial transcription of the *SOX*9 gene through the link of *SF1* to TESCO (McClelland *et al.*, 2012). With the progress of the sexual differentiation SOX9 is totally inactivated in the XX gonads while undergoing a fast up-regulation (thanks to the combined action of *SF1* and *SRY*) in the XY gonads (Ikeda *et al.*, 1994; Morais de Silva *et al.*, 1996) where it is responsible for the differentiation of the cells in Sertoli cells, that must reach a certain number to obtain an adequate testicular development. Sertoli cells can also originate from cell lines, that did not initially show the activation of the *SRY* gene: studies on chimerism (XX-XY) show that 10% of XX cells have differentiated into Sertoli cells (Palmer and Burgoune, 1991) while research performed with antibodies for *SRY* and *SOX*9 shows that not all Sertoli cells express the *SRY* gene, reinforcing the theory that there are paracrine signals responsible for *SOX*9 expression in these cells. Among the factors secreted by Sertoli cells in response to the activation of the *SOX*9 gene Prostaglandin D2 (PGD2) and fibroblast growth factor 9 (*FGF9*) activate the expression of *SOX*9 gene also in cells where *SRY* gene is not expressed, thus recruiting new future Sertoli cells (Kim *et al.*, 2007; Bagheri- Fam *et al.*, 2008). This further recruitment is necessary to increase the number of Sertoli cells as they are essential for the differentiation of other testicular cell lines (in particular Leydig cells and spermatozoa) and for the proper organization of the gonad. Loss of function (LOF) mutations of genes encoding *FGF9* or FGFR2 result in insufficient expression of *SOX*9 and the differentiation of an inadequate number of Sertoli cells, resulting in a blockage of gonadal development in the male sense (Colvin *et al.*, 2001; Kim *et al.*, 2007; Bagheri-Fam *et al.*,

2008). *SOX*9 in Sertoli cells, in synergy with *SF1* and *WT1* (-KTS), activates the transcription of the anti-Müllerian hormone (AMH) (Sekido and Lovell-Badge, 2013), member of the transforming growth factor-β superfamily (TGF -β) and responsible for the regression of the parathyonephric ducts in the XY embryo (Knower *et al.*, 2003). Alfred Jost, a French endocrinologist, in 1947 suggested the presence of an "active" genetic program for male phenotypic differentiation that obligatorily required the presence of the testicles and the existence of a pathway of "default" for the differentiation in the female that provoking the regression or the removal of pluripotent gonads from sexually undifferentiated embryos, both XX and XY individuals develop a female phenotype. In 1953 he predicted the future discovery of AMH (Picard and Josso, 1984) and T claiming that the fetal testes produce at least two morphogenetic substances for the complete development of the male reproductive system. With the discovery of the TDF (Testis-determining factor) on the Y chromosome, the Jost hypothesis was translated from the phenotypic sex to the gonadal one, justifying the existence of an active genetic mechanism for testicular development and a merely passive mechanism for the ovarian one.

The existence of sex reversal syndromes with the development of testes and male phenotype in XX *SRY*-negative subjects denied these theories indicating the existence of aa active genetic program in gonadal development of XX individuals. A correct ovarian differentiation requires the activation of specific genes (Nef *et al.*, 2005; Small *et al.*, 2005; Beverdam and Koopman, 2006), an error in any phase of this process can lead to the development of different phenotypic anomalies, including ovarian dysgenesis (Villagòmez *et al.*, 2009). The researchers are involved in identifying an alternative gene to the *SRY* one in the XX gonads able to suppress the transcription of *SOX*9 during ovarian development, over time different candidates were indicated, some of them will be reported below.

DAX1, whose malfunction determines in human XY sex reversal syndrome, hypogonadism with incomplete or absent sexual maturation, was initially considered the ovarian determining gene, it was later discovered that it is not fundamental for ovarian development, but its presence is important for a correct differentiation of the male gonad (Meeks *et al.*, 2003; Bouma *et al.*, 2005).

FOXL2, associated with female infertility, is specifically expressed in the XX gonads, especially in future granulosa cells (Schmidt *et al.*, 2004); however, it seems that null mutations of the *FOXL2* gene cause so much

problems in the early stages of ovarian differentiation and in folliculogenesis (Garcia-Ortiz *et al.*, 2009) since this gene is fundamental for the differentiation of granulosa cells. Mutations of the *FOXL2* or *PISRT1* gene in humans do not induce the development of testes in XX subjects (De Baere *et al.*, 2003). Recent studies show that *FOXL2* causes an up-regulation of *FST* expression (follistatin gene, member of the TGF-ß binding factor superfamily) (Garcia-Ortiz *et al.*, 2009); *FOXL2* ablation in ovaries of adult subjects results in trans-differentiation of granulosa cells into Sertoli cells, with the expression of specific markers for male gonads, including *SOX*9. This trans-differentiation seems to be possible thanks to the activation of TESCO, enhancer (promoter) of *SOX*9; this suggests that *FOXL2* binds to TESCO by blocking its activation and subsequent expression of *SOX*9 (Uhlenhaut *et al.*, 2009; Kimura *et al.*, 2014).

*WNT4*is one of the best studied ovarian differentiation markers; it belongs to *WNT* gene group that encodes proteins that have been implicated in oncogenesis and in a variety of developmental processes, including the regulation of cell fate and their structuring during embryogenesis. It is the first molecule that has been shown to influence sex determination. After the beginning of gonadal differentiation, the expression of this gene decreases in the XY gonad while it persists in the XX gonads and in the mesenchymal tissue surrounding Müller's ducts (Parma and Radi, 2012). This gene and its nuclear receptor are known to antagonize TDF and to play a role both in the control of female development and in the prevention of the formation of testicles (provided by RefSeq, Jul 2008).

WNT4 is involved in the development of Müller's ducts and in the inactivation of the development of testicular vascularization in the XX gonads but it is not identifiable as the primary and only gene responsible for ovarian differentiation and testicular arrest (Parma and Radi, 2012).

FOXL2 and *WNT4* could act in synergy to repress male genes during ovarian differentiation; nevertheless, ambiguous gonadal and phenotypic typology indicates the presence of other ovarian factors necessary to activate the development of the XX gonad and to oppose its development in the masculine sense (Parma and Radi, 2012).

R-SPONDIN 1 gene encodes an activating protein with two cystein-rich, furin-like domains and a trombospondin type 1 domain. The coded protein is a ligand for leucine-rich repeat-containing ligand G-protein coupled receptors (proteins LGR) and positively regulates the *Wnt* signaling pathway. The *RSPO1* malfunction can determine the development of hermaphrodite subjects.

RSPO1 belongs to the R-spondine family, proteins involved in the embryonic development of different organs (Nam *et al.*, 2007). These proteins act by stabilizing beta-catenins through the interaction with specific receptors. One of the functions of beta-catenins is to "migrate" from the cytoplasm to the cell nucleus to transduce the signal originating from the *WNT4* binding with the appropriate receptor (Kimelman and Xu, 2006). This transduction causes the inhibition of β-catenin degradation, in fact, if the cells are not exposed to the *WNT4* signal, the quantity of beta-catenins available is minimal as they are subjected to the degradation mechanism. These proteins, migrating within the nucleus, regulate the expression of specific genes important for female sexual development; moreover, the *WNT*/beta-catenin system probably antagonizes the binding of *SF1* with TES, thus participating in the activation of TESCO, promoter of *SOX*9 (Kimura *et al.*, 2014). The *SRY* gene activates the transcription of *SOX*9 (then maintained and reinforced by positive feedback by *FGF9* and PGD2) that ultimately activates the differentiation of Sertoli cells and thus testicular development. In the absence of *SRY*, *RSPO1* and *WNT4* trigger, through the stabilization of beta-catenin and in synergy with *FOXL2* and support the differentiation in the female direction and they antagonize the expression of *SOX*9 in somatic cells. The molecular pathways for the determination of the testes and ovaries cross each other in several moments. *FGF9* and *WNT4* act as antagonists: in male gonads, *SRY* stimulates an upregulation of *SOX*9 with a consequent up-regulation of *FGF9* and the arrest of *WNT4*. *FGF9* in turn exerts a positive feedback towards *SOX*9, in fact exogenous exposure of gonadal tissues to *FGF9* induces the expression of *SOX*9 and blocks that of *WNT4*: this suggests that *FGF9* acts both by directing gonadal cells towards male differentiation and blocking female signals. In the other hand, in the developing ovaries WNT4 inhibits the action of *FGF9* and *SOX*9 (Parma and Radi, 2012). In the pluripotent gonad *SOX*9 and beta-catenins appear to be the two main mechanisms involved in this sort of molecular duel able to direct somatic precursors towards differentiation in Sertoli cells or in granulosa cells, but further studies are needed to identify all genes fundamental for gonadal sexual differentiation (Villagomez *et al.*, 2009; Parma and Radi, 2012).

As regard endocrine sex, the situation is more complex. Biology predisposes all embryos to be potentially females in the first phase of their development but once that a genetic basis (XX or XY) is established they will be the hormones produced by certain regions of the organism to influence the development of gonadal gender.

In normal XY individuals, the masculinization process take place due to testosterone production by Leydig cells in the testis. Testosterone induces the differentiation of Wolff's ducts in epididymis and vas deferens. In the testes too, Sertoli cells secrete the anti-mullerian hormone (AMH), responsible in the male fetus of the regression of the Müller ducts, embryonic structures that are precursors of uterus, fallopian tubes and the upper part of the vagina. The secretion of these two hormones must take place within a critical time window during embryonic development (different for each species) to obtain normal masculinization. In the urogenital sinus and in the genital tubercle, testosterone is converted into dihydrotestosterone (DHT) by 5α-reductase, which causes the closure of the urethra and the development of prostate, penis and scrotum. The descent of the testicles into the scrotumis due to both hormonal and mechanical events and it starts in the first moments of the individual's extrauterine life and it completes the phenotypic development in male. In XX individuals the Mullerian ducts, the urogenital sinus and the genital tubercles can develop in internal and external female genitals because the absence of *SRY* determines the absence of the hormonal cascade of AMH, T and DHT. Muller's ducts develop into the oviducts, the uterus, the cervix and the cranial part of the vagina; the urogenital sinus determines the formation of caudal vagina and vestibule; the genital tubercle develops in the clitoris and in the vulva. An abnormal sexual differentiation could be due to errors in the constitution of the chromosomal, gonadal or phenotypic sex. A wide variety of subjects have been identified with ambiguous genitalia that are apparently normal and potentially fertile or infertile. Sexual differentiation in the different species begins in the embryo during the first 4-5 weeks of intrauterine life, even if the chromosomal sex is already defined in the zygote. One of the most important problems at birth is the attribution of sex. Therefore, the assignment of sex must certainly consider the somatic indices since the appearance of the genitals is crucial for registration, for the productive and reproductive future of the subject and for sexual self-identification. In general, but not always, the phenotypic sex corresponds to the genotypic one and it depend on the action of sex hormones. In humans too the alteration of the normal level of masculinizing or feminizing hormones (due to illness, malformation or administration) may lead to phenotypic sexual characteristics that differ from genotypic sex. The presence or absence of substances secreted by the testicles will influence the differentiation of internal and external genitalia. The genetic stability of

the fetus is one of the main factors of genital differentiation, to which they are added genes involved in the development of genital tubercle, the gonadal determination, the gonadal steroid synthesis (testosterone and dihydrotestosterone: DHT) and the sensitivity to these hormones (Okeke *et al.* 2001; Boisen *et al.*, 2004). The fetal and prepubertal Sertoli cells produce the anti-Müllerian hormone (AMH), which is responsible for the regression of the paramesonephric ducts through the interaction with specific receptors (MIS type I and II receptors) expressed by the target structures, in particular on the mesenchymal cells surrounding the aforementioned ducts (Nef and Parada, 2000). Although the molecules responsible for transcription of the gene encoding the AMH have not yet been all identified, scientific data indicate that a fundamental role is undoubtedly played by *SF1* (Ikeda *et al.*, 1994; Achermann *et al.*, 1999), *SOX*9 (De Santa Barbara *et al.*, 1998) and *WT1* (Nachtigal *et al.*, 1998). In the male the AMH represents a specific marker to identify the presence of immature Sertoli cells as its concentration is high in intra-uterine life and remains so until the beginning of puberty; the Sertoli cells in this phase do not have androgen receptors (AR). From the achievement of sexual maturity, the synthesis of AMH decreases due to the inhibitory action carried out by the increase in intratesticular testosterone associated with the expression of AR by Sertoli cells (Chemes *et al.*, 2008; Boukari *et al.*, 2009). Experiments conducted on adult mice with AMH hyperproduction have shown that this condition leads to the development of hyperplasia of Leydig cells, focal atrophy of the germinal epithelium, reduced serum testosterone and hypo-development of testosterone-dependent organs (Lyet *et al.*, 1995; Mishina *et al.*, 1996), reinforcing the thesis that the anti-Müllerian hormone performs an autocrine/paracrine inhibitory action on the steroidogenesis in the post-pubertal male gonad. However, AMH, although in a very low concentration, is also produced in the adult testis and it is therefore likely that it plays some functional roles (Tsafriri *et al.*, 1988). Leydig cells are responsible for the testosterone production; this hormone binds to androgen receptors (AR) in the target organs, blocking the apoptosis of Wolff's duct cells and stimulating further differentiation in epididymis, vas deferens and seminal vesicles. The expression of the enzyme 5alpha-reductase type2 in specific target organs leads to the conversion of testosterone into dihydrotestosterone (DHT). Through the binding with AR, DHT induces the differentiation of urogenital sinus in prostate and male urethra, the fusion of labioscrotal folds to form the scrotum and the differentiation of penis and prepuce from genital tubercle

(Josso *et al.*, 2013). Leydig cells secrete also INSL3 (Insulin-like factor 3), a hormone-containing substance of the insulin peptide hormone family (Adham *et al.*, 1993; Pusch *et al.*, 1996; Zimmermann *et al.*, 1999) it intervenes, together with testosterone, in the process of descent of the testicles inside the scrotum (Nef and Parada, 1999, 2000; Zimmermann *et al.*, 1999). In fact, testicular migration can be divided into two phases, a passive transabdominal and an active inguino-scrotal (Hutson *et al.*, 1994) (Nef and Parada, 2000). XY mice carrying a null mutation for *INSL3* exhibit bilateral cryptorchidism for abnormal and incomplete development of the gubernaculum testis (Zimmermann *et al.*, 1999), although testosterone production is normal; the same result was observed following estrogen administration to females (Nef *et al.*, 2000); the over-expression of the aromatase gene too may induce abdominal cryptorchidism (Klonisch *et al.*, 2004). Androgens play a major role in the second part of the gonadal descent: in this phase they induce the regression of the gubernaculum testis which, in combination with the increase of intra-abdominal pressure caused by the increase in organ size, causes the testis to be " pushed "through the inguinal canal into the scrotum (Frey *et al.*, 1983). Amman (2007) divides the inguino-scrotal migration phase into two distinct phases: the trans-inguinal migration for which the presence of intra-abdominal pressure is sufficient (Arighi, 2011) for the crossing of the inguinal canal by testis and epididymis, and the inguino-scrotal migration, that is the path that brings the gonad from the external inguinal ring to the definitive position inside the scrotum for which the presence of androgens and the relative receptors is instead fundamental. Testosterone is, in fact, responsible for the masculinization of the genitofemoral nerve at an early stage of embryogenesis; this structure presents a sexual dimorphism and in the male fetus it develops following the path of the gubernaculum testis. Following the binding of androgens with the receptors on its surface, this nerve starts to produce and secrete CGRP (calcitonin gene-related peptide), a substance that is able to induce elongation of the gubernaculum testis in the right direction to guide the descent of the testes (Hutson *et al.*, 1998; Hutson and Hasthorpe, 2005; Ng *et al.*, 2005); the CGRP also stimulates the expansion of the vaginal process, the development of the cremaster muscle and the final regression of the gubernaculum itself. In the absence of testicular secretions, the target organs develop in the feminine direction. Thanks to the production of estradiol, Wolff's ducts regress, while the paramesonephric ducts remain and form the oviduct, the uterus, the cervix and the cranial portion of the vagina. The urogenital sinus differs in the

caudal portion of the vagina and in the vestibule. The genital tubercles give rise to the clitoris and the labioscrotal folds remain open, forming the vulva. The ovaries do not migrate but remain in the sublombar abdominal position (Christensen and Meyers-Wallen, 2011).

1.2 The disorders of sexual development

Sexual Development Disorders (DSDs) are all those "congenital conditions associated with atypical development of chromosome, gonadal or phenotypic sex" (Lee *et al.*, 2006; Hughes, 2008).
DSDs have been reported in humans and in different animal species (Lear and McGee, 2012).
However, although numerous studies have described the possible chromosomal rearrangements that have diversified the karyotypes in animal species of zootechnical and veterinary interest, there are still few studies on the relationship between hereditary diseases and intrinsic genetic causes.
For this reason, in the veterinary and zootechnical reality, it is not unusual to observe congenital anomalies that are described only phenotypically in literature only the phenotypic, but no genetic mechanisms underlying the anomaly and its diffusion are known. The diagnostic process should be structured starting from the examination of anomalies based on their origin: anomalies of the chromosomal, gonadal or phenotypic sex. In this regard Meyers-Wallen proposes a classification based on the identification of the first phase in which sexual development deviates from its normal evolution (Table 1).

Anomaly	Karyotype	Gonads	Müller Ducts	Wolff Ducts	External Genitalia	Diagnosis
Sexual chromosome	XXY	Testicles	Nobody	Epididimus, deferens ducts	M	XXY syndrome
	X0	Gonadal disgenesis	Uterus, Oviduct, Vagina	Nothing	F	X0 syndrome
	XX/XY	Ovaries/ Ovotestis/ Testicles	Variable	Variable	F/ ambiguous/M	Chimera
Gonadal Sex	XX	Testicles /Ovotestis	Uterus +/- Oviduct	+/- epididius vasa deferents	M/ ambiguous/F	XX sex reversal XX male or XX true hermaphrodite
Phenotypic sex: Female pseudohermaphroditism	XX	Ovaries	Uterus, Oviduct	+/epididimus	Ambiguous or M	Androgens or exogenous progestagens
	XY	Testicles	Uterus Oviduct	Epididimus Ducts deferens	M +/- criptorchid	Persistence syndrome of Müller's ducts
Male pseudohermaphroditism	XY	Testicles	Nessuno	Nothing +/- Epididimus, ducts deferens	F/ ambiguous	Testicular feminization (TFM) syndrome complete or incomplete

Tab. 1.1 Baseline characteristics of DSDs with reference to small animals: use of the classical nomenclature (Mayers-Wallen, 1999).

The terms intersex, hermaphrodite, pseudo-hermaphrodita (with a phenotype tending to the male or female) are being replaced with more appropriate terminologies and that allow a better identification of the situation of the examined subject (Table 2). Since 2006, in human, there has been a main revision of the nomenclature of pathologies related to the alterations of sexual development, the term "disorders of sex development" is now used and it includes all those pathologies in which the genitals appear abnormal in relation to chromosomal or gonadal sex (Kun Suk Kim et al, 2012). In the proposed classification, the karyotype arrangement is the prefix to DSD and this terminology is used in place of the old nomenclature (intersex, hermaphroditism, pseudohermaphroditism).

