FACULTY OF VETERINARY MEDICINE
accredited by EAEVE

# REPRODUCTION IN EQUIDAE: A COMPARATIVE STUDY OF DONKEYS AND HORSES

Word count: 17.120

Emma Van den Branden
Student number: 01507050

Supervisor: Prof. dr. Peter Daels
Supervisor: Dr. Osvaldo Bogado

A dissertation submitted to Ghent University in partial fulfilment of the requirements for the degree of Master of Veterinary Medicine

Academic year: 2020 - 2021

GHENT UNIVERSITY

# Preface

This thesis is a dissertation as partial fulfillment of the requirements for the degree of Master of Veterinary Medicine at Ghent University. It has been a great opportunity and experience to study veterinary medicine at Ghent University and to write this thesis.

My keen interest in donkeys was sparked a few years ago when my parents adopted three Poitou donkeys. I realized that we don't learn a lot about donkeys during our courses and that most practitioners in the field treat donkeys as horses. With this thesis I hope to emphasize the important differences between donkeys and horses, not only in reproduction and obstetrics.

Writing a thesis takes a lot of time and effort, and the support of certain people is indispensable. I would first of all like to thank my supervisor, Professor dr. Peter Daels, for his enthusiasm for the subject and his guidance. I also wish to thank my parents for adopting these three donkeys, and especially my mother for proofreading every draft of this thesis. Finally, I would like to thank my friends and boyfriend for their help and support during the writing of this thesis.

I hope you enjoy your reading.

Emma Van den Branden

# Table of contents

# List of abbreviations

| | |
|---|---|
| AI | Artificial insemination |
| AV | Artificial vagina |
| BCS | Body condition score |
| CL | Corpus luteum |
| COCs | Cumulus-oocyte-complexes |
| DAPI staining | 4’,6-diamidino-2-phenylindole staining |
| dCG | Donkey chorionic gonadotrophin |
| DMEM | Dulbecco's modified Eagle's medium |
| DSO | Daily sperm output |
| E2 | Estradiol |
| eCG | Equine chorionic gonadotrophin |
| ET | Embryo transfer |
| FSH | Follicle stimulating hormone |
| GnRH | Gonadotropin-releasing hormone |
| hCG | Human chorionic gonadotrophin |
| HPG axis | Hypothalamic-pituitary-gonadal axis |
| ICSI | Intra cytoplasmatic sperm injection |
| IVM | In vitro maturation |
| LH | Luteinizing hormone |
| OPU | Ovum pick up |
| P4 | Progesterone |
| PBS | Phosphate buffered saline |
| PGF2α | Prostaglandin F2α |
| PGFM | Prostaglandin metabolites |

# 1. Abstract

Abstract

Donkeys and horses show reproductive similarities and differences. Jacks are frequently crossed with mares to produce mules and hinnies, but the difficulties associated with interspecies breeding emphasize how different donkeys are from horses. To treat donkeys properly, equine veterinarians should be educated about their reproductive idiosyncrasies. The estrus cycle of jennies is longer than of mares, and they exhibit some typical sexual behaviors like mouth clapping when in estrus. Jennies show a longer gestation, probably due to their lower placental efficiency. Compared to horses, the reproductive organs of both jennies and jacks are relatively larger. Due to the larger testicles and supplying blood vessels, castration of jacks leads to a higher incidence of hemorrhage complications. Jacks take longer to achieve erection and to ejaculate, which should be kept in mind when trying to collect semen. Pasture and hand breeding are most used when breeding donkeys. However, for the preservation of breeds, suitable protocols for applying reproductive biotechnologies, e.g. artificial insemination and embryo transfer, in donkeys are needed. Artificial insemination can be performed successfully in donkeys, although not widely accepted by owners at this time. Some protocols with cooled semen have been applied in donkeys; however, use of frozen-thawed semen still remains a challenge to this day. Embryo transfer has become more successful over the last years, but pregnancy rates need to improve to become an effective reproductive technique in donkeys. To develop more suitable protocols for artificial breeding, a more profound knowledge of the reproductive anatomy and physiology of the donkey is required.

Samenvatting

Ezels en paarden vertonen zowel gelijkenissen als verschillen op het vlak van voortplanting. Ezelhengsten worden vaak gekruist met merries voor het verkrijgen van muildieren en muilezels, maar de moeilijkheden gepaard met het fokken van muildieren benadrukken hoe verschillend ezels en paarden wel zijn. Als paardendierenarts moet je op de hoogte zijn van de eigenheden van ezels om ze op de juiste manier te kunnen behandelen. De oestruscyclus van de ezelin duurt langer dan bij merries, en het open en toe klappen van de mond is typisch voor hun hengstigheid. Ook hun drachtduur is langer, wat te verklaren valt door een minder efficiënte placenta. De voorplantingsorganen van zowel de ezelin als de ezelhengst zijn relatief groter dan die van paarden. Vanwege de grotere testikels, en bijhorende grotere bloedvaten, leidt castratie van ezelhengsten vaker tot hemorragie. Ezelhengsten hebben meer tijd nodig om een erectie te krijgen en te ejaculeren, iets waar rekening mee moet gehouden worden bij het verzamelen van sperma. Er wordt meestal met ezels gefokt ofwel door ze vrij op de weide te laten ofwel door ze handmatig te dekken. Voor het behoud van met uitsterven bedreigde rassen zijn echter geschikte protocollen voor het toepassen van reproductieve biotechnologie, zoals kunstmatige inseminatie en embryotransfer, nodig. Kunstmatige inseminatie kan met succes bij ezels worden uitgevoerd, hoewel dit nog niet algemeen gedaan wordt. Enkele protocollen zijn reeds beschreven voor inseminatie met gekoeld sperma, maar het gebruik van diepgevroren sperma blijft nog een uitdaging. Embryotransfers zijn de voorbije jaren meer succesvol geworden, maar de drachtigheidspercentages moeten verbeterd worden vooraleer dit als effectieve voortplantingstechniek kan toegepast worden. Voor het ontwikkelen van meer geschikte protocollen is een diepere kennis zijn van de reproductieve anatomie en fysiologie van de ezel nodig.

# 2. Introduction

Donkeys played an important role in society in the past (Burden and Thiemann, 2015; Miragaya et al., 2018). They were used as working animals in agriculture, commerce and militia and were a source of milk, hide and meat (Crisci et al., 2014; Camillo et al., 2018; Miragaya et al., 2018). Although nowadays their role and population has decreased in more industrialized countries, there is a new growing interest in the world for donkey milk, mainly for human consumption but also for production of beauty products (Crisci et al., 2014; Camillo et al., 2018; Miragaya et al., 2018). More recently, they also have acquired a role in recreation, therapeutic activities and production purposes e.g. production of antiantibodies and antivenom drugs (Ali et al., 2014; Burden and Thiemann, 2015; Miragaya et al., 2018). In low-income countries, donkeys are still extremely valuable as working animals and evidently, they are indispensable for the production of mules (Crisci et al., 2014; Burden and Thiemann, 2015; Miragaya et al., 2018).

Both donkeys (*Equus asinus*) and horses (*Equus caballus*) belong to the same taxonomic family, called Equidae (Miragaya et al., 2018). Despite shared heritage between both species, there are genetic, anatomical and medical differences (Burden and Thiemann, 2015). For example, horses have 64 chromosomes while donkeys only have 62, the reason why hinnies and mules are infertile (Burden and Thiemann, 2015).

Although equine veterinarians will rarely come across cases requiring treatment of reproductive disorders in donkeys, it is important that they can give qualitative advice to breeders and owners whenever needed. When treating donkeys, equine medical practices are commonly applied. However, donkeys are not just another type of horse; they are unique equines with their own specific characteristics. Donkeys have reproductive dissimilarities, e.g. a longer gestation length and estrus cycle, and some typical sexual behaviors like mouth clapping when in estrus (Miragaya et al., 2018). With a total global donkey population estimated to be 44 million, it is important to be aware of those differences in order to treat this species appropriately and effectively (Burden and Thiemann, 2015). Unfortunately, expertise in donkey reproduction is still limited (Crisci et al., 2014).

The donkey population in the more industrialized countries has declined dramatically, up to 80%, in the 20th century (Crisci et al.,2014; Camillo et al., 2018). During this period, no attention was paid to the breeding selection and biodiversity conservation, and nowadays most of the European breeds are considered endangered by the Food and Agriculture Organization of the United Nations (Camillo et al., 2018).

Another reason for the shrinkage in the global population is the increasing demand for donkey hides from China. Medicinal and rejuvenating effects have been attributed to the gelatin produced from donkey hides, called “ejiao” (The Donkey Sanctuary, 2019). Due to the Chinese economic growth, ejiao is now affordable for a much larger section of the population, causing the growing demand for donkey hides (The Donkey Sanctuary, 2019). With less than 50 million donkeys worldwide, this demand threatens a whole species towards global extinction and the scale of the trade is devastating rural communities worldwide and lacks any form of legislation and control (Köhle, 2018).

Reproductive biotechnology is one of the potential tools for the conservation of endangered species and breeds, hence the application of assisted reproductive techniques in donkeys such as artificial insemination, sperm cryopreservation, embryo transfer and newer technologies should be investigated in more depth in order to broaden our current knowledge of reproductive physiology and options for preserving endangered breeds or even an entire species (Miragaya et al., 2018).

In summary, two main problems are intertwined: on the one hand the decreasing population and on the other hand the lack of knowledge concerning donkey medicine and especially reproduction. The context of the globally decreasing donkey population strengthens the importance of improving and disseminating the current knowledge far beyond its relevance for individual breeders and owners. The objective of this literature review is to collect and compare available and scientifically substantiated information regarding reproduction in donkeys and identifying knowledge gaps requiring further investigation. The results of this study will enable equine veterinarians to have first-hand and readily-accessible information on reproductive topics of relevance when giving qualitative advice to breeders and owners.

# 3. Literature study

## 3.1 Female reproduction

### 3.1.1 Anatomy

Anatomical data regarding the reproductive organs of jennies are sparse. Based on published comparative studies, the genital organs of donkeys appear similar to those of mares, although their internal genitalia are proportionally larger (Renner-Martin et al., 2009; Canisso et al., 2019). The vascularization of the reproductive tract is very similar to that of mares (Abd-Elnaeim et al., 2001). The anatomy of the female reproductive organs is depicted in Figure 1.