Old Nomenclature	**Revised Nomenclature**
Intersex	Disorders of Sex Development (DSDs)
Male pseudohermaphroditism Hypovirilization of an XY male Hypomasculinization of an XY male	46,XY DSD
Female pseudohermaphroditism Hypervirilation of a female XX female Masculinization of a female XX	46,XX DSD
Hermaphroditism	DSD Ovotestes
XX male o XX sex reversal	46,XX DSD testicles
XY sex reversal	46,XY DSD with complete gonadic disgenesy

Tab. 1.2 Application of the new nomenclature in clinical situations in human medicine.

Sexual Chromosome DSD	**46, XY DSD**	**46, XX DSD**
45,X (Turner's Syndrome) 47,XXY (Klinefelter Syndrome) 45,X/46,XY (gonadic disgenesy, DSD ovotesticolar) 46,XX/46,XY (chimerism, DSD ovotesticolar)	Disorder of gonadic development (testicular): • Complete or partial gonadal dysgenesis • Gonadal regression • Ovotesticular DSD Disorders in the synthesis or action of androgens: • Defects in androgen biosynthesis CAIS, PAIS • Hypoplasia of Leyding cells Disorders in AMH or AMH receptors (persistence syndrome of Müller's ducts) • Hypospadias • Cloacal estrophy	Disorder of gonadic development (ovaric) • DSD ovotesticular; • DSD testicular (*SRY*+, dup *SOX*9); • Gonadic Dysgenesis Excess of Androgens • Fetal • Foetoplacental • Maternal (luteoma, exogenous) Cloacal estrophy Mullerian agenesis; Renal agenesis; Cervicothoracic Somite abnormalities (MURCS)

Tab. 1.3 DSD Classification of Chicago consensus. Human Medicine (Hughes, 2008).

Although there are still some critical points in the new nomenclature, the acronym DSD has now been accepted by the medical community, included the veterinary one, and is commonly used in scientific literature (Kun Suk Kim *et al*., 2012. The adaptation of the new terminology to veterinary medicine could be difficult, above all due to the lack of specific definitions of the various phenotypes and their etiology; nevertheless, some of the proposed terms are used with unequivocal meaning even in the veterinary clinic (Villagòmez *et al.*, 2009).

1.2.1 Chromosomal sex disorders

Defects in the number or structure of sex chromosomes result in abnormal chromosomal sex. Chromosomal sex abnormalities include XXY, XO and XXX syndrome, hermaphrodite chimeras, XX/XY chimeras with testes and XY/XY chimeras with testes. Most animals with chromosomal sex abnormalities have few clinical symptoms. When animals are breeded in a farm or are part of a breeding program, the early detection of these anomalies is much more likely. The analysis of the karyotype is necessary to define errors in chromosome structure and number and in particular of sex ones. In all patients with suspected sexual development dysfunctions a series of clinical-diagnostic examinations is required in order to describe the subject as completely as possible:

• clinical and obstetric visit
• description of the general appearance
• accurate description of external and internal genitalia
• histopathology of the gonads and of all the surgically removable structures

Gonadectomy and hysterectomy are recommended in order to improve the life of the animal.

XXY syndrome

XXY or Klinefelter syndrome is the most commonly reported abnormality of sex chromosomes (Meyers-Wallen *et al.*, 1986) although, as previously mentioned, the true incidence of this disorder in dogs is unknown, whereas for the equine species in literature there are not many reported cases and only 6 are confirmed as Klinefelter (Bouters *et al.*, 1975; Makinen *et al.*, 2000; Iannuzzi *et al.*, 2004; Kakoi *et al.*, 2005). Klinefelter animals have a 2n + 1, XXY karyotype, hypoplastic testicles, epididymis, deferent ducts, male external genitalia that can evolve into hypoplastic testicles over time and are sterile. The presence of testes able to produce AMH and testosterone allow to have a normal male phenotype but the presence of two X chromosomes prevents the normal spermatogenesis, with consequent sterility.

XO syndrome

A 2n-1 karyotype, XO, also known as Turner's Syndrome, causes certain sterility of the individual, dysgenetic ovaries, internal and external genitalia developed as those of an impuberal subject. The monosomy of

the X chromosome and related forms of mosaicism are the first type of chromosome anomaly to have been described in the horse and are the most common in this species (Power, 1990; Villagòmez *et al.*, 2009). In the dog two cases of X chromosome monosomies are reported in which this condition has also determined phenotypic problems as reduced stature (Smith *et al.*, 1989, Lofstedt *et al.*, 1992) and prolonged proestrus (Johnston *et al.*, 1985).

XXX Syndrome

XXX syndrome is very rare (Johnston *et al.*, 1985), it X occurs in 1 on 1000 live human births and the affected women generally have a normal sexual development and are generally infertile, there are reports of normal reproductive cycles and developmental abnormalities (Hoang *et al.*, 1999).

True hermaphrodite chimera

True chimerism occurs when two or more cell populations deriving from different zygotes are present within an individual. The merging of two or more zygotes with a different set of sex chromosomes gives rise to a single zygote-chimera XX/XY. True hermaphrodites have both ovarian and testicular tissue, sexual combinations can appear among the most disparate (unilateral ovotestis with contralateral ovary or testis, bilateral ovotestis, unilateral ovary and unilateral testis). Three canine cases of true hermaphrodite chimeras have been reported. The karyotype of these individuals was XX/XY or XX/XXY; all had female phenotype with an enlarged clitoris (Meyers-Wallen, 1986). In the horse chimerism is a very rare event, Anaya *et al.* 2017, in a study conducted on the PRE, hypothesizes that the incidence of this disease in a population of about 200,000 animals is about 1%.

1.2.2 Gonadal sex disorders

Also named reverse sex disorder (Sex Reversal-SR) is that anomaly involving individuals with an apparently normal sex chromosomal arrangement XX or XY in which gonadal sex is not consistent with the chromosomal sex. In the dog it has been reported only XX SR: the affected dogs have a karyotype 78, XX with variable degrees of differentiation of the gonads in the masculine sense.
XX SR includes individuals:

- XX true hermaphrodites
- XX males
- XX true hermaphrodites with ovotestis
- XX males with bilateral testicles.

Eighty percent of XX human males are *SRY* positive due to the autosomal translocation of the Y chromosome. However, the XX male dogs described so far that have been tested for *SRY* gene are negative (Meyers-Wallen, 2006). It has been described as a family disorder probably of autosomal recessive type in many dog breeds (Meyers-Wallen, 1999), in the equine species, the XY DSD is sporadic although it has been shown a hereditary component in some genetic lines of Arabian breed and Quarter Horse (Kieffer *et al.*, 1976; Kent *et al.*, 1986; Bugno *et al.*, 2003). SR individuals of both 64 XX and 64 XY have been described in the horse and, after the X chromosome monosomy, this is the most common chromosomal anomaly (Lear and Bailey, 2008; Villagòmez *et al.*, 2009). Raudsepp *et al.* (2010) report that in the context of the diagnosis of chromosomal abnormalities performed over eight years, 26% is represented by XY DSD. The affected subjects are generally sterile; infertility is due to gonadal dysgenesis or testicular hypoplasia (Kent *et al.*, 1986, 1988; Power, 1986, 1990; Pailohoux *et al.*, 1995; Abe *et al.*, 1999; Makinen *et al.*, 1999, 2001; Hasegawa *et al.*, 2000; Bugno *et al.*, 2003; Switonski *et al.*, 2005; Villagòmez *et al.*, 2009). A further subdivision of the XY or XX DSDs can be performed based on the presence or absence of the *SRY* gene (*SRY*-negative or *SRY*-positive respectively).
In dogs, as well as in horses, phenotypic manifestations are numerous, such as: females with primary anaestro, females with abnormal vulva or males with bilateral cryptorchidism and prepuce and abnormal penis. A peculiarity of the dog is that the XX real hermaphrodites have both ovaries

and testicles. The gonads can have different combinations: bilateral ovotestis, an ovotestis and an ovary, an ovotestis and a testicle, the latter being the least common combination. The amount of testicular tissue is related to the degree of masculinization of the internal and external genitalia. In phenotypically female subjects or partially masculinized female phenotypes, they were been described: normal or abnormal vulva, a normal or enlarged clitoris (commonly with a Clitoris bone), uterus, oviducts, epididymis and vas deferens. SR XX males may have testes, Wolff's ducts (epididymis and vas deferens) and prostate, oviducts are usually absent, but a bicorn uterus may be present. The foreskin is usually of abnormal shape and moved caudally. Most males XX have a hypoplastic penis, hypospadias or abnormal curvature of the penis. Veterinary surgeons recommend gonadectomy and hysterectomy in all cases. To assess SR XX, the analysis of the karyotype is required, combined with the clinical or surgical evaluation of the presence of testicular tissue (at least one ovotestis or a testicle). A serum evaluation of the increase of testosterone in response to gonadotropin (GnRH) stimulation with human chorionic gonadotropin (hCG) can reveal the presence of testicular tissue; however, a negative stimulation test does not completely exclude the presence of testicular tissue. A PCR (Polymerase Chain Reaction) to test the presence of *SRY* gene can give official confirmation. Most of the XX true hermaphrodites and all the XX SR males are sterile. There is no laboratory test able to identify the carriers, the best recommendation is to inform the owners of reproducing subjects and not to use any brother of affected individuals for breeding, if possible, parents should be eliminated from the breeding program.

1.2.3 Phenotypic sex disorders

They are individuals in whom we have a balance between chromosomal and gonadal sex, but phenotypic gender (internal genitalia, external or both) is not in balance with gonadal one, for example, a phenotypically male XY dog with defects in the genital conformation. The syndromes that were been described up to now include:

- Cryptorchidism
- Female pseudohermaphroditism
- Male pseudohermaphroditism
- Persistent Muller's duct syndrome

• Defects in androgen-dependent masculinization

Cryptorchidism
It is the failure of the testicles to descend into the scrotum which leads to the incomplete development of phenotypic sex. The genetic and hormonal control of the testicular descent is the basis of this anomaly that were recently been considered a phenotypic sex disorder.

Female pseudohermaphroditism
A female pseudohermaphrodita has a chromosomal XX arrangement with internal or external ovary and masculinized genitals. Female pseudohermaphroditism is due to the virilizing effects of endogenous exposure to androgens but were not been reported in dogs or cats.

Male pseudohermaphroditism
Male pseudohermaphrodites have a XY chromosomal constitution, testes and feminization of internal or external genitalia. Male pseudohermaphrodites include XY males in which Muller's ducts fail to regress and individuals with defects in androgen-dependent masculinization (Bigliardi *et al.*, 2011).

Persistent Muller's Duct Syndrome (PMDS)
Sexual development disorder that affects males who have normal male reproductive organs and normal male external genitalia. However, they also have uterus and tubas derived from Müller's ducts. Usually Muller's ducts regress in males but persist in individuals with PMDS. The first signs of PMDS may include cryptorchidism or inguinal hernias. During surgery to treat these conditions the uterus and fallopian tubes are often found. Other features of PMDS may include abnormal placement of testes and of reproductive organs; transverse testicular ectopia (when both testicles descend from the same side); and infertility. PMDS is caused by mutations in the *AMH* gene (PMDS type 1) or *AMHR2* (PMDS type 2). It is inherited in an autosomal recessive manner. In some cases, the genetic cause is unknown. Persistent Muller's syndrome (PMDS) is recognized as a form of male pseudohermaphroditism in the Dwarf Schnauzer in the United States (Marshall *et al.*, 1982). In Dwarf Schnauzer, affected individuals are XY males, bilateral testicles, external male genitalia, but all derivatives of the Muller and Wolff ducts are present.

Defects in androgen-dependent masculinization

Animals with defects in androgen-dependent masculinization have a XY sex chromosomal constitution, bilateral testes and derivatives of Muller's ducts. However, internal and external genitalia show variable degrees of feminization. The resulting phenotype can range from complete (severe) to incomplete (mild). The subjects are classified according to the main defect:

• Defects in androgen production
• Androgen resistance or insensitivity, which includes defects in type 2alfa-reductase isoenzyme type (failure to convert T into DHT).
• Androgen receptor defects (testicular feminization).

Hypospadias, an abnormal position of the urethral orifice, is considered a rare congenital malformation of the reproductive organs in male dogs and occurs when there is incomplete masculinization of the urogenital sinus (closing of the urethra). The incidence of canine hypospadias is 0.003% (Hayes *et al.*, 1986), although considering more variables and the possibility of having today, more data on the real incidence, this could range around 0.3% (Carmichael, 2003; Caione, 2009; Switonski *et al.*, 2018). In humans it can occur as an isolated disorder or in association with other abnormalities, including cryptorchidism and other malformations of the urogenital tract (Bouty *et al.*, 2016). The molecular mechanisms underlying this anomaly, particularly in the sporadic form, are largely unknown. Many cases show further congenital anomalies, including a rudimentary or underdeveloped penis, abnormal prepuce and cryptorchidism (Cassata *et al.*, 2008; Jurka *et al.*, 2009). Genetic defects of LH receptors result in hypoplasia of Leydig cells, with severe hypospadias. Insufficient testosterone secretion induces cryptorchidism and / or micropenis (Latronico *et al.*, 2012; Vezzoli *et al.*, 2015). Mutations of the hydroxy-delta-5-steroid dehydrogenase gene and of the 3-beta gene and steroid-delta-isomerase 2 have been reported (Codner *et al.*, 2004). Mutations in the 5-alpha reductase gene (*SDR5A2*) are associated with a reduced synthesis of DHT (Wang *et al.*, 2004; Huhtaniemi *et al.*, 2006; Nicoletti *et al.*, 2005; Ocal *et al.*, 2002). It is also believed that the polymorphisms of *SRD5A2* genes contribute to the occurrence of the malformation. *MAMLD1* is one of the molecules that contributes to a

normal synthesis of testosterone (Fukami *et al.*, 2008). Its mutations in humans have been associated with severe phenotypes (Fukami *et al.*, 2006; Igarashi *et al.*, 2015). A *MAMLD1* missense mutation was also described in a gonadal dysgenesis syndrome (p.P677L) with a severely reduced in vitro transactivation of the promoter of the *HES3* target gene (Ruiz-Arana *et al.*, 2015). In Leydig cells, *MAMLD1* increases the expression of *CYP17A1* and allows the production of a quantity of testosterone necessary for male differentiation (Nakamura *et al.*, 2011). Defects in hormonal genetic management during fetal life and normal plasma testosterone in adult hypospadic patients with modified *MAMLD1* exhibit transient impairment of the testicular function of Leydig cells before birth. Another possible candidate for the fetal development of hypospadias is the Androgen Receptor (AR) gene, placed on the Xq12 chromosome, since it mediates an important biological effect of testosterone and DHT (Wilhelm *et al.* 2007). Its function is fundamental in the developing male fetus (Sinclair *et al.* 2005) by regulating the expression of genes that deal with androgen receptor function once that the complex has been formed with DHT or with testosterone in the cytoplasm (Alam *et al.* 2007). The receptor function can be modified by mutations in the AR gene that can cause partial or complete androgen insensitivity syndrome and, in some cases, hypospadias (Meyers-Wallen 1999; Villagomez *et al.*, 2009; Parma *et al.*, 2006)

Agenesis and dysgenesis of the reproductive tract

Agenesis is the absence of a structure or a system of organs due to lack of formation during embryonic development. Dysgenesis is a partial defect in the development of a structure or an organ. The most observed malformations are: agenesis or dysgenesis of the gonads, Muller or Wolff's ducts, urogenital sinus, genital tubercle or genital sketches. Other examples include monorchidism and testicular hypoplasia; ovarian agenesis and ovarian hypoplasia; segmental aplasia of epididymis, vas deferens, oviducts, uterus and vagina; hypoplasia of the penis. In females, the agenesis of Muller caudal ducts or urogenital sinus can give rise to a wide variety of vaginal anatomical abnormalities.

1.3 DSD in the Dog and the Horse

The onset of DSDs is an emerging problem in dog breeding and among the main causes of subfertility or sterility of horses, involving a wide variety of phenotypes: from phenotypically normal females with gonadal dysgenesis to subjects with ambiguous external genitalia and male and female internal organs (Christensen BW 2012, Lear *et al.* 2012). The types of DSDs in the canine species that have been studied are cryptorchidism (multifactorial XY DSD) diagnosed only in advanced age in 78 XY *SRY*-Positive subjects and the presence of an abnormal reproductive apparatus in subjects 78 XX with presence or absence of the *SRY* gene. Recently, it has been shown that hypospadias (multifactorial XY DSD) occurs more frequently than previously hypothesized (Switonski *et al.*, 2018).
Up to now, four types of DSDs have been diagnosed in the equine species:
1) Sexual chromosomal abnormalities (63, X, 64, XX/64, XY, 65, XXX, 65, XXY, etc.);
2) 64, XX *SRY*-negative with DSD;
3) 64, XY *SRY*-positive with DSD;
4) 64, XY *SRY*-negative.
In genetically XX horses, unlike cats and humans (Wu QY *et al.*, 2014; Szczerbal *et al.*, 2015), the *SRY*-positive DSD has never been reported, probably due to the localization of the *SRY* gene. In fact, in the equine species the *SRY* gene locus is far from the pseudoautosomal region and may therefore be less susceptible to meiotic wrong translocations during gametogenesis (Raudsepp *et al.* 2016). Also, for this species large-scale DNA profiling or cytogenetic examination of equine populations (Bugno *et al.* 2007; Demyda-Peyras *et al.* 2013) and the lack of a good case registration system suggests that the available clinical data underestimate the real prevalence of these anomalies.

1.3.1 EXPERIMENTAL PART

The work carried out during the PhD period was concentrated in the study of phenotypic and genetic characterization of DSDs cases in the canine and equine species. The cases were recruited during the normal genetic counseling activity that the Laboratory of Veterinary Genetics and Biotechnology applied to Animal Productions of the University Federico II of Naples held at the University Veterinary Didactic Hospital (OVUD) of the Department of Veterinary Medicine and Production Animals (MVPA) and for freelancers operating throughout the national territory.
Some of the studied cases have been recruited thanks to a collaboration with the School of Biosciences and Veterinary Medicine, University of Camerino, Matelica (MC).
A part of the genetic analysis was carried out in collaboration with the Poznan University of Life Sciences, the Faculty of Veterinary Medicine and Animal Sciences, during the PhD internship period.

1.3.2 MATERIALS AND METHODS

Recruitment of cases

16 cases of DSDs were studied, 11 subjects belonging to the canine species (Table 4) and 5 subjects belonging to the equine one (Table 5). All subjects were phenotypically and genetically characterized. In Tables 5 and 6 are reported data, phenotypes and investigations carried out on the individual cases.

Case	Breed/Age	*Phenotypic Sex	Clinical Findings	Exams carried out
D1	Mixedrace – 4m	F	Penile Hypospadias/ micropenis	Karyotype
D2	Poodle – 12y	F	Penile Hypospadias	Karyotype, surgery, Histology
D3	Chihuahua – 1y	M	Penile Hypospadias	Karyotype,
D4	German Sheperd – 5m	M	Normal Reproductive Apparatus but urine by anus	Karyotype
D5	Mixedrace – 1y 9m	F	Penile Hypospadias	Karyotype
D6	Mixedrace – 1y 9m	F	Penile Hypospadias	Karyotype
D7	Jack Russell – 5m	F	Rudimentary penis	Karyotype
D8	Jack Russell – 1y	M	Perineal Hypospadias	Karyotype, surgery, Histology
D9	Pit Bull – 9m	F	Megaclitoride from which protrudes a rudimentary penis	Karyotype, surgery, Histology
D10	Bull Dog – 6m	F	Megaclitoride, micropenis and penis bone	Karyotype
D11	French Bull Dog – 8m	F	Megaclitoride	Karyotype

Tab 1.4 Summary table of Dog DSD cases; * phenotypic sex indicated before genetic diagnosis.