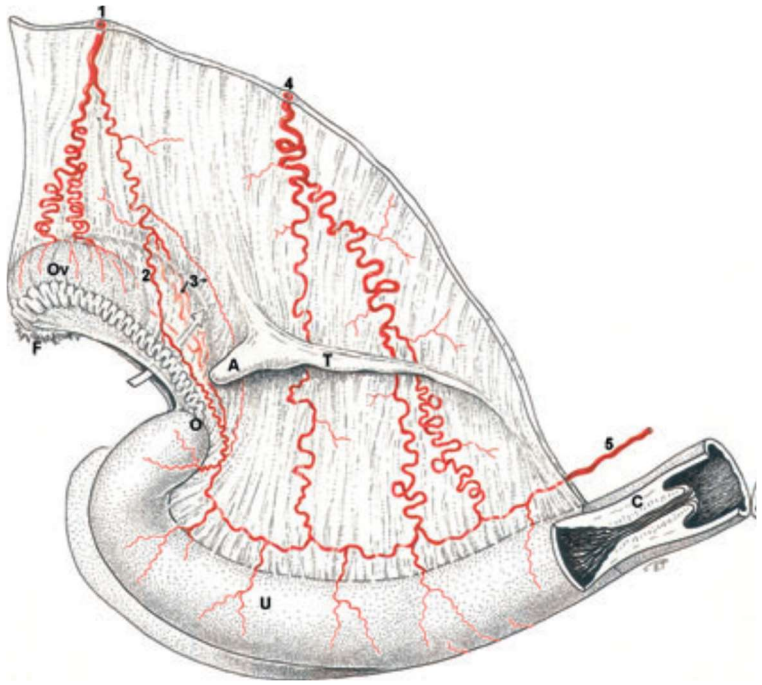


*Figure 1. Left lateral aspect of parts of the genital tract and of the ligamentum latum uteri (cut) in a jenny.*
*White arrow points into the ovarian bursa. 1. arteria ovarica; 2. ramus tubarius of arteria ovarica; 3. rami uterini of arteria ovarica; 4. arteria uterina; 5. ramus uterinus of arteria vaginalis; Ov. left ovary; F. fimbriae tubae uterinae; O. ostium uterinum tubae; U. cornu uteri sinistrum; A. appendix of ligamentum teres uteri; T. ligamentum teres uteri; C. cervix uteri.*
*Source: Renner-Martin et al. (2009)*

#### 3.1.1.1 Uterus and ovaries

The uterus is Y-shaped, when viewed dorsally in its normal anatomical position and is relatively larger than the standard equine size (Renner-Martin et al., 2009; Purdy, 2010). The diverging horns form cranioventral convexities and almost reach the caudal extremities of the ovaries where they incorporate the infundibulum of the oviduct (Renner-Martin et al., 2009). The tips of the horns are located at the level of the fifth lumbar vertebra (Renner-Martin et al., 2009). The ovaries are located at the level of the fourth to fifth lumbar vertebra, which is slightly further cranial than in mares, but their measurements do not differ noticeably from those of mares (Renner-Martin et al., 2009). Similar to mares, the ovaries are bean-shaped, bearing an ovulation fossa at their free border (Renner-Martin et al., 2009).

#### 3.1.1.2 Cervix and vagina

The cervix has a similar appearance to that of horses but points upwards, is longer and has a smaller diameter (Purdy, 2010). This, along with the fact that the donkey cervix protrudes deeper into the vagina compared to horses, may preclude intrauterine ejaculation and also represents a challenge for intrauterine procedures, particularly in small-frame maiden jennies (Pugh, 2002; Hagstrom, 2009; Canisso et al., 2019). The cervical channel exhibits longitudinal mucosal folds showing a tortuous course which seems to be a specific asinine feature, and these folds are continuous with the endometrial folds of the uterine body (Renner-Martin et al., 2009). These features make the donkey cervix predisposed to lacerations and associated post-dystocia cervical adhesions (Pugh, 2002; Canisso et al., 2019). There are also folds in the vaginal region, dorsally and ventrally, obstructing passage to the cervix (Burnham, 2002).

#### 3.1.1.3 Ligamentum latum uteri

The morphology of the ligamentum latum uteri differs in two aspects from horses: (1) the mesosalpinx is much wider, covering the lateral aspect of the ovary like a curtain and forming a spacious bursa ovarica; and (2) the ligamentum teres uteri is taut and more pronounced than in mares, and provided with a very prominent cranial end, called the appendix (Renner-Martin et al., 2009).

#### 3.1.1.4 Pelvis

The jenny's pelvis is more sloped downward (Hagstrom, 2009). This leads to the vulva being tipped such that the ventral commissure of the vulva is more cranial than the dorsal commissure. This is a more desirable angle because this slightly tilted ventrally slope makes contamination of the reproductive tract less likely. Thus a Caslick's procedure (vulvoplasty) is less frequently required and performed in jennies (Hagstrom, 2009; Canisso et al., 2019).

#### 3.1.1.5 Vulva and clitoris

The vulva is, as mentioned above, slightly tilted ventrally (Canisso et al., 2019). The vulva lies entirely below the pelvic brim, contrary to mares where the vulva is 1/3 above the pelvic brim (Canisso et al., 2019). The minor vulvar lips and clitoris are larger than in mares (Canisso et al., 2019).

### 3.1.2 Puberty

Puberty is usually reached at 1 to 2 years of age (Pugh, 2002). This variability can be attributed to diverse factors such as breed, nutrition, health, temperature, and photoperiod (Tibary, 2004). When these factors are all favorable for an early onset of puberty, then puberty can take place at only 1 year of age (Fielding, 1988). Although 1 year old jennies can be sexually mature, they should not be bred before 3 years of age (Yilmaz et al., 2012; Canisso et al., 2019). The most fertile age is 4 years (Yilmaz et al., 2012).

### 3.1.3 Estrus cycle

Knowledge of the estrus cycle can greatly influence breeding management, and an interchange of equine knowledge to donkeys is often inaccurate (Contri et al., 2014). Jennies are polyestrus and may cycle throughout the winter, and their estrus cycle seems to be different compared to mares (Purdy, 2010; Yilmaz et al., 2012; Contri et al., 2014).

In jennies, the estrus cycle ranges from 20-40 days with an average of 24 days, whereas mares have a slightly shorter cycle ranging from 18-24 days with an average of 21 days (Pugh, 2002; Hagstrom, 2009; Miragaya et al., 2018). The estrus cycle length of mules tends to be intermediary with an average of 22 days (Canisso et al., 2019). The wide variation in estrus cycle length in jennies results from an interaction between environmental and genetic factors, e.g. age and body condition score (BCS), with

older or obese jennies having longer interovulatory intervals (Fielding, 1988; Quaresma and Payan-Carreira, 2015). Similar to horses, the average estrus length can differ between breeds (Miragaya et al., 2018). The duration of estrus is similar between jennies and mares, whereas the duration of diestrus is longer in jennies (Blanchard et al., 1999). The estrus varies from 4 to 10 days, with ovulation occurring in the last 24 hours, and the diestrus varies from 15 to 19 days (Miragaya et al., 2018; Canisso et al., 2019).

The trends in hormonal plasma concentrations during the estrus cycle in the jenny are displayed in Figure 2. The trend of estradiol (E2) and progesterone (P4) plasma concentrations during the estrus cycle are similar to those reported for mares (Contri et al., 2014). An increased growth and activity of the granulosa cells within the dominant follicle lead to an increase in E2 concentrations, from 10 pg/ml during early estrus to a peak around 40 to 60 pg/ml at 2 days before ovulation (Contri et al., 2014; Canisso et al., 2019). At 2 days before ovulation, a sharp reduction in E2 concentrations is observed followed by a steadier decrease between days 1 and 4 post-ovulation, a well-known pattern in the mare (Contri et al., 2014).

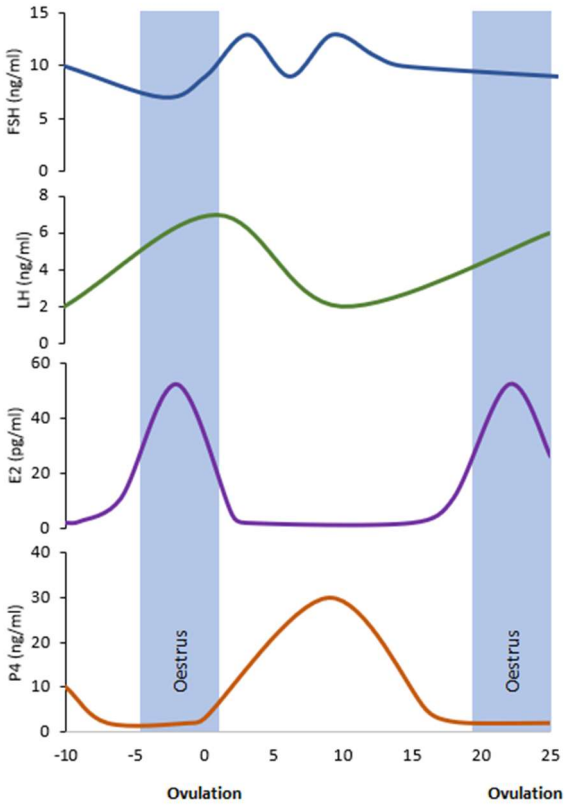


*Figure 2. Trends in hormonal concentrations in the peripheral circulation of the jenny during the estrus cycle. Sources: Contri et al. (2014), Vandeplassche et al. (1981)*

The mean P4 concentrations remain low up to the day after ovulation (Bansal et al., 2006; Canisso et al., 2019). From day 2, it slowly increases until day 4 to 6 post-ovulation, reaching its highest level approximately on day 11 (Bansal et al., 2006; Canisso et al., 2019). P4 concentrations start to drop, returning to baseline concentrations (< 1 ng/mL), 2 to 3 days before the onset of estrus (Quaresma and Payan-Carreira, 2015; Canisso et al, 2019). The mean P4 concentrations in diestrus are higher in cycles with multiple ovulations than in those with single ovulations (Quaresma and Payan-Carreira, 2015).

Beside E2 and P4, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) play an important role in the estrus cycle (Canisso et al., 2019). FSH concentrations remain low throughout the estrus cycle, and peak 3 and 9 days post-ovulation to stimulate the growth of follicles (Canisso et al., 2019). LH concentrations increase 8 days before ovulation to eventually initiate ovulation, peak 2 days after ovulation, and then decrease to baseline (Meira et al., 1995; Canisso et al., 2019).

Jennies normally have 1 follicular wave, whereas in mares mostly 2 follicular waves are observed (Canisso et al., 2019). However, a study by Lemma et al. (2006) described the occurrence of more than one wave in jennies. Multiple follicles of different sizes can be observed per ovary, depending on the stage of the estrus cycle, with small follicles mostly occurring during the post-ovulatory period and large follicle during the pre-ovulatory period (Meira et al., 1995; Miragaya et al., 2018). In a study by Lemma et al. (2006), the smallest ovarian follicle detected was 2 mm and the largest 40 mm. Follicular deviation, i.e. the moment that one of the follicles becomes the dominant follicle with a diameter of approximately 19 to 25 mm, takes place 8 to 9 days before ovulation (Miragaya et al., 2018; Canisso et al., 2019). Follicles with a diameter of 25-30 mm should be considered potentially pre-ovulatory, but

this may vary between breeds with a positive correlation between body frame and pre-ovulatory follicular diameter (Pugh, 2002; Miragaya et al., 2018; Canisso et al., 2019). A better BCS is associated with larger sizes of pre-ovulatory follicles (Kebede et al., 2012). The average follicular diameter before ovulation is smaller in cycles with multiple ovulations than in cycles with a single ovulation (Quaresma and Payan-Carreira, 2015). The dominant follicle grows 2 to 3 mm diameter per day; however, this is also breed-dependent with large breeds' follicles growing up to 4 mm per day (Canisso et al., 2019).

Ovulation takes place less than approximately 15 hours before the end of the estrus (Quaresma and Payan-Carreira, 2015). The best predictors for an imminent ovulation are estrus behavior, follicle size and follicular texture (Taberner et al., 2008). Close to ovulation, estrus behavior intensifies (this behavior will be described later) and continues for up to 15 hours after ovulation (Quaresma and Payan-Carreira, 2015; Canisso et al., 2019). Growth of the pre-ovulatory follicle stagnates the day before ovulation and circa 80% of pre-ovulatory follicles change in shape from spherical to oval, irregular or ellipsoid (Lemma et al., 2006; Taberner et al., 2008). The remaining 20% preserves its spherical shape until ovulation (Taberner et al., 2008). Close to ovulation, the follicle becomes fluctuant, with a decreased turgidity of the wall (Contri et al., 2014). A jenny showing both mouth clapping, urination and immobility, and having a follicle larger than 45 mm with a very soft texture, is very likely to ovulate within the next 24 hours (Taberner et al., 2008). During estrus, the uterus loses tonicity and becomes edematous, but there are no important day-to-day changes during estrus; therefore, uterine tone does not help predict ovulation (Taberner et al., 2008).

In several studies, there were slightly more ovulations in the left ovary than in the right (Miragaya et al., 2018). However, in a study by Quaresma and Payan-Carreira (2015), a non-significantly higher frequency of ovulations occurred in the right ovary. Occurrence of multiple ovulations is higher in donkeys and variable across breeds, similar to the differences observed in horse breeds (Pugh, 2002; Canisso et al., 2019). In a study by Galisteo and Perez-Marin (2010) the estrus duration tended to be longer when multiple ovulations occurred, perhaps due to the hormonal environment and follicular growth. However, this finding conflicts with another study by Hughes et al. (1975) reporting that multiple ovulations did not appear to affect the duration of the estrus cycle in mares.

In the absence of pregnancy, the corpus luteum (CL) remains active for 15.5 ± 5.11 days (Tibary, 2004; Lemma et al., 2006). The early CL may exhibit a homogeneous echotexture, but in 95% of the cases a white central hyperechoic zone is created before complete luteolysis. In less than 5% of cases, jennies show a non-echogenic central lacuna that gradually reduces in size until luteolysis (Lemma et al., 2006; Miragaya et al., 2018). The diameter of the CL and the vascularized area are affected by the P4 plasma concentrations, with the size of the CL reducing sharply after the decrease in the P4 plasma concentration (Meira et al., 1995; Panzani et al., 2018a). Color Doppler provides a rapid and easy way to examine the state of luteolysis (Miró et al., 2015). Miró et al. (2015) observed an increase in luteal blood flow followed by a progressive reduction after induced luteolysis, in contrast to mares where no increase in luteal blood flow is seen after induced luteolysis.

The hormones playing a role in the estrus cycle influence the jennies' reproductive tract. In estrus, the cervix relaxes and the vaginal mucus secretion increases (Miragaya et al., 2018). Endometrial edema and uterine size increase towards the day of ovulation (Lemma et al., 2006). There is a positive correlation between the size of the dominant follicle, the cross-sectional diameter of the uterus, and the edema score of the endometrial folds (scored on a scale of 0 = no folds, 1 = very slight folds, 2 = moderate folds, and 3 = extreme folds) (Lemma et al., 2006; Miragaya et al., 2018). However, the score of the endometrial folds is not a reliable predictor of an imminent ovulation (Miragaya et al., 2018).

### 3.1.4 Sexual behavior

*Figure 3. An interaction between a Poitou jenny (left) and jack (right). The jenny is showing mouth clapping.*
*Source: Author*

Estrus detection is generally easy in jennies as their typical behavior makes it very obvious to determine when they are ready to be bred (Hagstrom, 2009). When in estrus, usually only the sound of a jack braying prompts them to display estrus behavior (Hagstrom, 2009). Typical signs of a jenny in estrus are mouth clapping (the frequent vertical opening and closing of the mouth with ears pressed against the extended neck accompanied by a characteristic sound, as depicted in Figure 3), sialorrhea, clitoral winking, urinating, tail raising at a 45° angle, vulvar edema, and assuming the breeding stance with their hind legs spread apart (Pugh, 2002; Tibary, 2004; Taberner et al., 2008; Hagstrom, 2009; Yilmaz et al., 2012; Miragaya et al., 2018; Canisso et al., 2019). Jennies also hit the jacks to the chest with abbreviated double hind-leg lifts for several minutes, which is required for the jack to achieve full erection (Purdy, 2010; Miragaya et al., 2018). Interactions between jennies are also recorded, especially if another female is in heat as well, including herding/chasing, showing the flehmen response after smelling urogenital secretions from other jennies in estrus, and mounting each other like cows, which is rarely seen among mares (Taberner et al., 2008; Hagstrom, 2009; Purdy, 2010; Canisso et al., 2019). Females bray to call mates during estrus and are therefore more vocal than mares during their heat period (Hagstrom, 2009; Yilmaz et al., 2012). Some jennies do not show estrus behavior when there is no jack present, when nursing a foal, or when another female interferes (Purdy, 2010).

When they are in diestrus, jennies run away when approached by jacks and clamp the base of the tail against the perineum (Miragaya et al., 2018). Jennies in diestrus may be very aggressive towards the jack and may bite and kick the jack with full extension of both hind legs (Miragaya et al., 2018).

### 3.1.5 Seasonality

Seasonality is a controversial subject in various studies (Miragaya et al., 2018). It is influenced by photoperiod combined with other factors such as breed, BCS, nutrition, health, and environmental conditions (Miragaya et al., 2018). Some researchers describe the jenny as nonseasonal, while others claim the opposite (Fielding, 1988; Canisso et al., 2019). However, they all agree that donkeys seem to display less seasonality in comparison with horses (Fielding, 1988; Pugh, 2002; Miragaya et al., 2018; Canisso et al., 2019). Henry et al. (1987) reported that only 40% of the monitored jennies in their study showed a seasonal anestrus. Zakari et al. (2015) reported that all jennies included in their study cycled throughout the whole year. Hence, it is important to know that it is likely for jennies to cycle throughout winter (Henry et al., 1987; Purdy, 2010; Zakari et al., 2015). This could suggest a different seasonal effect on the hypothalamic-pituitary-gonadal (HPG) axis, in comparison with mares. Winter anestrus in mares is driven by the photoperiod, and means a complete stop of reproductive activity because the activity of the HPG axis stops (Contri et al., 2014).

Several studies report that the estrus cycle length in jennies is longer in summer compared with autumn and winter (Galisteo and Perez-Marin 2010; Contri et al., 2014; Miragaya et al., 2018). However, estrus tends to be shorter during spring and summer, and is characterized by the ovulation of a larger follicle compared to autumn and winter (Contri et al., 2014; Canisso et al., 2019). Diestrus is shorter in summer compared to the other seasons (Contri et al., 2014). High environmental

temperatures may negatively affect the reproduction of donkeys, but the role of the environment on the estrus cycle and reproductive endocrinology still has not been thoroughly investigated (Contri et al., 2014; Zakari et al., 2015). Studies performed in different locations worldwide observed variations in the frequency and length of the estrus cycle, which could be attributed to differences in climate (Pugh, 2002; Tibary, 2004; Miragaya et al, 2018). As mentioned previously, breed and BCS are also influencing factors on seasonality. Jennies with a good BCS continue to cycle throughout the year, whereas jennies with a poor BCS are more likely to stop cycling during the fall and winter (Canisso et al., 2019).

### 3.1.6 Rectal palpation and ultrasonography

Although the reproductive tract of jennies is proportionally larger than in mares, rectal palpation is challenging in small-framed donkeys (Canisso et al., 2019). A jenny's pelvis is more sloped downward from front to back, which makes rectal palpation more difficult (Hagstrom, 2009). Additionally, donkeys tend to have stronger rectal contractions (Hagstrom, 2009). The use of lubricant alone or in combination with butylscopolamine bromide may facilitate rectal palpation (Canisso et al., 2019).

The reproductive tract of jennies resembles that of the mare on ultrasound, with the exception that uterine edema is less pronounced (Canisso et al., 2019). The use of transrectal ultrasonography in jennies has been proven very useful in reproductive management, but animal character and economic considerations may impede transrectal ultrasonographic scanning (Lemma et al., 2006; Galisteo and Perez-Marin, 2010). Its application includes monitoring follicular changes, prediction and confirmation of ovulation, evaluation of CL, estimating the stage of the estrus cycle, diagnosis of ovarian and uterine pathology, and detection of early pregnancy and twins (Lemma et al., 2006).

### 3.1.7 Endometrial culture, cytology and biopsy

Culture and cytology can be performed as in mares, with a cotton-tip double-guarded swab, a cytobrush or a small-volume uterine lavage (Canisso et al., 2019). Cytology of a healthy jenny reveals the occasional presence of inflammatory cells (Canisso et al., 2019). After insemination, especially with frozen-thawed semen, jennies have a physiological post-breeding inflammatory response (Canisso et al., 2019). Persistently high inflammatory cell counts in the uterine cytology however may be associated with endometritis, but cutoffs for physiological versus pathological post-breeding inflammatory response have not been determined for jennies (Canisso et al., 2019).