Case	Breed-Age	*Phenotypic Sex	Clinical report	Exams carried out
H1	Thoroughbred Arabian – 5y	F	normal vulva; Uterus decreased in volume; Non-palpable gonads. nulliparous	Transrectal ultrasound; Exploratory Laparoscopy; Histological examinations; Cytogenetic and molecular examinations;
H2	Thoroughbred Arabian – 12y	F	Vulva of reduced dimensions; Uterus decreased in volume; Non-palpable gonads. Nulliparous; Irregular oval cycles	Transrectal ultrasound; Exploratory Laparoscopy; Cytogenetic and molecular examinations;
H3	Thoroughbred Arabian – 5y	M	micropenis; No scrotum; Gonadi not identifiable neither in the canal or in the abdomen. No libido	Transrectal and transinguinal ultrasound; Exploratory Laparoscopy; Histological examinations; Cytogenetic and molecular examinations;
H4	Cavallo Agricolo Italiano Tiro Pesante Rapido – 3y	M	Penis and foreskin in the norm; Left eutopic testis; Right testis not identifiable neither in channel inguinal or in the abdomen	Transrectal and transinguinal ultrasound; Exploratory Laparoscopy; Orchiectomy; Histological examinations; Cytogenetic and molecular examinations;
H5	Sella Italiano- 15 months	Ambiguous	No scrotum; Mammary glands modally developed; median rafe raised ventral to the anal sphincter and terminating with caudally oriented micropenis; inguinal gonads; Stallion Behaviors	Transrectal and transinguinal ultrasound. Orchiectomy Histological examinations Cytogenetic and molecular examinations

Tab 1.5 Summary table of Horse DSD cases; * phenotypic sex indicated before genetic diagnosis.

For each clinical case the following data were collected:

- photographic finds;
- medical history, clinical findings (general and particular objective examination of the reproductive apparatus) and instrumental examinations (ultrasound and radiography);
- blood samplings for cytogenetic, genetic and biochemical analyses;
- surgical findings, when possible
- Histological examinations.

Laboratory exams

Blood samples
For each case a blood sample was taken in heparinized vacutainer tubes (Sodium heparin or Lithium heparin) and vacutainer tubes with K3 - EDTA. The blood was transported as quickly as possible to the laboratory. Lymphocyte cell cultures were set up from heparinized blood, while genomic DNA was extracted from the blood in K3-EDTA tubes. Where possible, a sampling was also carried out for hormonal levels, in this case the blood transported at 0-4°C serum is centrifugated at 3000rpm for 10 minutes and sent to an external laboratory.

Histological examination
Where possible, histological examination of the gonads or similar structures and of the reproductive apparatus surgically removed was carried out, the tissues were fixed in 10% neutral buffered formalin for at least 48 hours, dehydrated and clarified using a processor, subsequently included in paraffin and cut. The sections obtained in this way were stained and observed under a microscope.

Cytogenetic analysis
From the heparinized blood the lymphocyte cell cultures were prepared according to the protocols described in Ciotola *et al.*, (2012) for the equine species and in Reimann-Berg *et al.* (2012) for the canine species. For each subject, C- and R-banding techniques, to highlight any structural anomalies, were applied. The karyotypes were arranged according to the

standard karyotype of the dog (Reimann *et al.;* 1999) and of the horse (Iannuzzi *et al.;* 2003).
For the horse CASE H5 the following probes were used for the FISH: BAC specific clone147K8 for ECAY from CHORI-241 Library (https://bacpacresources.org/) and BAC horse 102C09 and 111A23 from the INRA library (Milenkovic et to the. 2002). The protocol followed was the one described by Albarella *et al.* (2018).

Molecular genetic analysis
The DNA extracted with WIZARD Genomic DNA Purification Kit (code A1125, Promega) was used to study some specific genes for the identification of the Y chromosome, in particular the search of the *SRY* gene was performed on all subjects with DSD belonging to both equine and canine species. The used primers and PCR protocols are shown in Table 6. As for the equine species, in addition to the *SRY* gene they were evaluated: *ZFY*, *ZNF33bY*, and *EIF3CY*. Furthermore, in case 5 the *EIF2s3Y* and *HPRT* genes were examined. The used primers and PCR protocols are shown in Table 6. The fragments obtained with PCR were visualized by 1.5% agarose gel electrophoresis.

The 8 dog cases with chromosomal and genetic arrangement 2n=78, XY *SRY*-positive, affected by hypospadias, have been analyzed for some specific genes involved in the development of the reproductive system, *SRD5A2* and *MAMLD1*, already studied in dogs (Switonski *et al.*, 2012), and AR studied in cats (Nowacka-Woszuk *et al.*, 2014). In particular, for AR, it has been observed that an increasing number of CAG repeats results in a lengthening of the polyglutamine traits and causes a reduced transcriptional activity that can be associated in the XY males with a moderate to severe genitalia underdevelopment (Lim HN *et al.*, 2000).
The length of CAG repeats has been studied in different cases of hypospadias (Aschim *et al.*, 2004; Muroya *et al.*, 2001). 11 control subjects were also analyzed (Tab. 8, Tab. 9, Tab. 10) because the model sequences obtained from www.ncbi.nlm.nih.gov (07.2018) and from www.ensembl.org (07.2018) are often derived from the human genome and need to be corrected. Table 6 shows the primers and protocols for the analysis of *SRD5A2* and *MAMLD1* and AR genes.

Species	Gene	Region	Primer		Annealing t	Annealing T	Bp	References
ECA	***SRY***		F	5'TGCTATGTCCAGAGTATCCAACA3'	30"	60°	697bp	Han *et al.*, 2010
			R	5'TGAGAAAGTCCGGAGGGTAA3'				
CFA	***SRY***		F	5'CTTTCCAACTTCCCTCCGTA3'	30"	57°	813bp	
			R	5'GGACGTTTCGTTAGCCAGAG3'				
ECA	**ZFY**		F	5'AAATCAAAACCTTCATGCCAAT3'	30"	58°	553bpY/ 604bpX	Han *et al.*, 2010
			R	5'TTCCGGTTTTCAATTCCATC3'				
ECA	**ZNF33bY**		F	5'CCACAGCAAATACAGGAGCA3'	45"	58°	800bpY/ 3000bpX	Paria *et al.*, 2011
			R	5'GTCTGACTCCTCCCCCTTTC3'				
ECA	**EIF3CY**		F	5'CCCAAGCAGGGTACCTATGG3'	30"	58°	134bpY/ 230bpX	Paria *et al.*, 2011
			R	5'GGACAGAAGTGACGCAATCA3'				
ECA	***SRY***		F	TGCTATGTCCAGAGTATCCAACA		58°	697bp	Han *et al.* 2010
			R	TGAGAAAGTCCGGAGGGTAA				
ECA	**ZFX/Y**		F	AAATCAAAACCTTCATGCCAAT		58°	553bp/X 604bp	Han *et al.* 2010
			R	TTCCGGTTTTCAATTCCATC				
ECA	**EIF2s3Y**		F	GAGCCATCTGTGTGATCGTC		58°	223bp	Paria *et al.*, 2011
			R	TATTCCTGGCCCTAAGCACA				
ECA	**ZFY**		F	TGAGCTATGCTGACAAAAGGTG		58°	186bp	Paria *et al.*, 2011
			R	TCTTTCCCTTGTCTTGCTTGA				
ECA	***SRY***	**Q**	F	ACAGTCACAAACGGGAGGAG		58°	149bp	
			R	AAAGGGAACGTCTGCGTATG				
ECA	**HPRT**		F	GAGGCCATCACATTGTAGCA		58°	381bp	
			R	TCCCCACAGCAATTCTTACA				
CFA	**AR**	ex1a	F	ATCGCAGCCTGTTGAACTCT		58°	907bp	
			R	AGCGGGGCGTACATACAA				
CFA	**AR**	ex1b	F	CTAGGTGGCAGTTCGACCAT		58°	939bp	
			R	CACCGACTAGGGTGATTCAAA				
CFA	**MAMLD1**	ex3	F	CCCTAAGCCCTGTGTCTAGGT		58°	268bp	
			R	GCCAACAAGTTTGCACAGAG				
CFA	**MAMLD1**	ex4 1	F	TCTATCTGCCACAGGGAACC		60°	648bp	
			R	CCGAGGGGATCACGTAGTT				

CFA	**MAMLD1**	ex4 2	F	GCCACCTGCTATCTGCTCTG		58°	666bp	
			R	ACGCGAGTCGTCTTACCTGT				
CFA	**MAMLD1**	ex5	F	GCTACACCAAAGCCAAGTCC		58°	257bp	
			R	CTGTTGAACTGAGCCGACTG				
CFA	**MAMLD1**	ex6a	F	GGCAGTTTCGAAGAGTCGAG		58°	613bp	
			R	CCTGGGCCAAGACAGAGTT				
CFA	**MAMLD1**	ex6b	F	AGTCCCCACCAGCTCAGAC		58°	793bp	
			R	GGGTTGTGAATTTGCAGAGG				
CFA	**SRD5A2**	ex1	F	CGTCTAGCGCCCCATAAAG		58°	407bp	
			R	CAACCGCTACCTGTGGAAGT				
CFA	**SRD5A2**	ex2 1	F	GGGTTTGATGCAATTGTCTG		60°	459bp	
			R	ATTCTCAGGTGCGGAGTGAC				
CFA	**SRD5A2**	ex2	F	TCTTTCCTGTCTCCAGGACATT		60°	241bp	
			R	TTTAGGCTGTGGGAAGTGGT				
CFA	**SRD5A2**	ex3	F	TGGGACAATGGCTAATTGGT		60°	402bp	
			R	AGGAGAAGTCAGGGGCAAAT				
CFA	**SRD5A2**	ex4	F	AGCAGCAGTGGGTGCTACTC		60°	503bp	
			R	AGTTCAGTGAAAGCCCAGGA				
CFA	**SRD5A2**	ex5	F	GAGAAAGGAGGTGGGCAAAT		60°	831bp	
			R	AGCCAGAGGAGCTGAGACAG				

Tab. 1.6 Primers and protocols used for PCR.

The Sanger technique was used to sequencing. Amplification was performed with the Bigdye v3.1 Cycle Sequencing Kit terminator. Capillary electrophoresis was performed with the Genetic Analyzer 3130 (Applied Biosystems). The DNAstar software was used to search for polymorphisms.

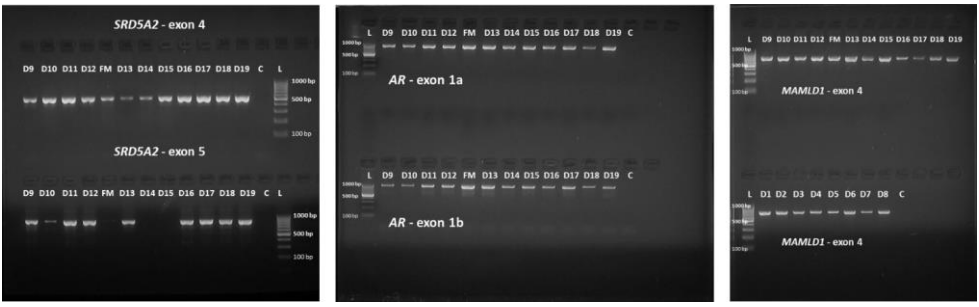


Fig.1.1 Example of MAMLD1, SRD5A2 and AR primers preformed during the experiment.

1.3.3 Description of DSD in the canine species

CASE D1

Anamnesis: half-breed dog born on 01/07/2011, medium-small size.
EOG: the subject is not affected by particular pathologies and is phenotypically feminine.
EOP: anomaly of the external genitalia characterized by the presence of a structure similar to a penis that protrudes from the vulva.
Laboratory tests: aneuploidy, karyotype analysis, study of *SRY*, SRD5A2 and MAMLD1 genes.

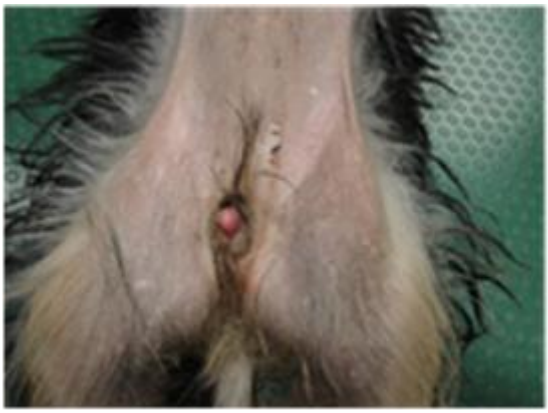


Fig. 1.2 Detail of external genitalia.

CASE D2

Anamnesis: Phenotypically feminine poodle born on 08/15/2001.
EOG: the subject is not affected by particular pathologies and is phenotypically feminine.
EOP: perineal hypospadias, the penis is rudimentary with urethral orifice in cranial position.
Surgery: surgically two structures similar to small testicles were removed.
Laboratory tests: aneupliody, karyotype analysis, study of *SRY*, SRD5A2 and MAMLD1 genes; histological examination of the removed gonads.

CASE D3

Anamnesis: Chihuahua, phenotypically referred to as a male, born on 02/07/2012.
EOG: the subject is not affected by particular pathologies.
EOP: penile hypospadias and bilateral cryptorchidism. There is a rudimentary penis in a dorsoventral position, with an incomplete prepuce and a urethral meatus in a caudal position.
Surgery: removal of the testes found in the abdomen.
Laboratory tests: aneupliody, karyotype analysis, study of *SRY*, SRD5A2 and MAMLD1 genes; histological examination of the removed gonads.

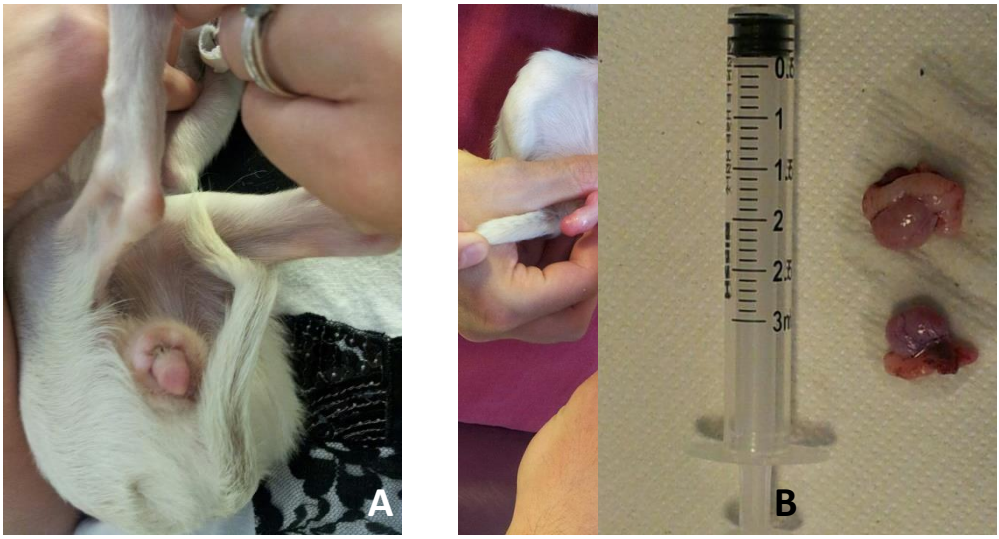


Fig.1.3 A e B detail of external genitalia. C, testes surgically removed.

CASE D4

Anamnesis: Phenotypically male German Shepherd, born 10/08/2013, brought to visit because the urine, in part, comes out from the anus.
EOG: It does not show pathological conditions.
EOP: the subject has a complete reproductive apparatus. Hypoplasia of the perianal wall muscles was found, and a duct was present starting from the urethra and with outlet at the level of the internal anal sphincter. The testicles are both present.
Laboratory tests: aneupliody, karyotype analysis, study of *SRY*, SRD5A2 and MAMLD1 genes.

CASE D5

Anamnesis: phenotypically female half-breed born on 15/02/2016, sister of case 6.
EOG: the subject is not affected by pathologies.
EOP: perineal hypospadias characterized by the presence of a rudimentary penis of reduced size with incomplete foreskin, for almost all its length is crossed by a deep and linear opening that originates from the perineum and continues cranially. This fissure crosses the two testicles medially, giving rise to a bifid scrotum. The urethral orifice is located in the perineum region, ventral to the anus, in the typical localization of the urethra of a female subject.
Instrumental examinations: abdominal ultrasound which excludes the presence of gonads or other female structures.
Laboratory tests: aneupliody, karyotype analysis, study of *SRY*, SRD5A2, MAMLD1 and AR genes.

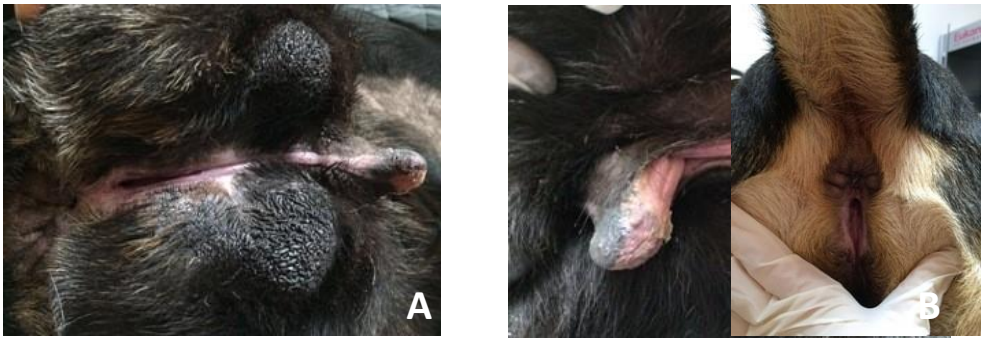


Fig. 1.4 A ventro-dorsal detail of external genitalia; B detail of Hypospadic penis; C, caudal view.

CASE D6

Anamnesis: phenotypically female half-breed born on 15/02/2016, sister of the case 5.
EOG: the subject is not affected by pathologies.
EOP: perineal hypospadias characterized by the presence of a rudimentary penis of reduced size with incomplete foreskin, for almost all its length is crossed by a deep and linear opening that originates from the perineum and continues cranially. This fissure crosses the two testicles medially, giving rise to a bifid scrotum. The urethral orifice is located in the perineum region, ventral to the anus, in the typical localization of the urethra of a female subject.
Instrumental examinations: abdominal ultrasound which excludes the presence of gonads or other female structures.
Laboratory tests: aneupliody, karyotype analysis, study of *SRY*, SRD5A2, MAMLD1 and AR genes.

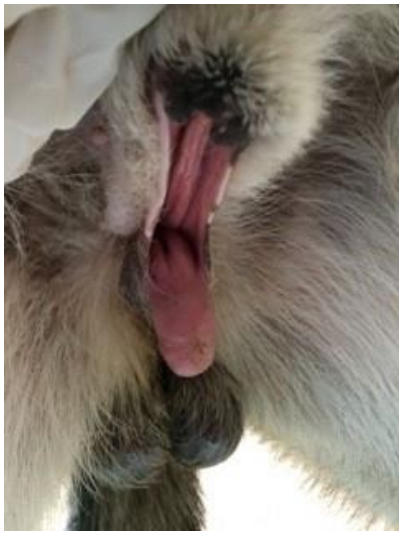


Fig. 1.5 Detail of hypospadic penis.

CASE D7

Anamnesis: Jack Russell Terrier, phenotypically female, born on 01/08/2017.
EOG: the subject is not affected by pathologies.
EOP: the subject has a structure similar to a rudimentary penis with urethral meatus in apical position. Estral manifestations with loss of blood and urine.
Instrumental examinations: abdominal ultrasound that highlights the presence in the abdomen of the left testicle, of a structure that resembles a uterus and the presence of penis bone; X-ray of the abdomen in latero-lateral projection confirms the presence of the penis bone; cystography detects a communication between the uterus and the bladder.
Surgery: a uterus-like structure and two testes-like structures have been surgically removed.
Histological examination: the structures removed during surgery have been histologically analysed.
Laboratory tests: aneupliody, karyotype analysis, study of *SRY*, SRD5A2, MAMLD1 and AR genes.

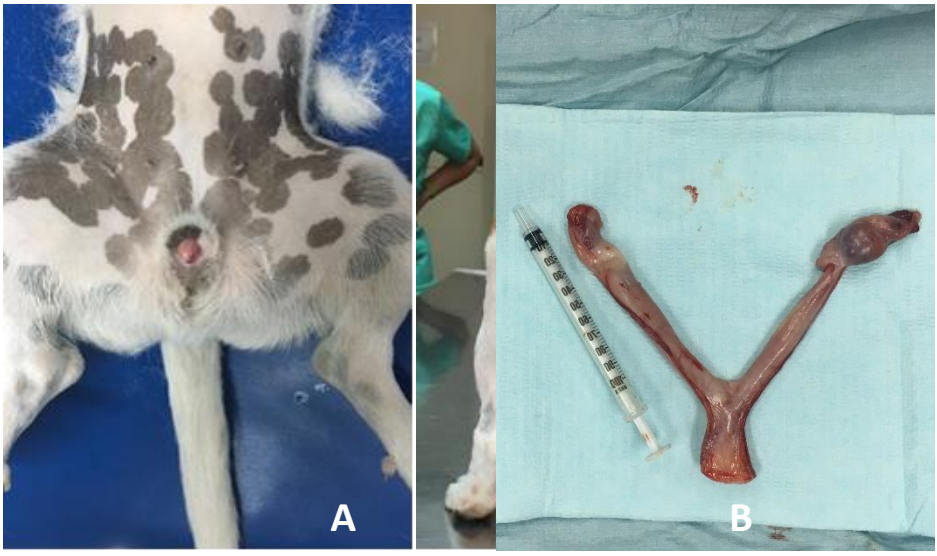


Fig. 1.6 A ventro-dorsal detail of external genitalia; B, caudal view; C, structure surgically removed.

CASE D8

Anamnesis: Jack Russell Terrier phenotypically male
EOG: the subject is not affected by particular pathologies.
EOP: the subject presents perineal hypospadias characterized by a rudimentary penis with urethral meatus in cranial position and a cavity positioned in a retro-scrotal position.
Instrumental examinations: abdominal ultrasound shows in the left side of the abdomen a testis-like structure.
Surgery: ectopic gonads are removed.
Histological examination: the structures removed during surgery have been histologically analysed.

Fig. 1.7 A e B detail of hypospadic penis.

CASE D9

History: American Pitt Bull terrier, phenotypically female, born on 02/02/2016.
EOG: the subject is not affected by particular pathologies.
EOP: presence of a protruding structure that is manually extracted like a small penis.
Instrumental examinations: abdominal X-ray in latero-lateral projection shows the presence of penis bone; Abdominal ultrasound does not detect gonads in the inguinal canal but highlights the presence of two structures caudally to the kidneys and similar to ovotestis.
Laboratory tests: aneupliody, karyotype analysis, study of *SRY* gene.

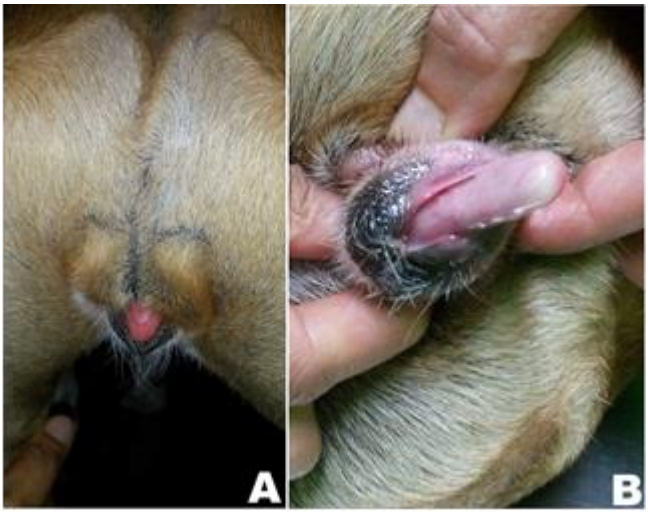


Fig. 1.8 A, caudal view; B, detail of protruding structure.

CASE D10

Anamnesis: Bulldog identified phenotypically as a female, born on 01/07/2016.
EOG: the subject is not affected by particular pathologies.
EOP: presence of a protruding structure with the appearance of micropenis with bone.
Laboratory tests: aneupliody, karyotype analysis, study of *SRY* gene.

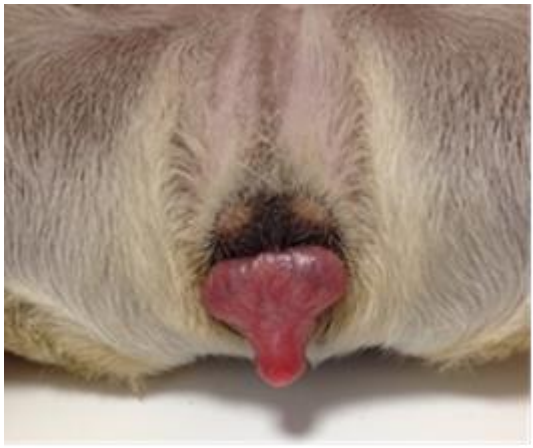


Fig. 1.9 Detail of protruding structure.

CASE D11

Anamnesis: French Bulldog with a phenotypically feminine appearance, born on 12/05/2017.
EOG: the subject is not affected by particular pathologies.
EOP: presence of a megaclitoride-like structures with probable presence of penis bone.
Surgery: tubular structure similar to a hypoplastic uterus is removed. It shows a left bifurcation that continued with a structure resembling a vas deferens connected to a testicle placed in the inguinal area on the left. The prostate was absent. The clitoris / penis contained a cartilaginous structure like a penis bone. The vagina communicated directly with the pseudo-uterus.
Histological examination: the structures removed during surgery have been histologically analysed.
Laboratory tests: aneupliody, karyotype analysis, study of *SRY* gene.

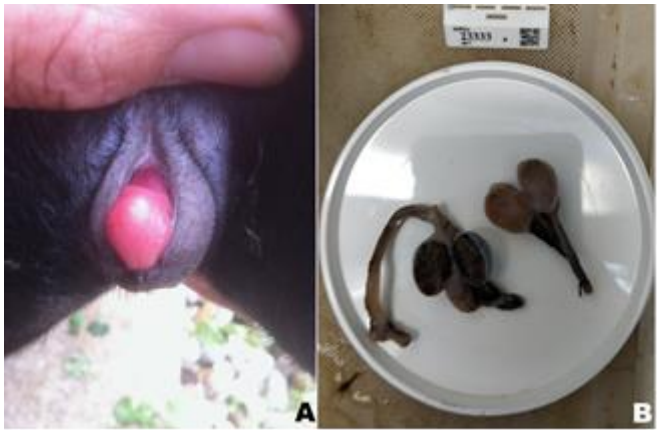


Fig. 1.10 A megaclitoride-like structures; B, structure surgically removed.

1.3.4 Results and discussions

The results of cytogenetic and molecular tests are shown in Table 1.7.

The results of the histological examinations are as follows:

CASE D2: in the sections it is possible to observe a normo-structured testicular parenchyma consisting of seminiferous tubules that conserve the germline. Tubules include roundish or oval interstitial cells with clear cytoplasm containing lipid vacuoles. The described morphological pattern is compatible with a normal testicular parenchyma.
Case D7: the histological examination shows that the removed structures are testicles and uterus; the presence of ovarian tissue is excluded.
CASE D8: the histological examination confirms the presence of testes and tissues similar to dermis and to cavernous bodies.
CASE D11: Both gonads showed a testicular structure characterized by seminiferous tubules lined by Sertoli cells without germ cells. Interstitial tissue was thick and many Leydig cells were present. The epididymis was present and showed empty tubules lined with columnar epithelium. The abdominal gonad showed a strong hematic component diffused in the interstitial tissue. The tubular structure showed a uterine feature with a lumen lined by simple columnar epithelium lifted in short papillae. Under the epithelium a cellular rich stroma surrounded glandular structures.

Clinical, cytogenetic and genetic data allowed to identify the type of DSD of the 11 cases, of which 8 presented a chromosomal and genetic arrangement 2n=78,XY *SRY* positive, and 3 are 2n=78,XX *SRY*-negative. Last three cases are united by the presence of a megaclitoride and penis bone but only case 11 is classifiable as female pseudohermaphrodita since it combines a chromosomal arrangement 2n=78,XX with the presence of internal and external masculinized gonads and genitals. The 8 cases with chromosomal and genetic arrangement 2n=78,XY *SRY* positive were classified as hypospadias with the presence of different types of underdeveloped penis. CASE D4 has been classified as hypospadias as possible because of the abnormal position of the urethral meatus that is a reference point in the diagnosis of this condition. Sequencing results of *SRD5A2*, *MAMLD1* and *AR* genes, carried out in dogs 2n=78,XY; *SRY*-positive and in 11 control cases are shown in Tables 8, 9 and 10.

Case	*Phenotypic sex	Genetic Sex	*SRY*	Diagnosis
D1	M	2n= 78, XY	*SRY*+	Penile Hypospadias
D2	M	2n= 78, XY	*SRY*+	Penile Hypospadias
D3	M	2n= 78, XY	*SRY*+	Penile Hypospadias
D4	M	2n= 78, XY	*SRY*+	Possible Hypospadias
D5	M	2n=78, XY	*SRY*+	Perineal Hypospadias
D6	M	2n=78, XY	*SRY*+	Perineal Hypospadias
D7	M	2n= 78, XY	*SRY*+	Penile Hypospadias
D8	M	2n= 78, XY	*SRY*+	Penile Hypospadias
D9	F	2n= 78, XX	*SRY*-	XX Real hermaphrodyte
D10	F	2n= 78, XY	*SRY*-	XX hermaphrodyte
D11	F	2n= 78, XY	*SRY*-	XX Real hermaphrodyte

Tab. 1.7 Cytogenetic and molecular results. *After genetic analyzes.

SRD5A2					
N.O.	**g:25027358G>A (exon 2) p.Ala120Thr**	**g:25027317C> T (exon 2) silent**	**g:25027268A> G (intron 2) rs853051170**	**g:25022560C> T (intron 3) rs852995854**	**g:25020322C> T (intron 3) rs22574661**
D1	GG	CC	AA	CC	**CT**
D2	GG	**CT**	AA	**CT**	**TT**
D3	GG	CC	AA	CC	**TT**
D4	**GA**	CC	**AG**	CC	**CT**
D5	GG	CC	AA	**CT**	**TT**
D6	GG	CC	AA	**CT**	**TT**
D7	GG	CC	**AG**	CC	**TT**
D8	GG	CC	**GG**	CC	**TT**
D9	GG	CC	**AG**	CC	**TT**
D10	GG	CC	AA	CC	**TT**
D11	**GA**	CC	**AG**	CC	**CT**
D12	GG	CC	AA	CC	**TT**
D13	**GA**	CC	**AG**	CC	**CT**
D14	GG	CC	AA	CC	**TT**
D15	GG	CC	AA	**CT**	**TT**
D16	GG	CC	AA	CC	**TT**
D17	GG	CC	AA	CC	**TT**
D18	GG	CC	AA	CC	**TT**
D19	GG	**CT**	AA	**CT**	**TT**

Tab. 1.8 Polymorphisms identified in *SRD5A2* gene: bold text evidence the homozygous genotype, underlined text evidence the heterozygous genotype.

MAMLD1			
Number case	**g.118737647A>G rs851570581 p.Asn43Ser**	**g.118774550C>G rs24609865 (silent)**	**g.118775244C>T rs851139956 (silent)**
D1	A	**G**	**T**
D2	A	**G**	**T**
D3	A	C	C
D4	A	**G**	**T**
D5	A	C	C
D6	A	**G**	**T**
D7	A	C	C
D8	**G**	**G**	**T**
D9	A	**G**	**T**
D10	A	**G**	**T**
D11	A	**G**	**T**
D12	A	**G**	**T**
D13	A	**G**	**T**
D14	A	C	C
D15	**G**	**G**	**T**
D16	A	**G**	**T**
D17	A	**G**	**T**
D18	A	C	C
D19	A	**G**	**T**

Tab. 1.9 Polymorphisms identified in *MAMLD1* gene: bold text evidence the alternative genotype.

AR			
N.O.	**g.51969945_51969946insGCA p.Leu54_Gln55insGln_rs851 250652**	**$(CAG)_{10/11}$**	**$(CAG)_{10}$/11/12/13(CA A)(CAG) (CAA)$(CAG)_6$(CA A)$(CAG)_2$**
D1	del	10	11
D2	**ins**	**11**	11
D3	**ins**	**11**	11
D4	**ins**	**11**	11
D5	del	10	11
D6	**ins**	**11**	11
D7	del	10	11
D8	del	10	11
D9	**ins**	**11**	11
D10	del	10	**12**
D11	**ins**	**11**	11
D12	**ins**	**11**	11
D13	**ins**	**11**	11
D14	**ins**	**11**	11
D15	del	10	**10**
D16	del	10	**10**
D17	del	10	11
D18	del	10	**13**
D19	del	10	11

Tab. 1.10 Polymorphisms identified in *AR* gene: bold text evidence the alternative genotype.

In both clinical and control cases, the sequencing of *SRD5A2* and of *MAMLD1* showed the presence of polymorphisms in intronic and exonic regions, respectively; however, such polymorphisms do not seem to have an impact on sexual development. The Odds Ratio test showed no statistically significant differences. Regarding *AR* sequencing, no association between the CAG repeat length and the investigated cases with respect to controls was found, and no mutations or polymorphisms associated with the altered phenotype were found. The Odds Ratio test showed no statistically significant differences.

1.4 Description of DSD in the equine species

CASE H1

Anamnesis: female born in 2010 (5 years old at the time of the visit), saura, PSA race, nullipara. The behavioral anamnesis does not report abnormalities. A gynecological examination is required to evaluate its reproductive potential as a mare.
EOG: the subject has an apparently feminine phenotype.
EOP: the vulva is of normal size but barely at the ventral commissure level. The mammary glands are in the physiological position and in a stage of development compatible with non-pregnant subjects.
Instrumental examinations: Transrectal ultrasound show a slightly smaller cervix and uterus; the gonads are not visible with ecography. Endometrial edema (score 1-2) is evident during estrus. The gonadal structure appears as an outline like a hypoplastic ovary; the wide ligament, the ligament proper to the ovary and a tubular structure like the uterine tuba are identifiable.
Laboratory tests: aneuploidy, karyotype analysis, study of the C bands, analysis of the *SRY* and ZFY Y, ZNF33bY and EIF3CY genes.

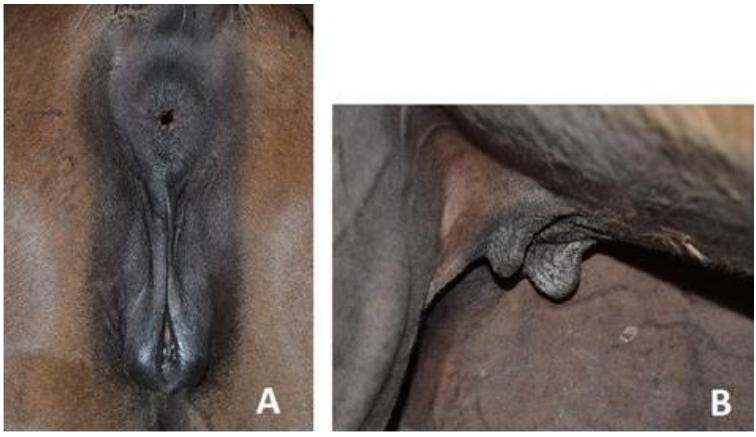


Figure 1.11 External genitalia of CASE H1. A vulva of normal size, with just-vented ventral commissure; B, breasts and nipples compatible with a nulliparus mare.

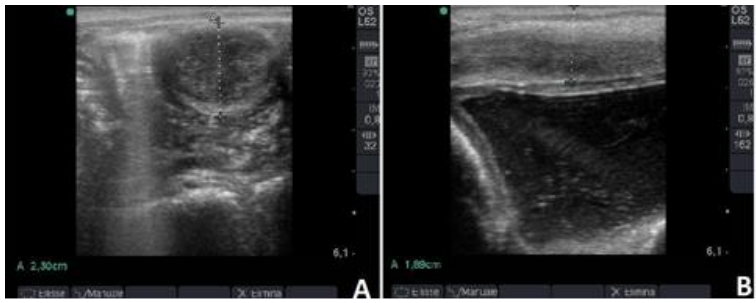


Figure 1.12 Ultrasound image of the uterus. A, uterine horn, scan in short axis; B, uterine body, long axis scan.

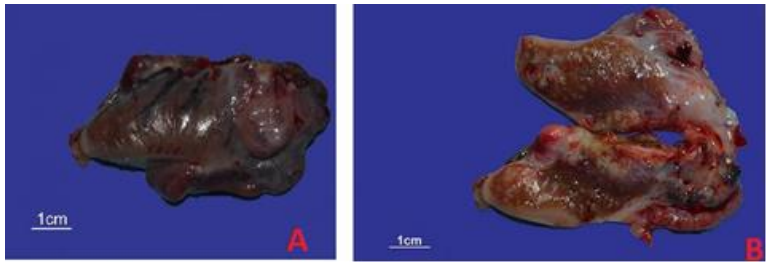


Figure 1.13 Gonads removed. A, external surface; B, gonadal parenchyma.

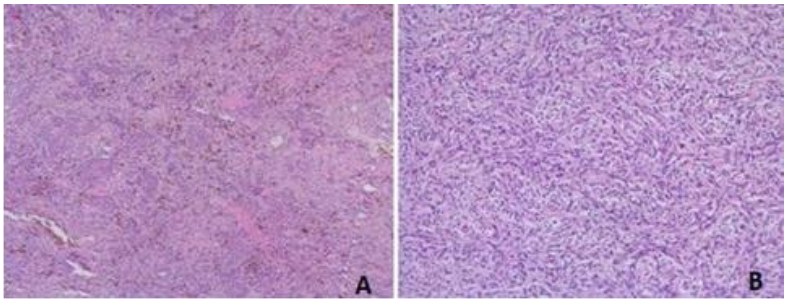


Figure 1.14 Severe diffuse ovarian hypoplasia, neither follicles nor luteal tissue are visible, the tissue is composed for the most part by disordered bundles of fibroblasts. EE. A. 4x; B.10x.