Endometrial biopsy can be used to assess inflammation and degenerative changes (Canisso et al., 2019). Based on the amount of fibrotic tissue and the extent and severity of inflammatory changes in the endometrium, jennies can be assigned to categories I, IIa, IIb or III, as used in a study in mares by Kenney and Doig (1986). Compared to mares, a healthy jenny endometrium has more neutrophils, eosinophils, and highly branched uterine glands (Canisso et al., 2019). These features contribute by default to a higher category on the Kenney and Doig (1986) classification, which should be kept in mind when categorizing jennies (Canisso et al., 2019). In jennies, there is no increase in total collagen and collagen Type 1 and no reduction in collagen Type 3 when the endometrium undergoes degenerative changes, as seen in mares (Canisso et al., 2019). This may explain why aged jennies are still able to conceive and carry pregnancies to term (Canisso et al., 2019).

### 3.1.8 Hormonal manipulation

An important goal of reproductive management is to maximize the production of live foals (Blanchard et al., 1999). The jenny should be bred near the time of ovulation, in order to use the breeding jack efficiently (Blanchard et al., 1999). Hormonal treatment plays an important role in the regulation of the time of ovulation, and thus the use of exogenous hormones is helpful for scheduling breeding at desired times (Blanchard et al., 1999). In mares, hormones commonly administered include prostaglandin F2α (PGF2α), human chorionic gonadotrophin (hCG), gonadotropin-releasing hormone

(GnRH), P4 and E2. Hormonal manipulation of the estrus cycle can also be used in donkeys for luteolysis, induction of ovulation and estrus synchronization.

#### 3.1.8.1 Prostaglandin F2α

The CL of mares is considered sensitive to PGF2α starting 5 days after ovulation, with the best luteolytic response obtained between 5 and 10 days after ovulation (Carluccio et al., 2006). PGF2α can also be used in jennies and mules to induce luteolysis, with an interval to estrus of approximately 3 to 6 days (Blanchard et al., 1999; Canisso et al., 2019). Compared to the spontaneous estrus cycle, the length of PGF2α-induced estrus is shorter (Carluccio et al., 2003).

There are minor to no adverse reactions associated with dinoprost, cloprostenol, alphaprostol, or luprostiol in jennies (Canisso et al., 2019). The dose of cloprostenol in jennies should be reduced, because side effects have been noted with the standard doses (Panzani et al., 2018; Canisso et al., 2019). There is still a lot of controversy regarding the earliest time-point that jennies will respond to a single PGF2α administration (Canisso et al., 2019). In a study by Carluccio et al. (2006), cloprostenol was administered to jennies at different time-points after ovulation and the results indicate that the CL is already sensitive to PGF2α three days after ovulation but these findings are contradicted by a study performed by Panzani et al. (2018a), where no shortening of the interovulatory interval following administration of alfaprostol 3 days post-ovulation was observed, which resulted in incomplete luteolysis in most of the jennies.

#### 3.1.8.2 Human chorionic gonadotropin and gonadotropin-releasing hormone

Spontaneous ovulation occurs at an unpredictable time during estrus, and thus induction of ovulation is a useful tool to narrow the ovulation window and facilitate assisted reproductive techniques such as embryo transplantation and artificial insemination with frozen-thawed semen (Carluccio et al., 2007; Camillo et al., 2014; Canisso et al., 2019). Both hCG and GnRH analogues are used to hasten ovulation (Canisso et al., 2019). hCG is the most efficient for induction of ovulation, but it is well known that the effectiveness of hCG in equids is reduced after multiple injections, due to the immunogenic properties of this human derived protein (Carluccio et al., 2007; Camillo et al., 2014). GnRH agonists, such as deslorelin acetate, lecirelin actetate and buserelin acetate, are also effective in inducing ovulation in donkeys (Camillo et al., 2014). With a single subcutaneous injection of buserelin acetate at a very low dose, it is possible to induce ovulation within 24 and 48 h in jennies (Camillo et al., 2014). A single administration of lecirelin acetate can also induce ovulation in jennies with a large follicle (> 30 mm), unlike in mares where the administration of only one or two administration of GnRH are mostly insufficient (Carluccio et al., 2007).

As mentioned, the size of the follicle is important when administering hCG or GnRH (Carluccio et al., 2007). The follicular diameter affects the interval of administration to ovulation, with smaller follicles requiring a longer interval to ovulation compared to larger follicles (Carluccio et al., 2007). The ideal follicular size for induction of ovulation depends on body size, with small breeds ovulating 28-32 mm follicles, and larger breeds ovulating 40-44 mm follicles (Canisso et al., 2019). Endometrial edema is a common criterion to induce ovulation in mares but is less pronounced in jennies (Canisso et al., 2019). Therefore, teasing is essential to determine if a jenny is ready to be induced or bred (Canisso et al., 2019).

#### 3.1.8.3 Estrus synchronization protocols

Estrus synchronization protocols used in horses are effective in donkeys (Blanchard et al., 1999). These protocols are useful in the reproductive management of equids because they reduce the need for multiple reproductive examinations before breeding (Canisso et al., 2019). There are two well-known estrus synchronization protocols used in jennies.

In the first protocol, the jennies are injected intramuscularly with PGF2α twice, with 16 to 17 days apart (Blanchard et al., 1999). The double injection is needed because a mature CL should respond to the first injection, and the jenny should return to estrus, ovulate, and 16 to 17 days later have another mature CL responsive to the second injection (Canisso et al., 2019). If the jenny does not have a mature CL at the time of the first injection, she should have a CL responsive to the second injection after 16 to 17 days (Canisso et al., 2019). This protocol can be modified by administering GnRH 7 days after the first PGF2α to ensure ovulation (Canisso et al., 2019). The second protocol is a combination of P4 (injectable or an intravaginal releasing device) and PGF2α to simulate a luteal phase followed by luteolysis (Blanchard et al., 1999; Canisso et al., 2019). P4 can be administered in combination with E2 for 10 days to provide a better synchronization (Canisso et al., 2019).

A study by Blanchard et al. (1999) compared the two protocols and found that the interval to ovulation was not reduced with the second protocol, making the additional time and expense of this protocol unnecessary, compared to the first protocol.

## 3.2 Male reproduction

### 3.2.1 Anatomy

Like the jenny, the jack has many similarities in the anatomy of the urogenital system to the horse; however, some differences exist (Pugh, 2002; Mai, 2014). The main difference is that their proportionally larger genitalia (Mai, 2014).

#### 3.2.1.1 Penis

The anatomy of the penis and prepuce of the jack is similar to the stallion. However, the penis is relatively longer, and a nipple can be seen on each side of the preputial sheath (Mai, 2014; Miragaya et al., 2018; Canisso et al., 2019). The glans penis presents a more pronounced dilation during erection and ejaculation (Miragaya et al., 2018). The prepuce's internal lamina forms the corona glandis and the collum glandis, which are not as prominent as in the stallion (Mai, 2014; Canisso et al., 2019).

#### 3.2.1.2 Scrotum and testes

The gross anatomy of the scrotum is similar to stallions, but jacks have a more pendulous scrotum (Canisso et al., 2019). The skin of the scrotum is softer and thicker, owing to the excess fat store, and covered with sweat glands which play a role in the thermal regulation of the testes (Miragaya et al., 2018; Canisso et al., 2019).

Equids have ovoid testes, with stallions having smaller testes that are narrowed laterally, whereas jacks have larger and more globular shaped testes (Miragaya et al., 2018; Canisso et al., 2019). The testes have a horizontal orientation or a slight cranio-dorsal inclination and are freely movable within the scrotum (Canisso et al., 2019). Testicular volume ranges from 250 to 500 $cm^3$ without differentiation between the left and right testis (Quartuccio et al., 2011). The average volume of testicular parenchyma, consisting of Sertoli and Leydig cells, increases three folds from 1.5 to 5 years of age, but nothing is known about changes at younger ages (Nipken and Wrobel, 1997; Rota et al., 2018a).

The spermatogenic cycle in jacks takes approximately 10.5 days, which is noticeably shorter than the approximately 12.2 days in stallions (Neves et al., 2002). The total duration of the spermatogenesis is estimated to take circa 47.2 days (Neves et al., 2002). The high spermatogenic efficiency and a relatively short length of spermatogenesis, in combination with large testes make the sperm maturation and storage in the jack the most efficient among domestic mammals (Canisso et al., 2019). This requires very large epididymides for sperm maturation and storage in jacks (Canisso et al., 2019).

#### 3.2.1.3 Spermatic cord

Donkeys have a very prominent ductus deferens with a diameter of 23-28 mm, making it easily palpable through the skin of the scrotum (Canisso et al., 2019). They also have a visibly larger central vein and a higher artery flow in the spermatic cord than horses, which could explain the better testicular efficiency (Canisso et al., 2019). The unique histologic features also facilitate a higher venous blood flow from the testis (Canisso et al., 2019).

#### 3.2.1.4 Accessory sex glands

The sexual glands include the ampulla, bulbourethral gland, vesicular gland and prostate gland (Contri et al., 2008; Canisso et al., 2019). In stallions, these glands secrete about 95% of total semen volume, but their contributions in jacks are still unknown (Contri et al., 2008; Canisso et al., 2019). The sex glands have the same shape as in stallions, but have a bigger diameter, which might result in the higher gel-free volume seen in jacks (Contri et al., 2008; Mai, 2014). The glands increase during sexual

stimulation and return to their original size after ejaculation, with the exception of the vesicular gland, which decreases in size after ejaculation in contrast to the stallion (Contri et al., 2008; Mai, 2014). The ampulla is more muscular and thicker in equids than other species, and is larger in donkeys than in horses, due to its more folded mucosa and perhaps due to the continuous stimulation provided by male interaction, as jacks are usually kept together (Miragaya et al., 2018; Canisso et al., 2019). Ampullary blockage, a frequently seen pathology in stallions, rarely affects jacks (Canisso et al., 2019).

### 3.2.2 Puberty

With onset of puberty in jacks being defined as the first ejaculate containing ≥ $50\times10^6$ spermatozoa with ≥ 10% total motility, puberty is only reached at 19 to 20 months of age, and it is generally accepted that almost all jacks are sexually mature by 2 years of age (Yilmaz et al., 2012; Rota et al., 2018a). However, when housing female and male yearlings together, it is important to keep in mind that some jacks can produce sperm as early as the age of 9 months (Yilmaz et al., 2012). Although jacks can produce spermatozoa at puberty, semen quality is still low (Rota et al., 2018a). The semen quality improves progressively over time, and is accompanied by changes in testicular size, testicular blood flow and testosterone plasma concentrations (Rota et al., 2018a).

### 3.2.3 Endocrinology

Many hormones are involved in the regulation of male reproductive functions, such as sexual behavior, libido, erection and ejaculation (Veronesi et al., 2011). However, reproductive endocrinology is still poorly understood in jacks (Canisso et al., 2019). Prostaglandin metabolites (PGFM), testosterone, LH, estrone sulfate and cortisol play a role in the endocrinology of the stallion, but the last three hormones are not as important in jacks.

PGFM stimulates the release of oxytocin and vasopressin and acts as a potent contractile agent on the male reproductive system (Veronesi et al., 2011). In jacks, there are no changes in PGFM concentrations during erection and ejaculation (Veronesi et al., 2011; Miragaya et al., 2018). However, an increase of PGFM is reported from the time of dismounting until approximately 22 minutes after ejaculation (Veronesi et al., 2011; Miragaya et al., 2018). This is in disagreement with the stallion, where PGFM increases progressively during erection and ejaculation, and remains at high concentrations up to 60 minutes after ejaculation (Veronesi et al., 2011; Miragaya et al., 2018).

Testosterone in jacks reaches maximum concentrations during ejaculation, and shows an increase during erection and dismounting, which can be explained by its influence on these processes and on sexual behavior (Veronesi et al., 2011; Rota et al., 2018b; Miragaya et al., 2018). In contrast, testosterone concentrations in stallions only increase within 5-30 minutes of ejaculation (Veronesi et al., 2011; Miragaya et al., 2018). The earlier increase in jacks could be due to the longer time needed to achieve erection (Veronesi et al., 2011).

Both luteinizing hormone and estrone sulfate concentrations did not show differences in the period before and around sexual stimulation in jacks whereas in stallions a post-ejaculation increase in estrone sulfate concentrations is observed (Veronesi et al., 2011; Miragaya et al., 2018). Cortisol plasma concentrations in the jack increase approximately 30 minutes after ejaculation, but there is a high individual variability (Veronesi et al., 2011). This is in contrast to the stallion, where cortisol plasma concentrations increase immediately after sexual stimulation (Veronesi et al., 2011).

### 3.2.4 Sexual behavior

Sexual behavior in jacks differs markedly from stallions (Tibary, 2004; Rota et al., 2018b). Domestic jacks kept free at pasture, show the characteristics of a territorial, non-harem breeder both with jennies and mares (Canisso et al., 2009a; Miragaya et al., 2018). The jack pays little ongoing attention to jennies not in heat (Miragaya et al., 2018).

In the presence of an estrus jenny the jack's normal response is vocalization and flehmen to initiate precopulatory interaction (Canisso et al., 2009a; Purdy, 2010; Yilmaz et al., 2012). The jack pursues a jenny in estrus sometimes very aggressively, and especially when first introduced (Purdy, 2010; Miragaya et al., 2018). He may bite the neck, back, and hind legs even until bleeding to make sure they are submissive (Taberner et al., 2008; Purdy, 2010; Miragaya et al., 2018). During the pre-copulatory and copulatory phases, jacks exhibit behaviors such as naso-nasal contact, mounting without erection, partial and total exposure of the penis, sniffing and biting specific areas of the female body, and lip clapping (Gastal et al., 1996). After multiple mounts, the jack retreats away from the jenny (Canisso et al., 2009a; Purdy, 2010). This period of disinterest and inactivity at short distance from the jenny is called male isolation, and is unique to jacks (Gastal et al., 1996; Purdy, 2010; Carluccio et al., 2013b). The male isolation is the time needed for pheromones to reach the vomeronasal organ through the flehmen response and activate the neuroendocrine system, and thus affect the limbic system (Carluccio et al., 2013b). Erection is typically achieved after this male isolation (Canisso et al., 2009a; Purdy, 2010). Within another few minutes the jack typically returns to the jenny, resumes teasing and then completes the breeding (Canisso et al., 2009a; Purdy, 2010).

### 3.2.5 Erection and ejaculation

Jacks take longer to achieve erection and ejaculation than stallions (Miragaya et al., 2018). The time from the first contact to ejaculation is variable between individual jacks and may range from 4-33 minutes with an average of 14-15 minutes, which is fairly long when compared to the approximately 10-11 minutes it takes stallions (Gastal et al., 1996; Canisso et al., 2009a; Veronesi et al., 2011; Carluccio et al., 2013a; Miragaya et al., 2018). Younger and older jacks in particular are slow breeders, and some jacks may take hours or never complete the breeding act at all (Canisso et al., 2009a; Hagstrom, 2009). Complete ejaculation in jacks takes approximately 6-12 seconds (Pugh, 2002).

### 3.2.6 Sperm characteristics

Sperm quality has an important role in determining fertility (Dorado et al., 2013a). Principal component analyses show sperm L-lactate production is a good indicator of sperm metabolism status (Miró et al., 2005). This, and the evaluation of seminal parameters and daily sperm output (DSO), are good predictors of sperm quality (Miró et al., 2005). The most important seminal parameters are total semen volume, sperm concentration, gel fraction, pH, viability, motility and morphology. Sperm motility, morphology, and pH are correlated with the BCS of the donkey, whereas none of the evaluated sperm characteristics are correlated with the age of the donkey (Dorado et al., 2013b).

#### 3.2.6.1 Daily sperm output (DSO)

An evaluation of the DSO could be indicative of the potential fertility, and is thus useful for maximization of the reproductive activity (Quartuccio et al., 2011). To calculate the DSO, daily semen collection for 10 consecutive days has been suggested (Quartuccio et al., 2011). In a study by Quartuccio et al. (2011), the correlation between testicular volume and DSO was poor and may suggest that both are not correlated in the donkey. However, the authors suggest that a larger number of included males may reveal a correlation.

3.2.6.2 Total volume, concentration and gel fraction

The semen volume and the total number of spermatozoa are relatively higher than in stallions (Tibary, 2004; Veronesi et al., 2011). Tibary et al. (2004) reported that the mean total volume of the ejaculate ranges from 10-250 ml, depending on the breed, whereas in stallions it ranges from 28-65 ml (Pugh, 2002; Contri et al., 2008; Contri et al., 2010; Veronesi et al., 2011; Carluccio et al., 2013a). Sperm concentration is assessed with a hemocytometer, spectrophotometer, or nucleocounter using horse settings (Canisso et al., 2019). The mean concentration of spermatozoa in jacks is 70-310×$10^6$/ml, which is similar to stallions (Contri et al., 2008; Quartuccio et al., 2011; Veronesi et al., 2011).

Both sperm volume and concentration are highly variable since both are influenced by the contribution of the accessory sex glands, which may differ from day to day even in the same jack (Quartuccio et al., 2011). Younger donkeys tend to produce ejaculates with lower sperm volume and higher concentrations than older jacks (Canisso et al., 2019). The amount of gel fraction depends on breed as well as individual differences, and thus is extremely variable with a wide range from 5-16 ml (Tibary, 2004; Contri et al., 2008; Quartuccio et al., 2011; Veronesi et al., 2011; Dorado et al., 2013a).

3.2.6.3 pH

The mean pH, evaluated within 5 minutes after semen collection, is 7.0-7.6, and is similar to the range reported in stallions. (Pugh, 2002; Contri et al., 2008; Contri et al., 2010; Veronesi et al., 2011; Carluccio et al., 2013a; Dorado et al., 2013a). Differences in pH could be attributed to the contribution of secretions from accessory glands fluids (Dorado et al., 2013a).

3.2.6.4 Viability and motility

Approximately 80-88% of spermatozoa are viable (Pugh, 2002; Contri et al., 2010; Quartuccio et al., 2011; Carluccio et al., 2013a). This percentage is much higher compared with values reported for stallions (approximately 55-65%) (Carluccio et al., 2013a). According to a study by Quartuccio et al. (2011), viability is correlated with testicular volume.

Sperm motility parameters can be subjectively assessed with a standard optical microscope or with a computer-assisted sperm analyzer (Canisso et al., 2019). Donkey spermatozoa are more rapid than stallion spermatozoa, although the progressiveness of their spermatozoa are relatively similar (Miró et al., 2005). Studies in jacks report wide-ranging results for the progressive motility, with a range of 65-80% (Pugh, 2002; Contri et al., 2008; Veronesi et al., 2011; Carluccio et al., 2013a). Sperm progressive motility shows a significant correlation with the testicular volume, and is correlated with fertility (Dorado et al., 2013a; Miragaya et al., 2018).

3.2.6.5 Morphology

Sperm morphology is typically assessed as part of a pre-purchase examination or infertility evaluation (Canisso et al., 2019). Most donkeys have 15% morphologic defects, but morphology is rarely linked with infertility in donkeys (Canisso et al., 2019). In the donkey, plasma membrane integrity can be evaluated using eosin-nigrosine, fluorophore propidium iodide, or the hypo-osmotic swelling test (HOS-test) (Rota et al., 2010). A HOS-test in donkeys can simply be done by simply diluting one part of semen with three parts of bi-distilled water followed by incubation for 5-45 minutes at 37°C (Rota et al., 2010).

### 3.2.7 Seasonality

Seasonal influences play a crucial role in optimizing reproductive management. However, a limited effect of season was reported in the jack (Carluccio et al., 2013a; Contri et al., 2014). A study by Pugh (2002) reported some seasonal differences in sexual behavior, with a more intense libido during spring and summer. This was confirmed in a study by Carluccio et al. (2013a), where differences in the time from exposure to the female to effective erection during the four seasons were observed, with a shorter time recorded in spring and summer compared to autumn and winter. However, both studies are in disagreement with a study by Gastal et al. (1996), where no seasonal effect was detected for the time required to the first mount, the time period prior to the first erection, or the time until ejaculation.