CASE H2

Anamnesis: female born in 2003 (12 years old at the time of the visit), saura, Quarter Horse breed, nullipara and never inseminated as previously used for sporting purposes; presents regular estral cycle. A gynecological examination is required to evaluate its reproductive potential as a mare.
EOG: the subject is phenotypically female. The stature corresponds to the breed standards while the skeletal muscle conformation is poor.
EOP: the vulva is small; the mammary glands are in the physiological position and in a stage of development compatible with that of a non-pregnant subject.
Instrumental examinations: transrectal ultrasounds shows cervix and uterus of slightly smaller dimensions while the gonads did not result ecographically visible. During the estrus there is a clear endometrial oedema (score 2).
Laparoscopy at the station: both the wide ligament and the ligament of the ovary are visible, while the gonads appear as roughly like hypoplastic ovaries. The owner does not allow the ovariectomy therefore no tissue samples are available for histological examination.
Laboratory tests: hormone dosage, aneupliody, karyotype analysis, study of C bands, analyses of *SRY* and ZFY Y, ZNF33bY and EIF3CY genes.

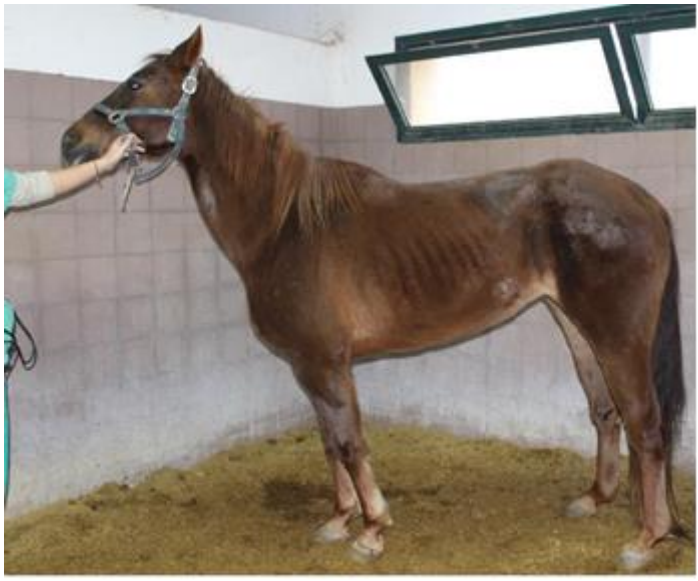


Figure 1.15 CASE H2, 12 years; the image shows reduced body weight and a poor general appearance.

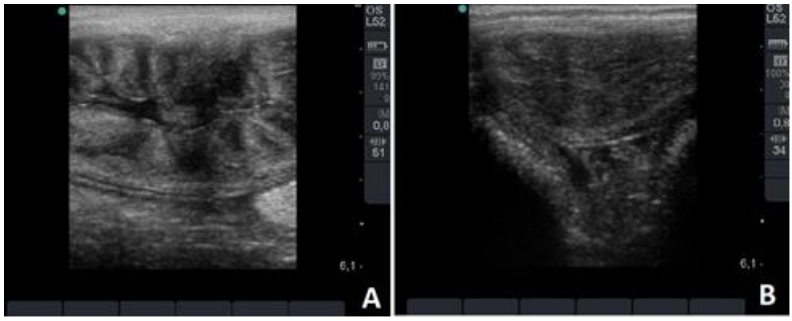


Figure 1.16 Ultrasound image of the uterus in a transverse scan. A, during the estrus; B, during the diestrus.

CASE H3

Anamnesis: male born in 2011 (5 years at the time of the visit), sauro, of the PSA race; examined for suspected bilateral cryptorchidism. The behavioral anamnesis refers the total absence of libido.
EOG: the subject has a general conformation corresponding to that of a male.
EOP: absence of scrotum, penis and foreskin of reduced dimensions. The palpatory examination of the inguinal region does not show any structure referable to the testis.

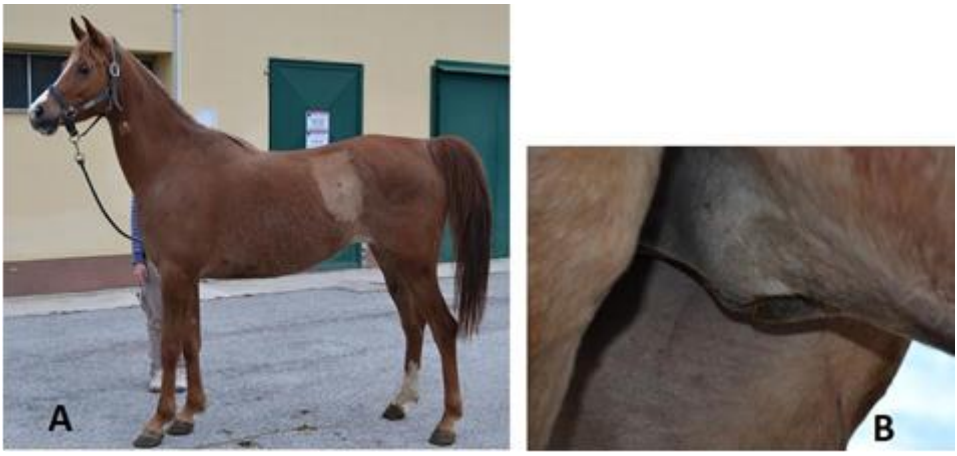


Figure 1.17 A, CASE H3, 5 years, skeletal muscle conformation and normal BCS; B, Small prepuce, micropenis and total absence of scrotal bag.

Instrumental examinations: transrectal ultrasound shows tubular structures dorsally to the bladder, presumably deferential ampoules. No testes were found in the abdominal cavity or within the inguinal canal. Laparoscopy at the station: the only elements potentially similar to gonads are represented by two small-sized structures (1.5x1 cm) positioned in the sublombar region, each supported by a thin and transparent mesorchio in which it is possible to visualize a newly sketched vascular system. The right gonadal-like sketch was removed laparoscopically through the ventral access and subsequently placed in 10% buffered formalin.
Laboratory tests: histological examination, aneupliody, karyotype analysis, study of the C bands, study of the *SRY*, ZFY Y, ZNF33bY and EIF3CY genes.

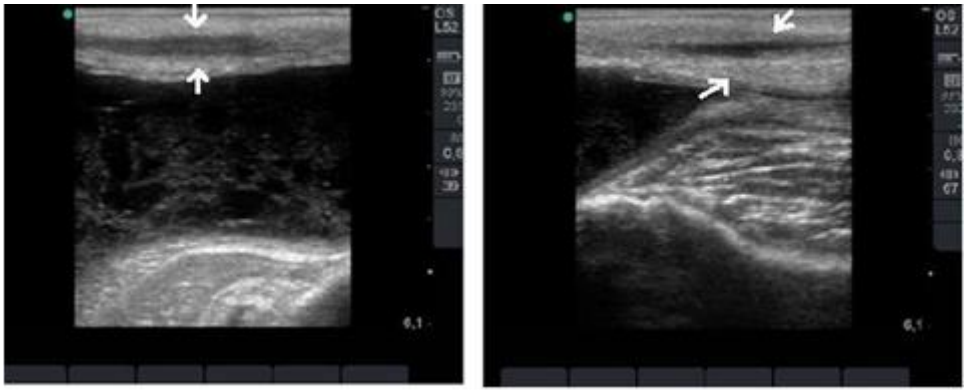


Figure 1.18 Ultrasound image of bladder and dorsal dorsal structures probably referable to vas deferens (arrows).

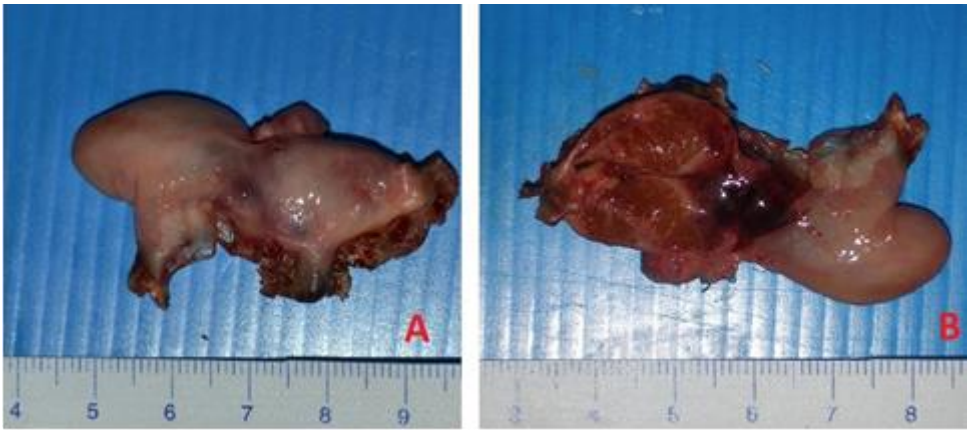


Figure 1.19. Gonadal sketch. A, external surface; B, a section.

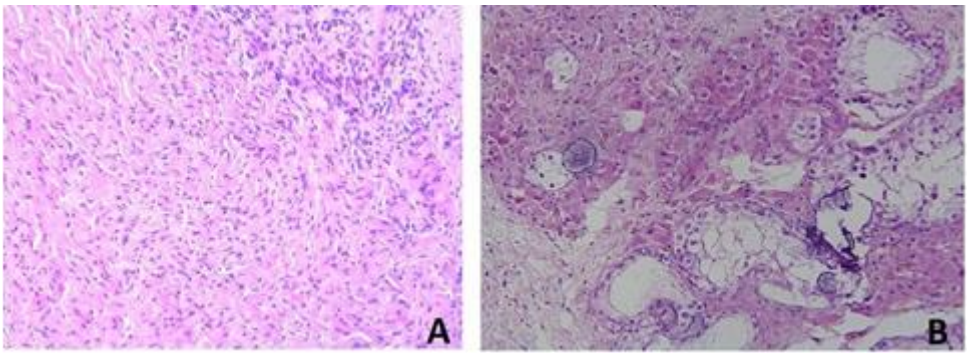


Figure 1.20 Severe testicular atrophy (hypoplasia). EE 10x. A, cellular fibrous tissue, consisting of disordered bundles of fibroblasts; B, seminiferous tubules with monolayered epithelium, without germination activity; focal calcification areas are visible.

CASE H4

Anamnesis: male subject born in 2014 (3 years at the time of the visit), bay, of TPR breed; no previous surgical procedures are reported. Examined for a suspect of monolateral cryptorchidism.
EOG: the subject has a general conformation corresponding to that of an entire male.
EOP: prepuce and penis are normal; the scrotum, in physiological position, contains only the left testis. By palpation the right inguinal region, it is not possible to perceive the right testicle.

Figure 1.21 A, CASE H4, 3 years, skeletal muscle conformation and BCS conforming to breed standards; B, prepuce, penis and left eutopic testis.

Instrumental examinations: Transrectal ultrasound that shows prostate, ampoules of the deferent ducts and bladder; the retained gonad is not detected either in the abdomen or inside the inguinal canal. Laparoscopy at the station: A structure similar has been found and extracted. It has a brownish color tissue, it has a round shape, dangling and with reduced dimensions positioned posterior to the caudal pole of the right kidney and connected to the abdominal wall from a thin band of fibrous tissue (mesorchio) in which the vascular component appears to be more developed than in Case H3.
Laboratory tests: histological examination, aneupliody, karyotype analysis, study of the C bands, study of the *SRY*, ZFY Y, ZNF33bY and EIF3CY genes.

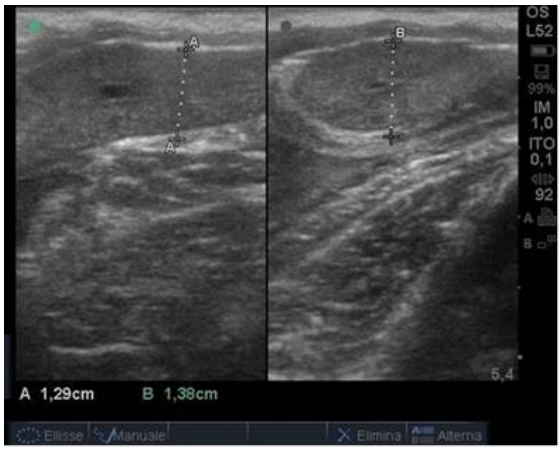


Figure 1.22 Ultrasound image of the ampules of the deferent ducts.

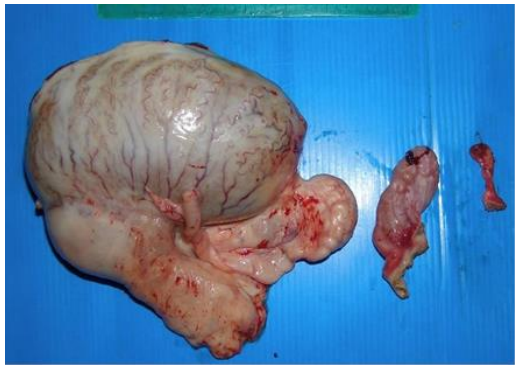


Figure 1.23 From left: eutopic testis, ductal structure and abdominal gonadal-like sketch.

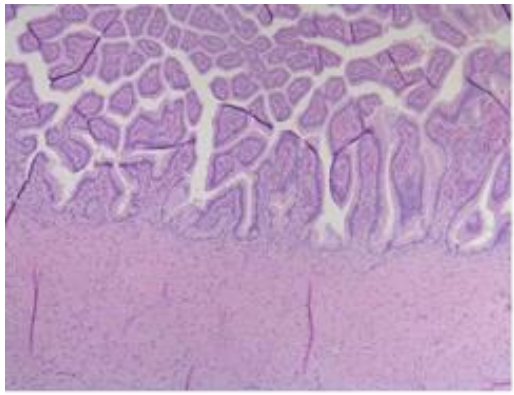
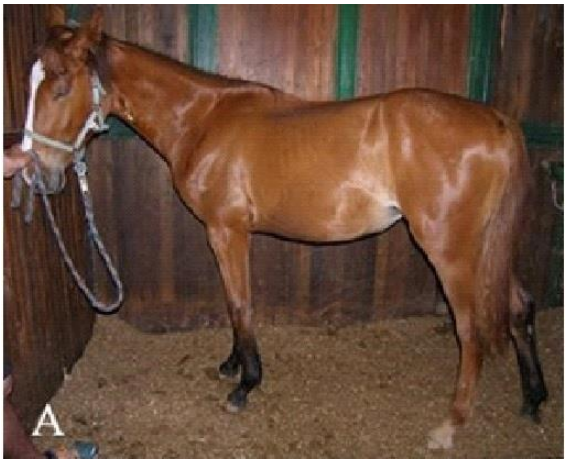


Figure 1.24 Ductal portion probably related to epididymis or vas deferens. EE 4x.

CASE H5

Anamnesis: subject registered as a female, born in 2015 (15 months at the time of the visit), bay above sauro, Sella Italian breed. Examined for phenotypic anomalies of the external genitalia and for the presence of stallion behaviors.
EOG: the foal has stature and physical conformation corresponding to the breed standard.
EOP: the absence of scrotum and a modest development of the mammary glands in the inguinal region are observable. Upon inspection of the perineal area, ventrally to the anal sphincter, a long (20 cm) raised median rafe is evidently similar to a process of fusion of the vulvar lips. This raphe ends in the ventral perineal region with a structure that can be traced back to a micropenis (11 cm x 3) with a caudal direction. The urination is abnormal and occurs through the external urethral sphincter located on the top of the urethral fossa. Instrumental examinations: transrectal ultrasound showed the absence of the gonads in the abdomen; Transinguinal ultrasound highlights the gonads at the level of the external inguinal ring.
Laboratory tests: histological examination, aneupliody, analysis of the karyotype, study of the C bands, study of the *SRY*, ZFY Y, ZNF33bY and EIF3CY genes.
Surgical approach: removal of the macroscopically identifiable gonads as hypoplastic testicles.



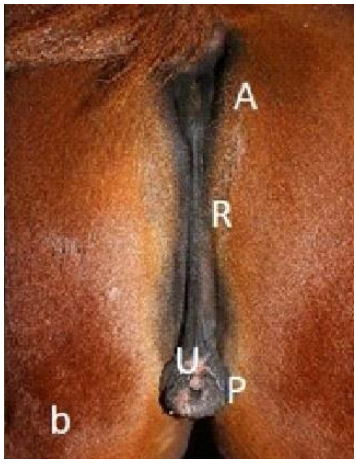


Figure 1.24 CASE H5, 15 months. Skeletal muscle conformation and BCS conforming to the breed standard; b. Perineal region. A= anus; P= small penic directed caudally; R= median rafe; U= external urethral ostium.

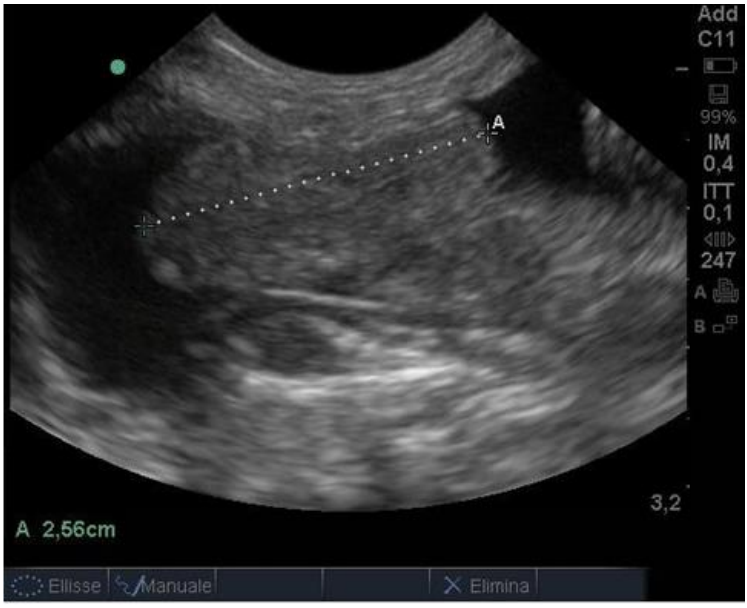


Figure 1.26 Trans-inguinal image of the gonad at the level of the external inguinal ring.

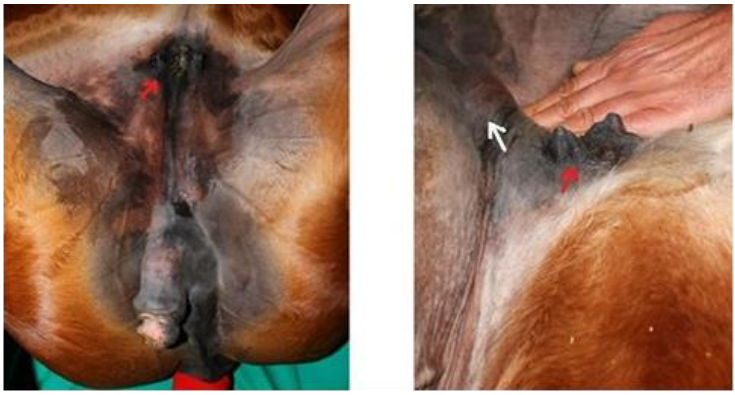


Figure 1.27 CASE H5 under general anesthesia and dorsal decubitus. Two well-developed nipples (red arrows) and gonads in subcutaneous position (white arrow) are evident.

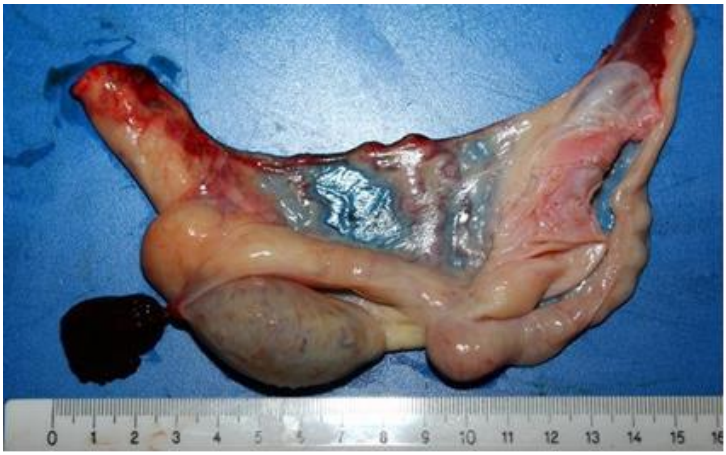


Figure 1.28 Hypoplastic testis.

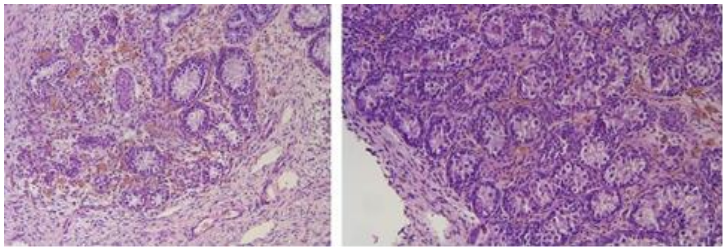


Figure 1.29 Testicular hypoplasia. The seminiferous tubules appear small and surrounded by a single layer of Sertoli cells. Leydig cells appear to be predominant due to the small size of the seminiferous tubules. EE 20x.

1.4.1 Results and Discussions

CASE H1

Histological examination: the gonad is characterized by total absence of follicles and / or luteal tissue; the tissue appears to be totally constituted by numerous fibroblasts interposed to scarce eosinophilic fibrillary extracellular matrix, arranged to form small disorderedly oriented bundles (connective tissue). Multifocally, capillary hyperemia and the presence of a few macrophages in erythrophagocytosis or containing small granules of hemosiderin are observed. The finding is compatible with severe diffuse ovarian hypoplasia with mild hemosiderosis and erythrophagocytosis.
Cytogenetic examination: The growth of the cell culture was not enough and the analyzable metaphase plates proved to be inadequate. Many metaphases with banding C are aneuploid and it has not been possible to identify the sex chromosomes due to their poor quality.
Molecular examination: the *SRY* and *ZFY* genes are absent while *ZNF33bY* and *EIF3CY* genes were normal. This result indicates the absence of genes typically and specifically located on the Y chromosome, supporting the hypothesis of its absence.
Discussions: The subject has gonadal dysgenesis with primary infertility, small ovaries, irregular or absent estrous cycles, hypoplastic genital apparatus, that is a clinical picture with a lot of phenotypic similarities with X0 subjects described in the literature. The height at the withers is normal while in many mares affected by Turner's Syndrome is lower than the breed standard. The cytogenetic results obtained did not allow us to perform an accurate evaluation of the subject's karyotype, to issue a diagnosis or to exclude a condition of chimerism 2n=63,X0/64,XX. Further analyses are needed, in fact, numerous studies show that a discrete percentage of sterile mares due to chromosomal abnormalities presents a condition of chromosomal mosaicism 63,X0/64,XX) (Blue *et al.*, 1978; Power, 1990). It is also necessary to evaluate the integrity of the X chromosome, in the literature are reported cases of mares with Xp deletion (Bowling *et al.*, 1978, Power, 1987, Lear, 2008 - unpublished data) that have a phenotype like the X0 subjects. To exclude even the presence of microdeletions it would be necessary the use of to more sophisticated molecular techniques such as FISH or microarray analysis (Powell-Hamilton, 2016).

The DSD 64, XY-*SRY* negative could be a further cause of ovarian dysgenesis: the subjects are phenotypically like X0 mares, with normal external genitalia but small, inactive or dysgenic ovaries; cervix and uterus may have a normal structure even if they are generally flaccid or completely hypoplastic. Estral manifestations are irregular or completely absent. With PCR, Y-chromosome primers other than the *SRY* gene can give positive results, indicating a deletion of the *SRY* gene on the Y chromosome. Although Case H1 belongs to the breeds most affected by this DSD, this hypothesis is currently excluded since the molecular DNA exams ruled out the presence of Y chromosome marker genes. Furthermore, the CBA and RB analyzed did not show any chromosome attributable to the Y one. If all the anomalies of the sex chromosomes described up to now were completely excluded as causes of the phenotype found in Case H1, other gene mutations must be considered. It is necessary to analyze genes (and their transcripts) responsible for ovarian development and differentiation like *FOXL2*, that in other animal species has shown mutation that can cause altered ovarian development, especially of granulosa cells.

CASE H2

Cytogenetic examination: C banding test allowed to visualize both X chromosomes in most metaphases; no studied plate showed the Y chromosome. Many metaphases had an aneuploidy condition due to the absence of one or more autosomes, an anomaly evident also in two of the three karyotypes performed. Molecular examination: the *SRY* and ZFY genes are absent; analyzing ZNF33bY the mare showed not only the female fragment but also an anomalous band of 1400 bp.
Discussions: Similarly, to Case H1, Case H2 presents gonadal dysgenesis with primary infertility, hypoplastic ovaries, irregular or absent estral cycles, hypoplastic genital apparatus, common to X0 subjects described in the literature. On the other hand, the cytogenetic results of Case H2 are more satisfactory and have not shown significant aneuploidies of the heterosomes. Also, in this case it would be necessary to deepen the presence of a chromosomal mosaicism with two cell lines present (63, X0 / 64, XX) (Blue *et al.*, 1978, Power, 1990) and an assessment of the integrity of the X chromosome. The analysis of the amplification fragments of the ZNF33bY gene, showed an abnormal band that does not correspond to the gene present on the Y chromosome and which was also found in two other

horses (unpublished data) affected by abnormalities of the reproductive apparatus. The study of this amplicon to understand its meaning is currently going on.

CASE H3

Histological report: the gonad is characterized by a testicular parenchyma largely characterized by atrophy and degeneration of the seminiferous tubules. It appears to be composed of few small and scattered lobules separated by a fibrovascular stroma; the lobules are made of tubules in turn separated by fibrous stroma. The tubules are internally delimited by flattened epithelium, totally devoid of germinative activity and multifocally expanded, as occupied by interstitial cells arranged in small islands or trabeculae; these cells have a polygonal to fusata morphology and rarely present vacuolated cytoplasm (with large vacuole). The described gonadal parenchyma appears to be incarcerated within a dense and very cellular fibrous stretch tissue sometimes consisting of numerous fibroblasts interposed with eosinophilic fibrillar extracellular matrix and arranged to form small disorderedly oriented bundles. Multifocally, within the fibrous tissue, there is a capillary hyperemia and the presence of macrophage infiltrates in erythrophagocytosis or containing small granules of hemosiderin mixed with groups of immature lymphocytes and macrophages containing lipofuscin. The picture is compatible with a severe testicular atrophy (hypoplasia).
Cytogenetic examination: the analysis of the metaphase plates with the R banding shows a karyotype 2n = 64, XY. 80% of the analyzed C-banded metaphasic plates have a chromosomal set 2n = 64, XY while 20% have an aneuploid set; moreover 99% of the metaphases are XY.
Molecular examination: *SRY*, ZFY, ZNF33bY and EIF3CY are positive. The results obtained indicate the presence of an integral Y chromosome and overlap those of the healthy male used as a control.
Discussions: this case has attracted our attention because it show a potentially similar phenotype to that of subjects suffering from Klinefelter Syndrome. The subject is presented as a male and is provided with immature male micropenis and gonads. Subjected to cytogenetic analysis, all the karyotypes have a chromosomal arrangement 2n = 64, XY and none of the analyzed CBA has a XXY condition. This chromosomal arrangement allowed us to exclude the mosaicism 64, XY / 65, XXY which occurs much more frequently than the pure form 65, XXY. Selders

et al. (2001) describe the case of a phenotypically normal stallion but without marked sexual behaviors and with suspected bilateral cryptorchidism; laparoscopic findings are very similar to those obtained in Case 3 surgery, with the sole exception of a potential vestige of the uterine horn identified by the author (but not removed and analyzed). On histological examination, the removed gonadal sketches are identified as hypoplastic testicles and the karyotype shows the presence of two cell lines 63, X0 / 64, XY of which the majority (27 out of 34) represented by line 63, X0. Sato *et al.* (2012) reported the case of a foal with caudo-ventrally directed micropenis and immature testes held in the inguinal canals; according to cytogenetic studies, the subject turned out to be affected by mosaicism 63, X0 / 64, XY with a ratio of 83:17. Despite the undeniable similarities between Case H3 and the case reported by Selders, the CBAs analyzed show 99% the presence of the Y chromosome, making this hypothesis very unlikely. In this case it will certainly be necessary, to highlight the causes of the phenotype found, to properly uncover the genes that may be responsible for the correct and complete male sexual development. Among these, however, we can already rule out alterations affecting the *SOX*9 gene since there are no anatomical structures like anomalous residues of the Müller ducts and therefore AMH must have been physiologically produced.

CASE H4

Histological examination: the eutopic testis appears normal as it presents a normotypical parenchyma, with active spermatogenesis inside the tubules; only focally thickened seminiferous tubules are observed for the proliferation of Sertoli cells. The tissue samples referred to as the testicle considered are instead constituted by a fragment in which the presence of a ductal structure with optically empty lumen and formed by a mucosa is raised and forms a short villi-like extroversion covered by pseudostratified epithelium; the mucosa is surrounded by a thick muscular tunic made up of smooth muscle fibers that form disordered bundles. Another fragment consists of stroma-rich tissue with many fibroblasts-fibrocytes and smooth muscle fibrocellulas forming a compact-solid tissue, focally surrounded by interstitial cells with moderate-abundant cytoplasm rich in lipofuscin and hyperchromatic round nuclei. The framework is compatible with the presence of a portion of the canalular ductal structure straddling the

epididymis and the vas deferens and with the presence of immature mesenchymal stromal tissue.
Cytogenetic examination: the analysis of the metaphase plates with the R banding shows a karyotype 2n = 64, XY. Of the 100 CBAs analyzed, 98% were found to be 64, XY and the remaining 2% 63, X0.
Molecular examination: the subject is *SRY*, ZFY, ZNF33bY and EIF3CY positive. These results are indicative of the presence of an integral Y chromosome and overlaps totally with the results obtained from the healthy male used as a control.
Discussions: monorchidismo is a term to indicate the agenesia of a testicle with or without partial development of the relative mesonephric duct or for a unilateral vascular problem. It is a rare condition in the horse and in most of the monorchidic subjects in the side in which the gonads is missing, a vaginal process is present, leading to the conclusion that the absence of the testis is due to a severe degeneration of the same during intrauterine life rather than a complete testicular agenesis (Arighi, 2011). In the literature few works are reported on this condition and in most of them the aetiological cause is identified in a vascular testicular damage during fetal life (Parks *et al.*, 1989; Kelmer *et al.*, 2006). In Case H4, since the histological results do not indicate the presence of scar tissue compatible with severe intrauterine testicular degeneration, cytogenetic and molecular analyzes were performed to evaluate whether the findings found could be due to aberrations of the heterosomes. Despite the 2% of CBA were aneuploids, this result is more likely to be due to iatrogenic alterations of the chromosomal set of some lymphocytes, also in light of the fact that in literature no X0 mosaic subjects have ever been reported presenting a phenotype comparable to Case H4. Furthermore, the molecular results indicate the presence of a normal Y chromosome. Much more likely, in this subject, the cause of the phenotype found could be a mutation affecting genes involved in the development of the reproductive apparatus and that are activated by the *SRY* gene, first, the *SOX*9 gene located on chromosome 11, since the protein encoded is responsible for the differentiation of somatic cells into Sertoli cells, whose presence in low number can lead to a more or less total block of testicular development.

CASE H5

Histological examination: the removed gonads are histologically constituted by numerous hypoplastic seminiferous tubules without germinative cells and/or spermatozoa and delimited by Sertoli cells, with cylindrical morphology, which extend from the undulating basal membrane and protrude inside the lumen. Between the tubules we can observe a stroma from scarce to more abundant (especially in the peripheral areas) with numerous interstitial cells tendentially fused, which rarely present vacuolated cytoplasm (with large vacuole) and with small hypercromatic nuclei; Vascular hyperemia and mild interstitial edema are present. Lymphocytes are rarely seen in the interstitium and the vascular component is well developed. The epididymis and the vas deferens are normal. The picture is compatible with severe atrophy (testicular hypoplasia).
Cytogenetic examination: The lymphocyte cultures were used for the preparation of slides intended for C and R bands or used for FISH mapping. The Cariotypes were organized according to the standard horse karyotype (Iannuzzi *et al.* 2003). The analysis of the karyotype with R bands does not show any anomalies affecting autosomes and heterosomes. The subject has a normal chromosomal arrangement 2n = 64, -XX. Of the 260 CBA analyzed, 94.6% are XX; 5.4% are X0 and 0.4% are 64, XY. It has also been consistently noted that, in each culture analyzed, an X chromosome has a centromere of much greater size than the homologous sexual chromosome.
Molecular examinations: the subject is *SRY* and ZNF33bY positive; ZFY and EIF3CY were negative at first but a further PCR performed with more specific primers for the above genes gave positive results.
Discussions: The reproductive system anomalies observed in this horse of 15 months have led to deepen the clinical case by performing clinical, ultrasound, surgical, histological, cytogenetic and genetic analyzes with particular attention. The observed histological results are consistent with severe testicular hypoplasia and atrophy of Leydig cells. Sertoli cells showed a diffuse and intense cytoplasmic immunolabelling for AMH. The anatomical and histopathological findings of this horse indicate that during embryonic development, the pathway of formation of the male genital apparatus has been correctly activated. This led to the formation of testicles and their migration into the inguinal canals. However, the genital tubercle has developed in the direction of the external male genitalia

without achieving a complete and correct conformation. The diffuse expression of AMH within Sertoli cells is like that of a previous study in which a positive immunostaining of AMH was found in intersected gonads and cryptorchid testes (Dunn *et al.* 1981) and complementary to the absence of Mullerian derivatives. This may be due to the postzygotic fusion of two distinct embryos rather than an early anastomosis between the twin vascular systems (one of which was then reabsorbed). In the latter case, in general, no abnormalities of the reproductive organs are observed in both twins because when vascular anastomoses are formed, sexual differentiation has occurred (Lear *et al.* 2012; Juras *et al.* 2010; Moreno-Millan *et al.* 1991). On the contrary, and in contrast with previously reported cases (Bugno *et al.* 2007; Han *et al.* 2010; Paria *et al.* 2011), the case in question does not show derivatives of female reproductive organs, while the male organs are almost completely developed. This phenotype may be due to the prevalence of XX cells on XY cells during critical stages of sex determination and sexual differentiation, so that although the Y chromosome initiates the *SRY* pathway, the low amount of XY genetic products may not be sufficient for a correct and complete male development. On the other hand, the percentage of different cell clones present in an animal's blood does not allow to trace the growth trend of all the different cellular clones during embryonic development. The presence of both XX and XY cells in the blood lymphocytes was further confirmed by FISH with BAC. PCR analysis with specific Y markers confirmed its presence, albeit at a low rate compared to normal male control. FISH experiments on metaphasis and interphase chromosomes have confirmed a very low chimerism. To our knowledge, this is the first case of a chimeric horse in which such a low percentage of XY cells in the blood (0.68%) is associated with the total absence of female structures. Genotyping of microsatellites across the entire genome performed on DNA from the blood did not reveal the presence of two cell clones due to the low percentage of XY cells. Instead, the same analysis performed on DNA by gonadal tissue revealed the presence of more than 2 alleles for some markers suggesting that this horse 64, XX / 64, XY is a chimera probably derived from the postzygotic fusion of two distinct embryos (tetragametic chimera) (Malan *et al.* 2006). This finding shows that when microsatellite genotyping is performed in a tissue with a very low percentage (<1%) of a particular clone cell chimerism, chimerism can remain undiagnosed and only discovered when the affected animal is old enough to show reproductive problems. Routinely, the karyotypes stained with Giemsa

(without bands) and CBA techniques seem to be the most sensitive method, thus indicating the need to always perform them more correctly, whether it is a farm animal or a case clinical. Furthermore, the early identification of individuals with cellular chimerism will allow the improvement of knowledge on the development of reproductive organs, in particular the molecular mechanisms underlying this biological event.

1.4.2 Conclusions

DSDs occur when there are errors in the constitution of chromosomal, gonadal or phenotypic sex; they are joined by the fact that they can appear phenotypically similar, only some are transmissible, and it is important to correctly understand the type of disorder.
Although for all the cases studied in the dog it was possible to identify the type of DSD, the molecular analyses excluded the involvement of the genes already known to be involved in the evolutionary dynamics of these DSDs. For this reason, further studies are needed to disclose the causes of the altered development of the reproductive system. The greater frequency of these diseases in recent times can be linked to multiple causes, from environmental to wrong reproduction practices of breeding farms. It is important to make this consideration because the manifestation of a DSD can have a different influence depending on the use of the affected animal, from a simple behavioral alteration of a pet animal to the sterility of a subject of high value or, for the horse, to altered anti-doping test results during sports competitions (Lear and McGee, 2012). Historically, the horse has played essential roles in human evolution. Today it is mainly used for recreational purposes, as an animal for affection and for sports use. In the clinical cases studied, despite the clinical features attributable to specific DSD, only in one subject it has been possible to obtain a reliable diagnosis with the diagnostic tools currently available (Albarella *et al.* 2018). The analysis of metaphase plates, in fact, is a process to be carried out with due attention, keeping in mind that due to the manipulation of cells necessary to obtain the metaphase plates to be evaluated the diagnostic value of the finding of a low percentage of chromosomal numerical abnormalities must be evaluated very carefully and by professionals. As in other animal species, the genome of the horse has been sequenced few years ago and the annotations of all the genes for the 31 autosomes and the X chromosome are not yet available, and data on the whole sequence of Y chromosome are not yet available. In general, the knowledge of the molecular genetics that controls the development of the reproductive system and the fertility in mammals is still insufficient. It is thought that there are more than one thousand genes involved from the time of oocyte fertilization until the sexual maturity and, subsequently, in the control of the development and functionality of testes and ovaries (Cederroth *et al.*, 2007; Wilhelm *et al.*, 2007). As for the Equidae, the road ahead is still long and full of challenges. DSDs remain among the main causes of primary infertility and

anatomical anomalies of the reproductive system. The task of the researchers is and will be to implement cytogenetic and biomolecular studies in the field of equine medicine to define the causes with greater certainty and thus reduce their incidence. The task of the sector technicians, in addition to that of a scientific study, must go towards monitoring and advicing to ensure that the influence of the selection desired by man would not be the main causes of the manifestation of this type of pathology. Veterinary consultancy, correct breeding management, screening tests in foals, can avoid important economic losses by early identification of the abilities and of the limits of the affected animals and allowing to allocate them to a life fitting with their status.