Jacks do not show significant differences in testicular morphometric characteristics throughout the year, in contrast to stallions where an increase in testicular size during spring and summer is reported (Carluccio et al., 2013a; Miragaya et al., 2018). There are no apparent seasonal variations in semen quality in jacks, although a seasonal influence was seen on some seminal parameters (Pugh, 2002; Miragaya et al., 2018; Canisso et al., 2019). In winter, there is an increase in total semen volume and gel-free volume in contrast to stallions, where a larger volume of total and gel-free semen in spring and summer is reported (Carluccio et al., 2013a; Canisso et al., 2019). A reduced sperm concentration is reported in winter, which is also in contrast to data reported in stallions, and could be the result of a greater secretion by the sexual glands in jacks (Carluccio et al., 2013a; Miragaya et al., 2018). This seems corroborated by the greater volume of semen reported in winter, and by the sperm output of the jack which does not vary during the seasons (Quartuccio et al., 2011; Carluccio et al., 2013a). Gastal et al. (1996) described a decrease in sperm morphologic defects in winter in donkeys, but no differences were found in a study by Carluccio et al. (2013a). However, Canisso et al. (2019) suggest there is an increase in sperm morphologic defects in the spring and summer. This would be similar to horses, where seasonal differences in sperm morphology were found in previous studies (Carluccio et al., 2013a). Canisso et al. (2019) also suggest that there is a reduction in sperm motility in the spring and summer. However, a study by Carluccio et al. (2013a) showed no differences in total and progressive motility during the four seasons.

### 3.2.8 Castration

Donkeys have larger testicles, a larger penis, and a thicker scrotum than stallions, which is important to take in account when castrating jacks (Burden and Thiemann, 2015; Miragaya et al., 2018). Many references suggest that castration of jacks is associated with a higher risk of hemorrhage complications (Tibary, 2004; Miragaya et al., 2018). According to Tibary (2004), this is not the case for mules; however, Burden and Thiemann (2015) believe that mules, just as donkeys, have a greater tendency to bleed after castration. The underlying reason for this higher incidence of hemorrhage complications in jacks has not yet been identified, but there is a strong believe that the larger testicles are associated with larger supplying blood vessels and thus a greater amount of tissue to be removed during castration and a subsequent greater chance of bleeding (Hagstrom, 2009; Burden and Thiemann, 2015). As a result, a standing castration is not recommended and an inguinal approach may be preferred in mature jacks to ensure minimal risk of infection (Hagstrom, 2009; Burden and Thiemann, 2015). Additionally, the spermatic cord ruptures more easily in jacks and thus it is advised to crush and ligate it during castration, which also minimizes the risk of bleeding (Hagstrom, 2009; Burden and Thiemann, 2015).

## 3.3 Breeding

### 3.3.1 Natural cover

Despite the popularity of artificial insemination in horses, natural cover is still frequently used in the donkey industry (Canisso et al, 2019). Natural cover in jennies can be attained in two ways: (1) by pasture breeding and (2) by hand breeding (Pugh, 2002).

Donkey owners apply pasture breeding by placing one jack in a field with multiple jennies (Purdy, 2010). Contrary to stallions, jacks are non-harem, territorial breeders (Canisso et al., 2019). At pasture, the jack establishes an area, typically close to water, shade and food (Miragaya et al., 2018; Canisso et al., 2019). Jennies attracted to the jack, approach the jack and play an active role during the teasing periods (Miragaya et al., 2018; Canisso et al., 2019). The jack selects the most receptive jenny by checking the urine and feces for pheromones (Purdy, 2010). Breeding activity at pasture will gradually increase up to 2 days before females ovulate (Yilmaz et al., 2012). Accidents with pasture breeding are rare because jennies are less violent than mares (Canisso et al., 2019).

Hand breeding allows the recording of exact breeding dates (Pugh, 2002; Purdy, 2010). An effective teasing program or serial transrectal ultrasound examinations of the ovaries will enhance the success by monitoring the growth of the pre-ovulatory follicle and thus the best time to breed (Pugh, 2002; Purdy, 2010). If hand breeding is used, the jenny should be mated the second day of estrus, and then at 48-hour intervals until the end of heat (Pugh, 2002; Purdy, 2010). Estrus jennies require minimal to no physical restraint during hand breeding (Canisso et al., 2019). Some jacks may be timid and will not breed when new people are present, while other jacks may be overly aggressive (Purdy, 2010). As a consequence, some of them will have to wear a muzzle to avoid injury to the jenny (Purdy, 2010). Interestingly, jacks used to mate only mares may refuse to breed jennies and require training to mate them (Canisso et al., 2019).

### 3.3.2 Artificial breeding

#### 3.3.2.1 Management of the female

When using transported semen the goal is to breed only once per estrus cycle, hence it is crucial to select the perfect timing for artificial insemination (Purdy, 2005). If a stud is available, heat can be determined by teasing the jenny (Purdy, 2005). If a stud is not available, the timing of insemination can be manipulated by using induction of ovulation and synchronization protocols, as described in a previous chapter (Purdy, 2005; Miragaya et al., 2018). Transrectal ultrasound evaluation of the ovaries and uterus and a vaginal speculum exam can be used to corroborate information gained from teasing and/or hormonal protocols (Purdy, 2005).

#### 3.3.2.2 Artificial insemination

Artificial insemination (AI) is a workable technique in donkeys (Purdy, 2005). Although jacks are easy to train for semen collection, this is currently not applied on a widespread basis (Purdy, 2010). Donkey semen is very concentrated and in general has good fertility (Purdy, 2010). Standard equine equipment can be employed to collect, evaluate, transport, and inseminate semen in donkeys (Purdy, 2005; Miragaya et al., 2018). Experience with cooling and freezing sperm is still limited in donkeys (Purdy, 2005; Canisso et al., 2019).

*3.3.2.2.1 Semen collection and evaluation*

As with stallions, jacks can be trained rather quickly for semen collection using a jump jenny in estrus and an artificial vagina, even jacks that are used to pasture breeding will adopt this method rather rapidly (Purdy, 2005; Hagstrom, 2009; Miragaya et al., 2018). The collection process is different from stallions due to the difference in reproductive behavior of the jack (Purdy, 2005). Patience is required from handlers and collectors because semen collection from jacks can take up to 30-60 minutes, with younger jacks being slower than mature jacks (Purdy, 2005; Canisso et al., 2019).

The jack's penis should be washed prior to collection to reduce contaminants (Canisso et al., 2019). Timid jacks may lose interest when washing the penis with a cup or a hose, therefore it is best to use wet cotton after letting the jack mount (Canisso et al., 2019). Semen from jacks can be collected with any type of artificial vagina (AV) (Canisso et al., 2019). Most donkeys can be collected with a regular length AV, but miniature donkeys may need a shorter AV (Canisso et al., 2019). The rubber AV liner is filled with warm water (approximately 50°C) and then the AV should be lubricated with non-spermicidal sterile lubricant (Purdy, 2005; Canisso et al., 2019). The jenny may be tied in a corner or controlled by a handler (Purdy, 2005). When the jack is fully erected and allowed to mount, the collector handling the AV quickly directs the jack's penis into the AV (Purdy, 2005). The other hand is placed at the base of the penis to feel for ejaculation (Purdy, 2005).

After collection, semen is processed similarly to stallions (Canisso et al., 2019). The evaluation procedures should be performed in rapid succession to preserve a maximum amount of viable semen (Purdy, 2005). The evaluation procedures have been described in a previous chapter.

*3.3.2.2.2 Cooling and shipping sperm*

Jacks typically have excellent semen quality and the semen can be handled in a manner similar to that of the stallion, using the same extenders and storage techniques (Pugh, 2002; Hagstrom, 2009; Canisso et al., 2019). Cooling rates of -0,6°C/min and -1,0°C/min until 4-6°C are better than rates of -3°C/min (Tibary, 2004).

Semen centrifugation and removal of seminal plasma before cooling increases the longevity of donkey semen (Miragaya et al., 2018; Canisso et al., 2019). It is reported that donkey seminal plasma contains proteins that remove cholesterol from the plasma membrane, which reduces sperm longevity during cooling (Canisso et al., 2019). The effect of seminal plasma may be breed and individual dependent because different studies show variable results (Tibary, 2004; Miragaya et al., 2018). Serres et al. (2002) observed that centrifugation and removal of seminal plasma has a significantly positive effect on motility and plasma membrane integrity of spermatozoa. In a study by Rota et al. (2008), the almost complete removal of seminal plasma had a negative effect on donkey sperm motility; however, centrifugation alone without removal of seminal plasma had no significant effect on donkey sperm motility.

For preservation by cooling to be successful, semen needs to be extended with an appropriate semen extender (Rota et al., 2008). Semen extenders usually contain antibiotics and nutrients to help maintain semen viability and to protect the spermatozoa from cold shock, negative effects of seminal plasma and excessive bacterial growth (Purdy, 2005; Rota et al., 2008). Many equine extenders can be used successfully to preserve donkey semen at 4°C for 48 hours, with motility decreasing to approximately 10% by 72 to 96 hours (Canisso et al., 2019). The number of studies comparing semen extenders in donkeys is limited, but all studies suggest that the use of semen extenders containing egg yolk is more efficient (Rota et al., 2008). The skimmed milk-based extender INRA82 with 2% centrifuged egg yolk added (INRA82-Y) ensures better sperm motility characteristics during cooled storage than

INRA82 without egg yolk or INRA96 (Rota et al., 2008; Miragaya et al., 2018). INRA96 is in turn more effective for semen cooling than E-Z Mixin, a more general milk-based extender (Contri et al., 2010).

Limited experience and data on pregnancy rates are available with regard to the efficacy of shipping donkey semen (Purdy, 2005). Until optimal transportation practices for donkey semen are determined, horse guidelines are used (Purdy, 2005, Canisso et al., 2019). Hence donkey semen can be cooled and shipped in passive cooling semen containers, such as the Equitainer, Styrofoam semen boxes, and other devices (Canisso et al., 2019).

*3.3.2.2.3 Sperm freezing and cryopreservation*

Presently, limited data and experience are available regarding frozen semen in donkeys (Purdy, 2005). Protocols used to process donkey semen for freezing have been adapted from stallions (Canisso et al., 2019). A suitable method for donkey semen cryopreservation would be very valuable for the ex situ management of genetic diversity (Rota et al., 2012; Sabatini et al., 2014; Oliveira et al., 2016; Miragaya et al., 2018).

Sperm concentration is determined with a nucleocounter and adjusted to 200 million spermatzoa/ml (Canisso et al., 2019). Extended semen is automatically loaded and sealed in 0.5ml straws (Canisso et al., 2019). Straws are cooled at 5°C for 20 minutes, then placed 4 to 6 cm above liquid nitrogen for 15 minutes or inserted into an automated freezing machine (Canisso et al., 2019). Semen is thawed at 37°C for 30-60 seconds, or 42°C for 7 seconds, followed by 20-30 seconds at 37°C (Canisso et al., 2019). AI using cryopreserved semen should be timed accurately because cryopreservation reduces sperm longevity in the female genital tract in comparison to fresh semen (Rota et al., 2012). The optimal interval for insemination ranges from 12 hours before and 12 hours after ovulation (Rota et al., 2012).