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CHAPTER I: References

ABSTRACT

The gains deriving from the use of stallions in reproduction depend on the quantity and quality of the spermatozoa present in their semen. As regard instrumental insemination the success depend also on other important factors like veterinary management and the mare's clinical condition. The improvement of the quantity and the quality of sperm using food supplements free of prohibited or harmful substances is an important aim in the breeding of livestock and pets. Maca (Lepidium meyenii) is a plant that has been used in the Andes for centuries to improve nutrition and fertility in humans and animals. This study aimed to evaluate if supplementing stallions' food with maca during breeding season can not only increase their sperm count and motility, but also other parameters like sperm shape and DNA fragmentation, which are two good predictive indexes of spermatozoa functionality. For this purpose, ejaculate volume, gel-free semen volume, sperm concentration and motility, total sperm count, maximum and minimum diameters, perimeter, area, shape factor, roughness of sperm head and sperm DNA fragmentation have been evaluated. According to our results, maca food supplementation in stallions during breeding season exerts an elongation of the spermatozoa head, a condition that is believed to improve spermatozoa fertilisation ability, and reduce the percentage of spermatozoa with fragmented DNA, indicating that maca could be an effective, healthy supplement useful in stallion's breeding.

Introduction

In recent decades the search for new plant-based food supplements that can improve male and female reproductive performance both in humans and livestock has increased. Close attention has been payed to the Peruvian plant maca (Lepidium meyenii), the hypocotyl of which has been used for centuries in the Andes for nutrition and fertility enhancement in humans and animals (Gonzales, 2011). The main effects observed in mammals orally supplemented with maca are increased sperm count and motility, and an improved DNA fragmentation index (Clément et al., 2012). All these beneficial effects are probably due to the antioxidant-oxidant balance induced by macamides and the lipid-extractable fraction of maca with an unknown mechanism of action (Melnikovova et al., 2015).
This study aimed to check morphometric measures and DNA fragmentation index modifications in the spermatozoa of Italian Thoroughbred stallions orally treated with maca during the breeding season. All these parameters have never been used to evaluate maca's effects on *Equus caballus* sperm quality, even though they provide useful data. Sperm motility and fertilisation ability are mainly due to the shape of the head (as well as structures like acrosome and flagellum); in fact, in a variety of mammal species it has been proven that sperm head morphometry is correlated with fertility (Hirai et al., 2001; Ostermeier et al., 2001; Vicente-Fiel et al., 2014; Waheed et al., 2015). Malo et al. (2006) affirmed that spermatozoa with more elongated heads may reach a higher swimming speed due to being more hydrodynamically efficient. According to Yàniz et al. (2015) morphometric analyses could be a useful predictive tool for semen fertility and freezability, once the technique for performing them is standardised (Yániz et al., 2015). Length, width, area and perimeter are the morphometric measures mainly used to objectively characterise sperm head shape. Sperm is a specialized cell in which chromatin is the main constituent, and its integrity is essential for successful fertilisation and normal embryo development. Sperm DNA fragmentation (SDF) is the index used to evaluate chromatin integrity, and it is inversely related to fertility (Agarwal et al., 2016). Unlike conventional semen analyses for quality assessment, such as concentration, motility and morphology, SDF allows for evaluating sperm genetic integrity; moreover, its rate is not necessarily linked to other sperm parameters. In fact, infertile men with normal semen may show a poor SDF index. The spreading of artificial insemination (AI), mainly

performed with cryopreserved semen, has given a strong boost to genetic selection and improvement in livestock breeding. A good outcome of the semen cryopreservation process requires a starting semen of good quality and quantity; thus, to confirm that nutraceutical substances like maca (Lepidium meyenii) can improve, if only transiently, ejaculate quality may have important economic consequences not only for equine reproduction, but also for reproduction of other livestock species.

CHAPTER 2
DNA fragmentation and morphometric changes in sperm of stallions supplemented with maca (Lepidium meyenii) during breeding season.

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2. **D'Anza E**., Albarella S., Ciotola F., Cocchia N., Del Prete C., Ciani F., Tafuri S., Frate D., Peretti V. (2017) "Evaluation of morphometric parameters and integrity of chromatin in the sperm of stallions feeding with maca's integration". **23° International Congress SIVE**, t 6-7th Oct 2017, Naples, Italy

2.1 Material and methods

2.1.1 Experimental Design

The study was planned so that maca administration was performed for one full horse spermatogenic cycle (57 days: spermatocytogenesis, meiosis and spermiogenesis) (Johnson et al., 1997), and its effects were checked for the next two cycles. Oral maca administration was performed for 60 days starting in April 2016, and sampling went on for 5 months after the end of treatment (October 2016). The first semen collection was planned 1 week before the beginning of maca administration, and its parameters were used as baseline control (T0); the subsequent collections were planned at 15 (T1), 35 (T2), 60 (T3), 75 (T4), 90 (T5) and 180 (T6) days after the first maca administration, for a total of 28 samples.

2.1.2 Animals

Fig. 2.1 Standardbred Horses (Photo By Thibaut – Fotolia).

Four healthy Italian Standardbred stallions, aged between 9 and 16 years, were selected for this study. All the stallions were housed in a farm, in the same breeding condition and used for AI. The animals were fed twice daily with hay and concentrates, and they had water *ad libitum*.

2.1.3 Source and supplementation of maca

Fig. 2.2 Peruvian Maca (Photo by Google).

Yellow maca (Fig. 2) hypocotyls used for this experiment were harvested in the district of Junín, in the Andean highlands of Peru (4.100 m above sea level), and exposed for 2 months at extreme temperature cycles, strong light conditions and atmospheric pressure typical of a high-altitude environment (> 3,500 m), thus reproducing traditional open-field drying. Hypocotyls were then selected, washed, milled to a powder with a particle size of 0.8 mm and packaged to be used.
Each stallion received a daily dosage of 4g of maca/100 kg body weight. The dose was chosen according to that found to show beneficial effects on spermatogenesis in humans (Gonzales et al., 2001) and rats (Cicero et al.; 2001, Zheng et al., 2000). Lepidium meyenii walp improves sexual behaviour in male rats indipendently from its action on spontaneous locomotor activity, (Cicero et al., 2002; Gonzales et al., 2004). The total glucosinolates content of dry extract from maca powder used for this work was 6.67% of which 3.33% was benzyl glucosinolate, 0.34% was m-methoxybenzyl glucosinolate and 3% was 3-Oxo-2-(2-entenyl) cyclopentane octanoic acid.

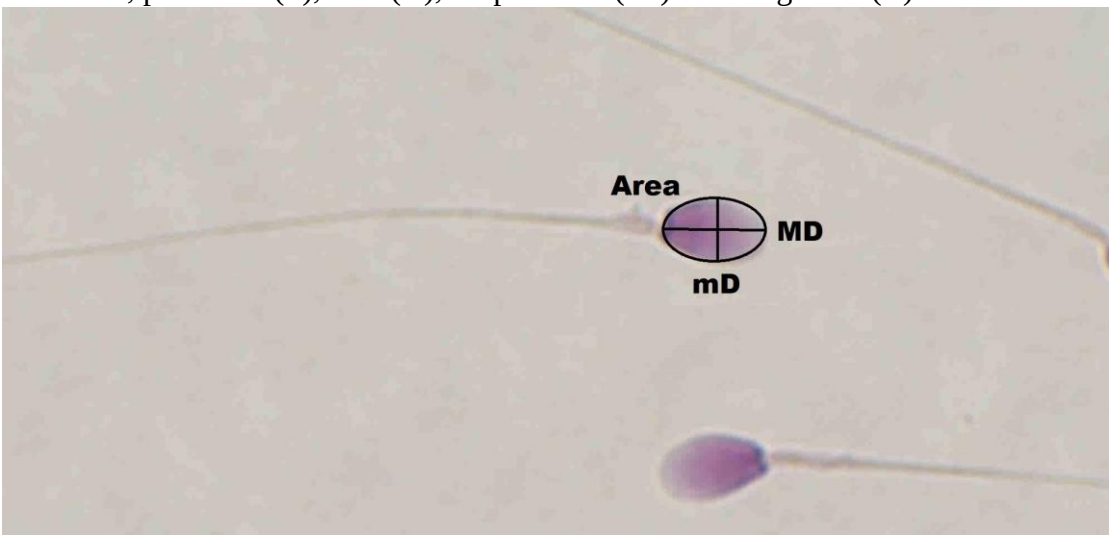
2.1.4 Ejaculate and semen processing

Immediately after collection, the total amount of ejaculate (semen and gel) was established using a graduated laboratory bottle (Sigma, Italy), the gel fraction was removed by a nylon semen filter (Minitube, Germany), the semen was filtered through a semen filter pouch (Minitube, Germany) and the quantity was measured. Sperm motility was visually assessed under a phase contrast microscope (Nikon Eclipse 80i) at 100x and 200x. Sperm

count was determined by a biophotometer (Eppendorf) there after the total sperm count (TSC) was calculated.
For morphometric evaluation, fresh semen samples were washed by centrifugation in a physiological solution at 1.000 x *g* for 5 min, and then re-extended to a concentration of $100x10^6$ cells/ml. Amounts measuring 10µl of the sperm suspension were fixed on slides and stained with a modified hematoxylin-eosin protocol: 10 min in Mayer's haematoxylin (Bio-Optica Milano SpA), and 5 min in eosin and disodium Salt solution (Fisher Scientific). The excess stain was removed, and the slides were dried and permanently mounted with Eukitt (Chem-Lab NV).

2.1.5 Morphometric analysis

At least 200 spermatozoa from each ejaculate were observed in a bright field under a Nikon Eclipse 80i microscope (100x), captured with a digital camera (Nikon DS-Ri1) and analysed with the software Nis Elements Imaging Software 4.00.02 (Nikon). Morphometric parameters (Fig.2.3) measured for each spermatozoon were major (MD) and minor (mD) diameters, perimeter (P), area (A), shape factor (SF) and roughness (R).

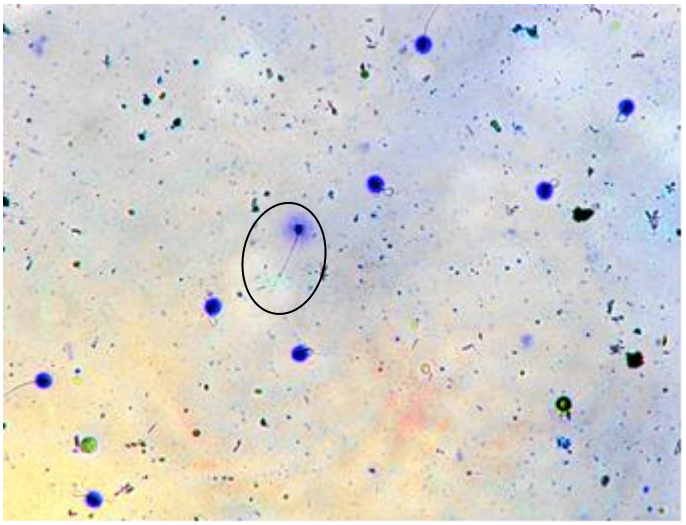


Dr E. D'Anza

Fig. 2.3 Example of measures performed on the spermatozoon.

2.1.6 Sperm DNA Fragmentation (SDF) analysis

Sperm chromatin fragmentation (Fig. 4) was evaluated for each semen sample by using a Halomax kit for *Equus caballus* (Halotech® DNA) according to the user manual. Slides were observed in a bright field under the Nikon Eclipse 80i microscope (20x), and 300 spermatozoa from each semen sample were captured with a digital camera (Nikon DS-Ri1) and analysed with Nis Elements Imaging Software 4.00.02 (Nikon).



Dr E. D'Anza

Fig. 2.4 Example of Sperm Chromatin Fragmentation.

2.1.7 Statistical analysis

Morphometric data were statistically analysed in two ways: considering 4 stallions like 1 in the different periods and then analysing the stallions individually in the different periods. All data follows the normal distribution. Data for the evaluation of morphometric measurements were analyzed with Anova Test. For statistical analysis, the IBM SPSS® Statistics software (version 20.0, IBM Corporation, Armonk, NY) was used.

2.2 Results

According to the data on semen quantity and quality parameters data of the T0 collection, among the four stallions used for this study, case 2 had the poorest semen (Table 1).

2.2.1 Semen quantity and quality

Mean (± sd) values of all semen quality and quantity parameters in the seven periods are shown in Table 1 and Figure 1. Although there are no statistically significant differences between the values of the samples taken over time, a general increase in motility, sperm concentration in the gel-free semen, total sperm count and volume of total ejaculate (spermatozoa + seminal fluid) can be observed.

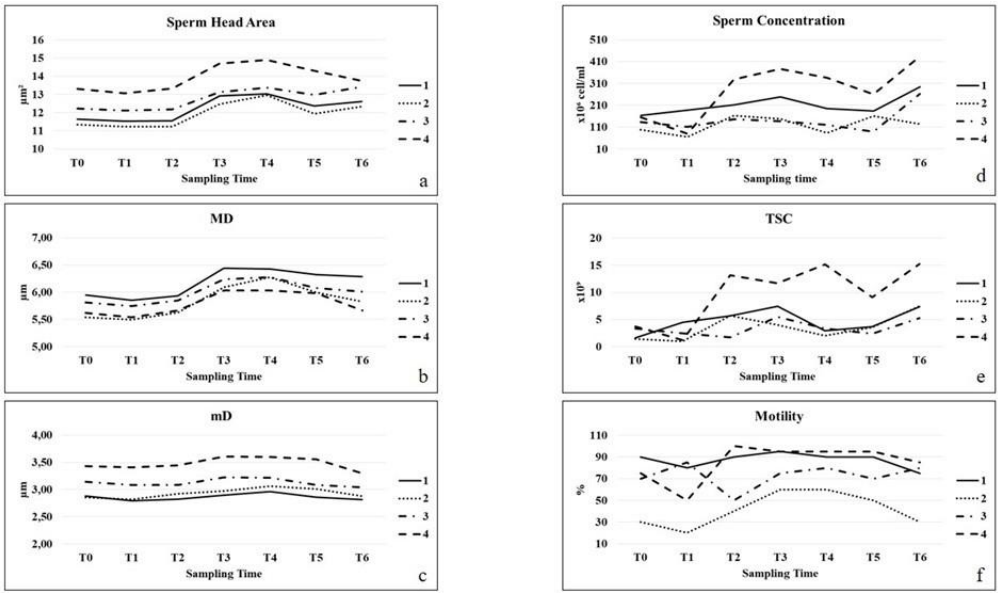


Fig. 2.5**.** Spermatozoa morphometric and quality parameters of the four stallions treated with maca at each sampling time.
a) Sperm head area (µm²). b) MD - Maximum Feret Diameter (µm). c) mD - minimum Feret Diameter (µm). d) Sperm Concentration (x10⁶). e) TSC Total Sperm Count (x10⁹). f) Motility (%).
T0 = 1 week before maca administration, T1 = Day 15 of maca administration (m.a.), T2 = Day 35 of m.a., T3 = Day 60 of m.a., T4 = Day 75 of m.a., T5 = Day 90 of m.a., T6 = Day 180 of m.a.

Samples	**T0**	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**
Ejaculate (ml)	36,75	40	38,75	57,5	65	56,75	47,5
Semen gel-free volume (ml)	18,5	19	28,5	32	28	22,25	35
Motility %	66,25	58,75	70	81,25	81,25	76,25	67,5
Sperm concentration ($x10^6$/ml)	137,5	111,25	212,5	227,5	184	173,75	279

Tab. 2.1 Mean (± sd) values of all semen quality and quantity parameters in the seven periods.

2.*2.2 Morphometric analysis*

Mean values (± sd) of all parameters for morphometry of horses' spermatozoa are shown in Table 2. All the measures and in particular the sperm areas analyzed combining the measurements of the four stallions show a statistically significant difference in the comparison between T0 (control period) and T3, T4, T5, T6 ($p < 0,05$). All the measures and the sperm areas analyzed merging the data of the measurements of the four stallions show a statistically significant difference in the comparison between T0 (control period) and T3, T4, T5, T6 ($p < 0,05$). Anova Test on sperm areas analyzed by single stallion shows the same result: a statistically significant difference between T0 (control period) and T3, T4, T5, T6 ($p < 0.05$). As regard maximum and minimum diameters, a statistically significant difference is observed ($p < 0.05$) between T0 (control period) and T3, T4, T5, T6. As Regard the SDF no statistically significant differences are observed over time.

Morphometric Measures	T0	T1	T2	T3	T4	T5	T6
Area (µm²)	12,13±1,33*	11,99±1,26	12,07±1,34	13,3±1,54*	13,56±1,4*	12,89±1,5*	13,03±1,17*
Perimeter(µm)	14,32±1,62*	14,13±1,47	14,65±2,74	15,87±2,85*	15,41±2,13*	15,10±1,8*	14,40±0,74*
Dia Max (µm)	5,73±0,37*	5,66±0,36	5,77±0,36	6,20±0,39*	6,25±0,37*	6,09±0,37*	5,95±0,42*
dia min (µm)	3,08±0,32*	3,02±0,34	3,07±0,34	3,17±0,38*	3,21±0,37*	3,12±0,36*	3,01±0,29*
Roughness	1,02±0,02*	1,02±0,02	1,03±0,03	1,03±0,02*	1,03±0,03*	1,03±0,02*	1,01±0,01*
ShapeFactor	0,79±0,09*	0,79 ± 0,09	0,77±0,20	0,74±0,24*	0,77±0,22*	0,76±0,22*	0,81±0,06*

Tab. 2.2 Morphometric measures in sampling timing and Anova Test showing a *SSD between T0-T3, T0-T4, T0-T5, T0-T6.

Area µm²	T0	T1	T2	T3	T4	T5	T6
Stallion 1	11,65±1,13*	11,53±1,05	11,55±1,02	12,92±1,32*	13,03±1,08*	12,36±1,20*	12,60±1,11*
Stallion 2	11,33±1,03*	11,23±0,97	11,23±0,91	12,46±1,30*	12,95±1,23*	11,93±1,15*	12,34±0,95*
Stallion 3	12,22±1,04*	12,11±1,11	12,18±1,09	13,13±1,26*	13,38±1,15*	12,98±1,21*	13,41±1,03*
Stallion 4	13,30±1,19*	13,07±1,04	13,33±1,26	14,70±1,31*	14,90±1,22*	14,30±1,26*	13,75±1,00*

Tab. 2.3 Anova Test of sperm head areas analysed individually; *SSD between T0-T3, T0-T4, T0-T5, T0-T6.

MFD	T0	T1	T2	T3	T4	T5	T6
Stallion 1	5,95±0,36*	5,85±0,33	5,93±0,36	6,44±0,34*	6,43±0,36*	6,33±0,34*	6,29±0,39*
Stallion 2	5,54±0,35*	5,50±0,36	5,62±0,31	6,09±0,35*	6,27±0,32*	6,00±0,35*	5,82±0,33*
Stallion 3	5,81±0,32*	5,75±0,32	5,84±0,32	6,24±0,36*	6,27±0,32*	6,08±0,33*	6,01±0,35*
Stallion 4	5,62±0,31*	5,54±0,31	5,67±0,36	6,03±0,34*	6,04±0,34*	5,98±0,30*	5,66±0,33*

Tab. 2.4 Maximum Diameter analysed individually; *SSD between T0-T3, T0-T4, T0-T5, T0-T6.

mFD	T0	T1	T2	T3	T4	T5	T6
Stallion 1	2,88±022*	2,79±0,21	2,82±0,21	2,89±0,23*	2,96±0,25*	2,86±0,24*	2,82±0,22*
Stallion 2	2,85±0,22*	2,82±0,23	2,92±0,20	2,97±0,25*	3,06±0,25*	3,01±0,22*	2,88±0,20*
Stallion 3	3,14±0,22*	3,08±0,25	3,08±0,21	3,23±0,25*	3,22±0,25*	3,09±0,25*	3,04±0,24*
Stallion 4	3,43±0,21*	3,41±0,25	3,44±0,33	3,61±0,26*	3,60±0,29*	3,56±0,24*	3,30±0,23*

Tab. 2.5 Minimum Diameter analysed individually; *SSD between T0-T3, T0-T4, T0-T5, T0-T6.