To date, various authors have reported low fertility rates with donkey cryopreserved semen using different extenders and amounts of cryoprotectant, compared to the horse (Rota et al., 2012; Sabatini et al., 2014; Oliveira et al., 2016; Miragaya et al., 2018). An explanation for the poor conception rates could be the more pronounced post-breeding inflammatory response and fluid accumulation following insemination with frozen-thawed semen in jennies than mares (Rota et al., 2012; Canisso et al., 2019). A pregnancy rate of 28% can be achieved when performing deep-horn insemination with multiple insemination doses of $1000\times10^6$ sperm (Miragaya et al., 2018). It is possible that deep horn inseminations allows for a lower time of interaction between spermatozoa and the endometrium, leading to reduced uterine inflammatory response and increased number of viable sperm and a closer proximity to the site of sperm storage and fertilization (Oliveira et al., 2016).

Improvement of cryopreservation in donkeys has been achieved in three ways: (1) By reducing the amount of glycerol. It has been hypothesized that glycerol may be toxic for jack spermatozoa or have a negative effect on the fertility of jennies (Tibary, 2004; Rota et al., 2012; Miragaya et al., 2018). The hypothesis of an effect of glycerol on the jenny genital tract rather than on the sperm cell could explain the higher pregnancy rates obtained in mares after inseminating with frozen donkey semen, when compared to jennies (Tibary, 2004; Rota et al., 2012; Miragaya et al., 2018). (2) By the addition of glutamine and replacing hen egg yolk with quail egg yolk. Glutamine improved the percentage of oocytes penetrated by sperm, and thus may act to prevent lipid peroxidation by protecting the sperm enzymatic defense systems (Trimeche et al., 1998; Tibary, 2004; Miragaya et al., 2018). (3) By adding autologous seminal plasma to frozen-thawed semen. Removal of seminal plasma by centrifugation is required for semen freezing (Sabatini et al., 2014; Miragaya et al., 2018; Canisso et al., 2019). However, seminal plasma contains factors modulating the uterine inflammatory response by affecting uterine clearance and altering chemotaxis; it also plays several roles in sperm metabolism and fertilization (Rota et al., 2012; Oliveira et al., 2016; Canisso et al., 2019).

*3.3.2.2.4 Insemination technique*

The technique of AI is similar in all equine species (Purdy, 2005). However, it can be challenging to pass a pipette into a donkey cervix, which tends to be located on the floor of the vagina, pointing slightly upwards (Purdy, 2005). In small donkey breeds, the jenny's vagina may be too small to insert a hand, which makes the technique more difficult to perform (Purdy, 2005).

*3.3.2.2.5 Conception rates*

AI of mares with fresh semen results in pregnancy rates varying from 40-80% (Canisso et al., 2019). The pregnancy rate after the use of frozen semen in donkeys is approximately only 6%, but a few studies report up to 35-60% if uterine lavage is performed or with post-thawing re-extension of frozen semen with autologous seminal plasma (Fanelli et al., 2019).

3.3.2.3 Embryo transfer

Embryo transfer (ET) is an assisted reproductive technique that consists in the transfer of one or more embryos from a female to one or more females (Panzani et al., 2018b). The approach to ET in donkeys was initially derived from equine consolidated protocols; however, initial attempts were disappointing when compared with those in horses (Panzani et al., 2018b; Canisso et al., 2019). The development of assisted reproductive techniques for donkeys remains a challenge, and more research is needed before ET can be considered a reliable tool to preserve endangered donkey breeds (Camillo et al., 2010; Panzani et al., 2018b).

*3.3.2.3.1 Embryo flush*

Despite the smaller size and the tight cervix, nonsurgical embryo flushing in donkeys is similar to the current technique in horses (Camillo et al., 2010; Canisso et al., 2019). The jenny uterus should be flushed at least 3 times with 0.5l lactated Ringer's solution, in maiden jennies, and up to 1l, in multiparous jennies (Canisso et al., 2019). In a study by Camillo et al. (2010), more embryos were recovered when flushing the uterus on days 8 and 9 compared with flushing on day 7. This could be due either to the larger diameter of older embryos, resulting in an easier recovery, or to a delay in the descent in the uterus of donkey embryos (Camillo et al., 2010).

*3.3.2.3.2 Embryo recovery rate and embryo viability*

The embryo recovery rate in donkeys is 53-76%, which is comparable with what is normally observed in mares. Factors affecting the embryo recovery in donkeys have never been evaluated, whereas in horses the embryo recovery rate is influenced by the number of ovulations per cycle, the day of recovery, the age of the donor mare, and the quality of the semen (Camillo et al., 2010; Panzani et al., 2018b).

The method most commonly used to grossly assess embryo viability is evaluating the morphology of the embryo (Panzani et al., 2012). Another and more accurate method is the estimation of proportion of dead per total cells by DAPI (4',6-diamidino-2-phenylindole) staining, a fluorescent stain that binds to chromatin (Panzani et al., 2012). The embryo viability in donkeys is similar to mares (Panzani et al., 2012).

*3.3.2.3.3. Holding media*

Embryos from each species need specific washing and holding media because of their specific characteristics (Panzani et al., 2018b). A study by Camillo et al. (2010) seems to indicate that Ringer lactate and Emcare Holding Solution is a better choice for donkey embryos than phosphate buffered

saline (PBS). It has been reported that it could be possible that donkey embryos are more susceptible to the inhibition of embryo development by phosphate (Camillo et al., 2010). Moreover, it is not possible to exclude an irritating effect of the holding media on the receptive jenny's uterus (Camillo et al., 2010).

*3.3.2.3.4 Embryo transfer technique*

The ET technique in donkeys is similar to horses (Canisso et al., 2019). The cervix should be grabbed and pulled backward, then the tip of the gun can be inserted in the cervical ostium (Canisso et al., 2019). Once the gun is in the cervix, the sanitary sheath can be broken (Canisso et al., 2019). Afterwards the cervix should be manipulated to help insert the gun in the uterus (Canisso et al., 2019). However, transcervical catheterization in jennies requires more manipulation due to the smaller, longer and tighter cervix (Camillo et al., 2010). Administration of acepromazine to recipients before ET may help cervical relaxation (Canisso et al., 2019).

Cervical manipulation is associated with a release of PGF2α, followed by a decrease in progesterone plasma concentration, which could negatively affect luteal and embryonic survival after transfer (Camillo et al., 2010; Canisso et al., 2019). However in a study by Panzani et al. (2012), the elevation of PGF2α did not induce a decrease in P4 plasma concentration within 4 days after ET, disproving the hypothesis that the nonsurgical technique of ET could be responsible for low recipient pregnancy rates in donkeys.

*3.3.2.3.5 Pregnancy rate*

Pregnancy rate in equines after ET is influenced by the quality of the embryos and the recipients (Camillo et al., 2010). Surgical and nonsurgical ET in the donkey usually results in low pregnancy rates, varying from 17% to 23% (Panzani et al., 2012; Camillo et al., 2019). The reasons for the low pregnancy rate remain unknown, but the ET techniques used in donkeys are simply transferred from horses, with the possibility that several factors could possibly negatively affect the outcome (Camillo et al., 2010). However, the transcervical technique is proven not to be the reasons for the low pregnancy rates in donkeys (Panzani et al., 2012).

3.3.2.4 Ovum pick-up and intracytoplasmic sperm injection

*3.3.2.4.1 Ovum Pick-Up (OPU)*

Oocytes are recovered with the same method used in mares, by transvaginal ultrasound-guided aspiration of follicles with an 18 or 20 G needle or by ovarian slicing and follicular scraping (Miragaya et al., 2018). After three seasons, the reported number of immature oocytes recovered from jennies is approximately 2,75-11,0 oocytes and the mean oocyte recovery rate per immature follicle is 34-71% (Deleuze et al., 2018). Hence, it can be considered that collection rates are similar to those reported in the mare (Deleuze et al., 2018).

*3.3.2.4.2 In Vitro Maturation (IVM)*

The immature oocytes collected by OPU require further IVM, as in horses (Deleuze et al., 2018). Cumulus-oocyte-complexes (COCs) are in vitro matured, yielding a 44% maturation rate, seeming to have a longer optimal duration for IVM (34 hours) than reported in mares (24-30 hours) (Deleuze et al., 2018; Miragaya et al., 2018).

Different media for IVM of donkey embryos have been tested: Dulbecco's modified Eagle's medium (DMEM) with high glucose; DMEM with low glucose medium; DMEM-nutrient mixture F-12 medium (DMEM-F12); tissue culture medium 199 (TCM199); TCM199-F12 medium which consisted of equal

volume of TCM199 and Hams nutrient mixture F-12 medium; Charles Rosenkrans medium with free amino acids and vitamins (Zhao et al., 2011; Miragaya et al., 2018). All these media are supplemented with 10% fetal calf serum, FSH, hCG, and gentamicin (Miragaya et al., 2018). One laboratory reported an advantage in the use of DMEM-F12 for oocyte maturation, not only in cleavage rate after intracytoplasmic sperm injection, but also in the in vitro embryo development to the blastocyst stage, whereas other laboratories reported even higher blastocyst development using TCM199 for oocyte maturation (Zhao et al., 2011). In a study by Miragaya et al. (2018), rates of cumulus expansion and nuclear maturation to metaphase II were higher when IVM was carried out in Charles Rosenkrans medium with free amino acids and vitamins and TCM199 media, compared to DMEM.

*3.3.2.4.3 Intracytoplasmic Sperm Injection (ICSI)*

To date, no conventional IVF technique is available in Equidae (Deleuze et al., 2018). Consequently, ICSI has been widely adopted to produce horse and donkey embryos in vitro (Deleuze et al., 2018).

## 3.4 Gestation

### 3.4.1 Gestation length

It is generally accepted that gestation in donkeys lasts approximately 12 months, but there are no references reporting a range of acceptable variation, as there is available for mares (Galisteo and Perez-Marin, 2010; Canisso et al., 2019). The gestation length in donkeys appears to be highly variable since studies reported normal foals being born after 331 days up to 421 days of gestation (Hagstrom, 2009; Yilmaz et al., 2012; Crisci et al., 2014; Carluccio et al., 2015; Miragaya et al., 2018; Canisso et al., 2019; Nervo et al., 2019).