% SDF	T0	T1	T2	T3	T4	T5	T6
Stallion 1	8,38	4,4	7,4	9,7	6,7	5,6	3,04
Stallion 2	12,1	10,6	18,4	12,12	12,1	9,97	11,3
Stallion 3	7,4	8,6	6,5	4,4	6,4	4,4	4,3
Stallion 4	7,9	6,9	15,8	9,8	9,4	15,6	4,3
Media	8,95	7,0	12,02	9,00	8,65	8,89	5,73

Tab. 2.6 Sperm DNA Fragmentation analysed individually and mean values.

2.3 Discussion

When stallion quantitative semen parameter is analyzed, many variables must be considered, from the physical condition of the subject to the time of collection, the skill of the sampler. The mean values observed in this study for the quantity and quality parameters conventionally used to evaluate stallion reproductive ability (gel-free semen volume, sperm concentration, TSC and sperm motility) confirm results already reported in the literature: maca food supplementation improves semen characteristics in horses. The SDF always remains below critical threshold values and decreases progressively starting from T3, the assumed starting moment of maca effect, which could indicate that the active substances of maca prevent the sperm from damaging the DNA due to the aging. The decrease in the percentage of spermatozoa with fragmented DNA starting from T4 may indicate that the stability of the spermatozoa DNA is at higher risk during the early stages of gametogenesis; therefore, the maca components are more effective in preventing DNA damage if they are present in the seminal tract from the early stages of gamete differentiation. According to Waheed et al. (2015) the morphometric parameters of the stallion's spermatozoa (MFD, mFD, P and A) remain unchanged during the spring and summer; this study has instead shown a certain variability of sperm measures that do not depend on the season. In fact, from T0 to T5, which corresponded to spring and summer seasons, there was an increase in all morphometric measurements (Table 2, Figure 1) that could be due to maca food supplementation. Among all the morphometric parameters measured, MD was the one that mainly increased (Table 4), indicating an elongation of the sperm head, which is a shape that can positively affect sperm's fluid-dynamic behavior, making it more efficiently in the movements. In fact, a recent study of the Iberian reed deer (Ramón et.al, 2013) showed that spermatozoa with rapid and linear movements were strongly correlated with spermatozoa having a small and elongated head; TSC, motility, MD, mD and A, independently analyzed for the four stallions are improved if associated with the integration with maca food. Fig. 1 shows how the improvements in the quantity and quality parameters of the sperm were different in the four stallions indicating a marked individual response to this food supplement. However, among all the animals the highest values were in T3-T5, corresponding to the period of the maca effect. Interestingly the stallion with the worst starting parameters showed an important improvement in sperm concentration and motility, suggesting

that maca food supplementation is ideal in stallions that have very low fertility ability due to poor semen parameters.

2.4 Conclusion

In this study, the use of maca as a food supplement to improve stallion sperm parameters has been evaluated using not only classical quality and quantity sperm parameters, but also morphometric measures and DNA fragmentation test. Data obtained about sperm quantity and quality confirm that maca administration also improves stallion semen quality during a stressful period such as the reproductive season. Sperm morphometry has also been successfully used in sperm competition studies. Sperm competition has been associated with an increase in total sperm dimensions and in sperm head elongation, and both aspects have been related to an improved sperm migratory efficiency (Sánchez et al., 2013). When spermatozoa increase in size, all sperm components increase in size simultaneously (Tourmente et al., 2011), and can produce more energy and swim faster (Sánchez et al., 2013). According to data reported in this study, maca food supplementation also improves spermatozoa morphometric parameters; thus, it appears to be a very economically effective treatment for stallions that are of high genealogy but have poor semen quality, making them good sires.

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Abstract

For some decades, the world has been facing major losses in the number of honey bees, with a major threat to the agro-bio-economy and global biodiversity. This is due to the concomitant action of different stress factors: pathogens, chemicals, environmental changes, soil reduction available for flowering plants. It is known that these factors can act by compromising the fertility of queens and drones. The aim of this study is to evaluate the morphological characteristics and morphometric parameters of Italian Apis mellifera ligustica spermatozoa to define its standard features and to compare them with possible pathological changes. Total length of the sperm (ToL), core length (NL), width (NW) and area (NA), tail (TaL), perforator (PL) and head length (HL) were measured in 1000 spermatozoa. The results were reported as mean and standard deviation. Since the head is considered the main transporter of the genome and its modifications may reflect anomalies of the DNA content, the correlations among the average morphometric parameters of the head have been analyzed. Morphologically, some spermatozoa have revealed visible signs of pathological changes, such as broken tails. However, more detailed studies are needed to understand this data and to standardize the normal morphology and morphometry of spermatozoa of this subspecies.

Introduction

In the last decade the decline in the number of honey bees on the planet is a topic that is treated both from the scientific and the mass-media points of view. The variables analysed to study the causes of the loss of numerous colonies in a relatively short time are defined by the international scientific community as Colony collapse Disease CCD (Vanengelsdorp et Al. 2009). The drastic reduction in the number of bees is seen as a major threat to the agro-zootechnical economy that revolves around beekeeping and, even more so, for biodiversity due to the pollination function that the bee covers in the old as well as in the new continent. However, this process is named "pollination potential" because they are involved domestic and wild bees and other wild pollinating insects. In the last decades bees and other pollinators are progressively diminishing globally, this is particularly in North America and Europe (Potts et al., 2010). This loss is attributed to the CCD and it is characterized by the disappearance of worker bees (Lebuhn et al. 2013). To give an idea of the economic importance of natural pollination, it is enough to think that the value of pollination in some European regions, in North America and in East Asia, can reach 1,200 euros per hectare, thus the diminishing of pollinating insects causes a significant money loss for farmers and the agricultural economy. In Italy and in Greece, there is a high degree of biodiversity due to the presence of the *Macchia Mediterranea*, and the value of pollination is even higher. The same happens in large regions of Spain, France, the United Kingdom, Germany, the Netherlands, Switzerland and Austria, which in turn have hot spots of significant value (Lautenbach et al., 2012). This death of honey bees does not have a specific cause identified and, now, it is agreed that the interaction of several factors, such as: the import of alien species, diseases and pests, climate and environmental changes, more habitats poor in flowers, changing of the agricultural practices, crop protection products, micro-dust and heavy metals (Goulson et al. 2015). Some of these factors have already been shown to negatively affect fertility in humans and many other species, either directly or through modifications of the endocrine system (endocrine disruptors), which plays a central role in reproductive functions (Straub, L. et al., 2016). In the same way these factors can create problems for the female and male reproductive ability of bees, compromising the fertility of the queens and drones (Tyler, C. R. et al.1998). However, most of the studies focus on the reproductive system of females and only in recent years more attention is paid to male

reproduction. It has been shown that male subfertility/infertility is complex as that of females (Kumar and Singh 2015). The Drone, male of the HoneyBee, has long been considered an idle and idle figure destined only to inseminate the Queen. Recently it has been showed that during his life, before arriving at the fertilization act, it performs many tasks to support the daily life of the beehive such as: warming the brood, operating the trofallassi and attracting the females of Varroa destructor limiting the damage to other areas of the hive. However, his main function remains the reproduction. The bees breed and respect their drones, the means by which a queen genetic value can be transmitted to the future generations. Coupling is a fundamental moment for the continuation of the species, for safety reasons everything happens in flight at about twenty meters in height. The drone, to mate, can travel about 20 kilometers driven by pheromones that the queen produces and only 15/18 will be the lucky ones destined for the "wedding flight". It has no sting, it is the only bee with a penis and, in the hive, only the queen bee is fecund, in fact, all the workers are sterile, so that nothing can jeopardize the phase of mating. The coupling takes place during the "wedding flight" and lasts up to 5 seconds. Ejaculation is explosive and accompanied by a sound that can be heard by the human ear; the penis explodes and remains inside the queen as a kind of cap that prevents the spillage of the seminal fluid until the next drone blocks the queen, removes the penis residues of the previous drone and goes on with a new fertility act. The last drone will leave the "final cap". The ejaculated sperm are received in the queen spermateca, an organ predisposed to the custody of spermatozoa, and it will remain at its disposal alive and vital throughout her life. Aim of this study is to evaluate the morphological characteristics and morphometric parameters of Italian bee spermatozoa (A. mellifera ligustica) to define its standard features, to compare them with eventually pathological changes and to try to contribute to the preservation of this insect.

This project was carried out in collaboration with the unity of general pathology and pathological anatomy of the Department of Veterinary medicine and Animal Production. I would like to thank dr Karen Power for the important contribution.

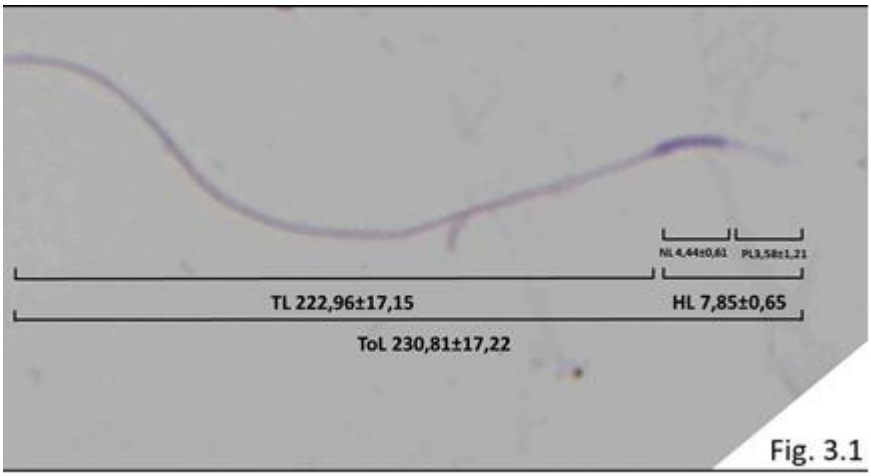
CHAPTER 3
Morphological and morphometric evaluation of Italian Apis mellifera ligustica spermatozoa

1. **D'Anza E**., Power K., Martano M., Maiolino P., Albarella S., Ciotola F., Peretti V. (2017) "Morphological and morphometric analysis of the Italian honeybee (Apis malliferaligustica) spermatozoa: a preliminary study". **X International Symposium on Wild Fauna (WAVES)**, Sep 21-23th 2017, Utad, Vila Real, Portugal. **(Contribution).**

2. Power, K., **D'Anza E**., Martano, M., Albarella S., Ciotola F., Peretti V., Maiolino P. "Analisi morfologica e morfometrica degli spermatozoi di Apis mellifera ligustica: uno studio preliminare per la valutazione delle alterazioni". **II CONVEGNO NAZIONALE SVETAP** "APICOLTURA, AMBIENTE E SICUREZZA DEI PRODOTTI DELL'ALVEARE". PAESTUM 12-13 APRILE 2018. **(Contribution).**

3.1 Drone honey bee (Apis mellifera Italian ligustica) reproductive aspects

The beekeepers select the drones for natural and artificial insemination of the queens observing some of their morphological and morphometric characteristics at the time of the presumed sexual maturity. In general, good body structure, color, hydration and the size (compared to that of the others) are observed.



Dr E. D'Anza

Fig. 3.1 Morphometric measures of A. M. Ligustica spermatozoon.

Since 1950, it has begun to give scientific and technological basis to many practices that were used in an empirical way. The drone size is an essential parameter to evaluate its reproductive potential (Gencer and Firatli 2005), large drones derive from large cells predisposed to produce males from unfertilized eggs and therefore destined to mating (Winston, 1987). The sexual maturity of the drone starts around fifteen days old when it reachs a weight that, depending on the species, will oscillate between 270 and 280 mg (Duay et al., 2003). The drone size too affects sperm production, large drones produce more sperm and more mucus than the small ones (Berg et al 1997, Schlüns et al., 2003). On average, each drone produces about $10x10^6$ spermatozoa (Meckensen 1955) and about $7.5x10^6$ per microliter of sperm (Meckensen 1964). Bee spermatozoa have a particular morphology. The cranial portion is constituted by a thin protuberance called *perforatorium*, it follows the head, also named nucleus, in which the

genetic information is stored. The tail reaches considerable size (Fig.1). In the literature, there are various indications on sperm measurements that are slightly different among the subspecies. The head is not ovoid in shape and sperm are mainly developed in length. The most commonly reported average total length is 270 μm (Woyke 1983a). An accurate and recent morphometric study, shown in table 3.1, was performed on A.M. Carnica (Gontarz A., et al., 2016). As regard Apis melliphera Ligustica there are no reference data for the sperm size.

3.2 Materials and methods

100 drones of A. mellifera ligustica were taken from 5 different apiaries located in the Campania Region from March to June 2017. The drones were chosen according to the size, selected by the beekeeper as the largest available at the collection time, older more than 15 days. Before the removing of the gonads the drones were narcotized. The testes were taken prior to dissection of the abdomen that was performed using a stereomicroscope (Microscope Axioscope HBO50, Zeiss, Milan, Italy). The reproductive system was exposed by gently removing the dorsal tergites of the abdomen and the gut. Testes, seminal vesicles and mucus glands were removed and carefully put into a 1.5 ml Eppendorf tube containing 500 μl of 0.9% sodium chloride solution. After centrifugation, a drop of pellet was swiped on a slide, stained with hematoxylin eosin and observed under an optical microscope (Nikon ECLIPSE 80i microscope) at 100X. Morphological and morphometric analyzes were performed on 10 spermatozoa per subject, 200 per apiary, 1000 spermatozoa in total. Punch, head, core, tail and sperm total length and the area of the core were measured using an image analysis software (Nikon NIS Elements 4.0). Data were analyzed using the unidirectional ANOVA and Pearson's correlation test.

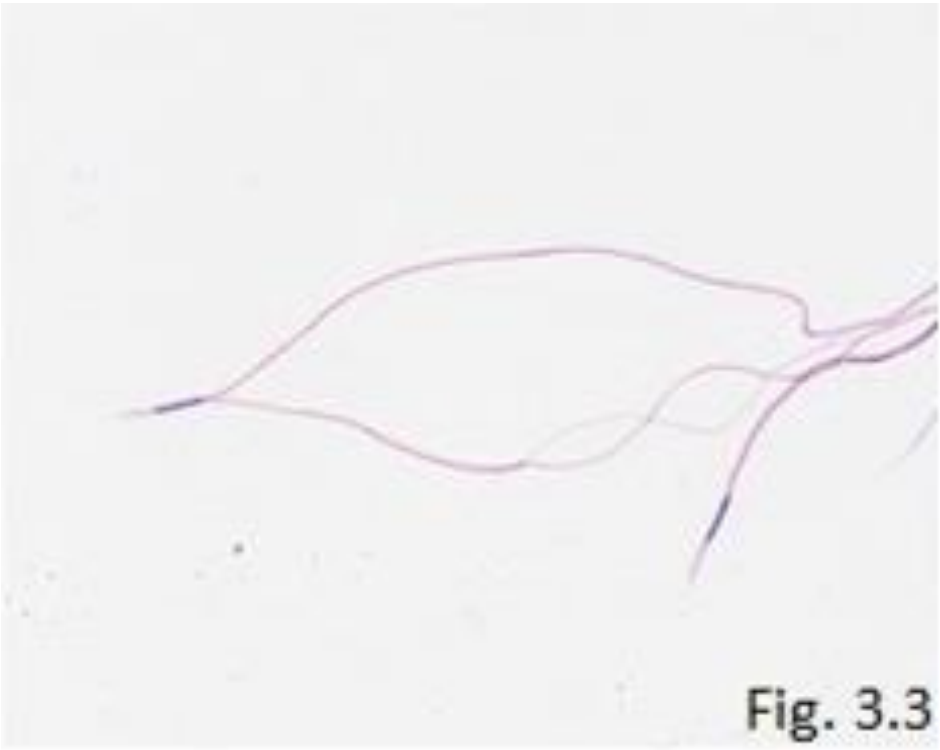
3.2.1 Results and discussions

The average total length of the spermatozoa of Apis Mellifera Ligustica is 230,81±17,22 μm. The measurements of the various structures of the spermatozoon (Figure 3.1, Table 3.1) were statistically analyzed allowing to detect the following: the value of the nucleus length is correlated to the value of the perforator length ($p<0.01$), both measures are inversely related to tail length; this may indicate that an increase in the nucleus or perforator

length implies a decrease in the tail length and vice versa. As regard morphological analyzes, most of the observed spermatozoa showed no evident morphometric changes (Fig. 3.2); only the 7% (70/1000) showed alterations such as broken tails and doubling of the tail and head (Fig. 3.3). The morphometric parameters (mean values and standard deviations) are reported in Table 3.1. The analyzed spermatozoa showed a SD variability of the mean values of the total and the tail length (Table1).

Dr Karen Power

Fig. 3.2 Normal honeybee spermatozoa.



Dr Karen Power

Fig. 3.3 Alteration of the sperm normal morphology.

The morphological and morphometric characteristics of Apis mellifera ligustica spermatozoa were smaller (Table 1) than those of other subspecies (Gontarz A., et al., 2016; Bushrow, ES et.al. 2006) also showing variability in the total length and in particular that of the tail. The reason for this variability is not known, but it is supposed to be linked to sperm competition. Moreover, the presence of morphologically abnormal spermatozoa, with less chance of being able to reach and fertilize the ovum, could be an index of reduced fertility, due to genetic and environmental factors, which could modify the normal gametogenesis process. It should be emphasized that, contrary to mammals, the drones develop from their embryo with the entire sperm pool already formed and sperm cells will no longer be produced during adult life, so the quality of the sperm can not be restored during his life.

Morphometric parameters (MV ±SD)	***A. M. Ligustica***	***A. M. Carnica*** **(Gontarz et al.,2016)**
Perforator µm	3,58±1,21	n.d
Nucleus µm	4,44±0,61	4,78 ± 0,25
Head µm	7,85±0,65	9,43 ± 0,38
Tail µm	222,96±17,15	264,07 ± 16,57
Total µm	230,81±17,22	273,50 ± 16,58
Area nucleus µm²	2,17±0,42	n.d.

Tab. 3.1 Difference on morphometric parameters between A.M. Ligustica and A.M. Carnica.

3.3 Conclusions

In conclusion, the results obtained, despite the low number of samples analyzed, allow a first definition of the standard measures of Apis mellifera ligustica spermatozoa and allow to develop a protocol analysis, which proved to be economic, quick and simple, to study the morphology and morphometry of sperm drones and detect any alterations. Considering the bee like a livestock, the reproduction of this animal must be well considered. The productions of the bee can be two, one direct and one indirect. The first is honey production, Europe produce more than 250,000 tons of honey sold at about 5 euro per kg. The second is, as previously mentioned, the pollination capacity that has an economic impact, in Europe, of about 1,200 euros per hectare. This type of study, therefore, beyond the environmental monitoring, could support other activities of safeguarding the Italian bees of Ligustica, showing that the suffering of these insects could allow to contribute to direct the global market towards an ecological agriculture and the maintenance of a high biodiversity by abolishing the use of pesticides and chemical fertilizers and thus bringing benefits to pollinating insects, their health and their fertility. This translates into benefits for crop pollination and in agriculture that is potentially less subject to the use of harmful substances.

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