Causes for such a wide range are uncertain (Yilmaz et al., 2012). Some factors attributable to small variations in gestation length have been described, such as foal gender, with longer gestations observed in jennies carrying male fetuses (Carluccio et al., 2015; Miragaya et al., 2018; Nervo et al., 2019). The date of ovulation also seems to affect gestation length in a similar way as in mares, with a longer gestation length when conception occurs at the beginning of the year, and decreasing thereafter (Galisteo and Perez-Marin, 2010; Carluccio et al., 2015; Miragaya et al., 2018; Nervo et al., 2019). Other environmental factors, such as temperature, might also influence gestation length, although their precise role remains unclear (Galisteo and Perez-Marin, 2010). The birthweight of the foal, donkey breed and the age of the jenny seem to have no effect on pregnancy length (Galisteo and Perez-Marin, 2010; Carluccio et al., 2015; Miragaya et al., 2018).

### 3.4.2 Pregnancy rates

With an average conception rate of 78%, fertility in donkeys seems to be higher than in mares (average of 65%) (Tibary, 2004; Hagstrom, 2009). The pregnancy rate appears to be affected by season and by age: it declines during the autumn and winter and in jennies older than 6 years old (Tosi et al., 2013).

### 3.4.3 Twinning

Compared with mares, a higher frequency of multiple ovulations has been reported in donkeys and as a result, twinning occurs more frequently (Hagstrom, 2009; Nervo et al., 2019). Incidents of twins have been reported to be as high as 40% at day 21 of pregnancy (Hagstrom, 2009).

The approach to manage twins in donkeys should be similar to mares and early diagnosis allows to choose the best treatment option, conservative or not, since spontaneous regression of one of the two is a likely scenario (Tibary, 2004; Hagstrom, 2009; Nervo et al., 2019). In a study by Nervo et al. (2019), they decided not to intervene in cases of twin pregnancies, and this always resulted in the resorption of one or both of the vesicles during the first 30 days of pregnancy. However, similar as mares having complications with carrying twin pregnancies to term, donkeys also experience problems and both twins survive in only about 14% of cases (Hagstrom, 2009; Yilmaz et al., 2012).

### 3.4.4 Endocrinology

The endocrinologic profiles during donkey pregnancies do not differ greatly from those reported for mares (Meira et al., 1998; Crisci et al., 2014). The most important hormones during pregnancy in donkeys are FSH, donkey chorionic gonadotrophin (dCG), P4 and E2.

Follicular activities persist during the second month of pregnancy, with some follicles even reaching a size of > 30 mm (Meira et al., 1998; Lemma et al., 2006). Secretion of dCG starts around day 40 of pregnancy, leading to secondary corpora lutea formation (Meira et al., 1998; Canisso et al., 2019). The secondary corpora lutea are no longer visible by day 150 (Meira et al., 1998).

During pregnancy, P4 plasma concentrations initially increase, from ± 0.9 ng/ml on day 0 to ± 20 ng/ml on day 10 and then gradually decrease to ± 12 ng/mL by day 30 due to partial regression of the primary corpora lutea, followed thereafter by an increase to ± 17 ng/ml on day 40 (Meira et al., 1998; Tibary, 2004; Bansal et al., 2006; Canisso et al., 2019). The increase between days 30 and 40 (prior to appearance of secondary corpora lutea) may be caused by a luteotrophic effect of dCG, similar to the effects of equine chorionic gonadotrophin (eCG) in the mare (Meira et al., 1998). Between days 40 and 110, P4 plasma concentrations remain relatively constant (from ± 17 to ± 110 ng/ml) due to the formation of secondary corpora lutea (Meira et al., 1998; Tibary, 2004; Bansal et al., 2006; Canisso et al., 2019). This plateau is followed by a gradual decline until day 160 after which it remains at approximately 4 to 7 ng/ml until a few days before parturition, when it increases again (Meira et al., 1998; Tibary, 2004; Bansal et al., 2006; Canisso et al., 2019). On the day of foaling, P4 plasma concentrations start decreasing again to < 4 ng/ml to reach the lowest level on day 9 post-foaling (Bansal et al., 2006; Crisci et al., 2014). Similar profiles have been reported in mares, although concentrations are somewhat higher in jennies (Meira et al., 1998).

E2 plasma concentrations increase to 100 ng/ml on day 90 (Meira et al., 1998; Canisso et al., 2019). The maximum concentrations of E2 plasma concentrations in jennies (> 1000 ng/ml) is reached between day 150 and 210, followed by a decrease to very low concentrations prior to parturition (Meira et al., 1998; Canisso et al., 2019). Overall, the E2 plasma profile is similar to that of mares, but E2 plasma concentrations are more elevated in the jenny (Meira et al., 1998; Crisci et al., 2014).

In mares, P4 and E2 assays have been used as diagnostic tools to assess well-being during pregnancy but such studies are lacking for jennies (Canisso et al., 2019). Estrone sulfates are excreted in high concentrations in the urine, and the Cuboni reaction, which is a fluorescent chemical reaction to detect estrone sulfates in the urine, has been successfully used for pregnancy diagnosis in jennies (Canisso et al., 2019). Although the barium chloride test has also been used for pregnancy diagnosis, it is less reliable and is highly influenced by season (Canisso et al., 2019).

### 3.4.5 Embryonic and early fetal development

The timing of transrectal ultrasonographical detectable features in the gestation of the jenny are summarized in Table 1. As from 10 days after ovulation, an embryonic vesicle with a mean diameter of 4 mm may be detectable by transrectal ultrasonography as a spherical structure, although the probability of detecting a pregnancy this early is very small (Gastal et al., 1993; Lemma et al., 2006; Canisso et al., 2019). In clinical practice, the first pregnancy diagnosis should be performed 12-15 days after ovulation (Canisso et al., 2019). The embryonic vesicle is detectable by rectal palpation between day 15 and 21 after ovulation (Gastal et al., 1993). Maternal recognition of pregnancy in donkeys seems to be similar to horses, with the embryonic vesicle remaining mobile within the uterine lumen until day 16, at which point the embryonic vesicle with a diameter of approximately 16-29 mm fixes at the base of a uterine horn (Gastal et al., 1993; Canisso et al., 2019). The embryonic vesicle starts losing its spherical shape around day 18 and the wall of the embryonic vesicle assumes an irregular shape (Gastal et al., 1993; Lemma et al., 2006; Canisso et al., 2019). The growth rate of the embryonic vesicle is similar as in horses (Crsici et al., 2014). The embryonic vesicle grows approximately 3 mm/day during the mobility phase, 0.1 to 0.7 mm/day between days 18 and 31, and 1.6 to 2.5 mm/day until day 60 (Gastal et al., 1993; Crisci et al., 2014; Canisso et al., 2019). The presence of a plateau between 20 and 30 days of is due to the fixation of the embryonic vesicle in the uterine horn and the loss of spherical shape (Gastal et al., 1993; Crisci et al., 2014; Canisso et al., 2019).

The embryo, visible as a small echogenic spot, appears at the ventral pole of the vesicle around days 19 to 22 (Gastal et al., 1993; Lemma et al., 2006; Canisso et al., 2019). Embryonic heartbeat can be detected from approximately day 20-25 (Gastal et al., 1993; Tibary, 2004; Crisci et al., 2014; Nervo et

al., 2019). The allantoic sac can be first seen between days 19 and 23 (Gastal et al., 1993). The embryo moves gradually toward its dorsal pole between days 21 and 35, reaches the dorsal pole by day 35, and begins to descend again after day 37 when it is attached to the dorsal pole by the developing an umbilical cord (Gastal et al., 1993). The descent is accomplished by day 44 (Gastal et al., 1993).

| Characteristics | Days of gestation | | |
|---|---|---|---|
| | Canisso et al., 2019 | Crisci et al., 2014 | Gastal et al., 1993 |
| Detection of embryonic vesicle | 10-14 | 10-14 | 10-12 |
| Fixation of embryonic vesicle | 13-21 | 16-20 | 12-21 |
| Loss of spherical shape | 17-23 | 18-20 | 17-23 |
| Detection of embryo | 18-24 | 20-24 | 18-21 |
| Detection of heartbeat | 20-26 | 24-26 | 20-25 |
| Detection of allantoic sac | 19-28 | 26-28 | 19-23 |
| Detection of umbilical cord | 31-47 | 45-47 | 31-47 |
| Embryo at dorsal pole | 21-44 | | 28-44 |
| Beginning descent of the embryo | 31-42 | | 31-42 |
| Concluding descent of fetus | 35-53 | | 35-53 |
| Detection of chest | 54-64 | 54-64 | |
| Detection of stomach | 60-71 | 60-71 | |
| Detection of eyeball | 71-96 | 71-96 | |
| Detection of aorta | 79-109 | 79-109 | |

*Table 1. First day of ultrasonographically identifiable gestational characteristics in the jenny according to 3 different sources (in days after conception).*
*Sources: Gastal et al. (1993), Crisci et al. (2014), Canisso et al. (2019)*

The diameters of the chest, eye orbit, and aorta are frequently measured to estimate fetal size and are strongly related with gestational age, as depicted in Figure 4 (Crisci et al., 2014). These measurements are in agreement with the horse, but all three parameters are, at the end of the pregnancy, smaller in the asinine fetus, because of the obvious interspecies size differences (Crisci et al., 2014). The orbital diameter is the most reliable parameter for monitoring the physiological development of the fetus (Nervo et al., 2019).

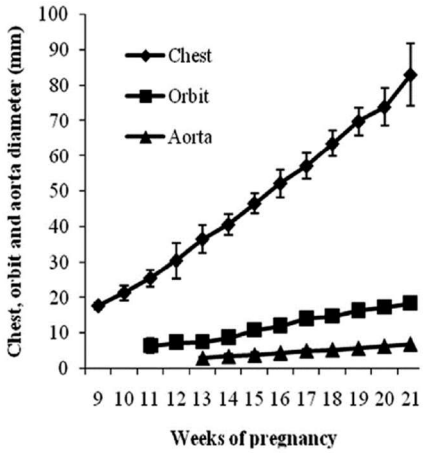


*Figure 4. Mean diameter of the fetal chest, eye orbit, and aorta from week 9 to week 21 of pregnancy measured using transrectal ultrasound in eight jenny pregnancies. Source: Crisco et al. (2014)*

Although, fetal sexing using transabdominal ultrasound is possible from week 22, the highest rate of successful determinations of foal gender is from 240-265 days of pregnancy onwards, later than what is described for mares (Crisci et al., 2014). This may be caused by the smaller size of the fetus and the longer gestation (Crisci et al., 2014). In a study by Mancuso et al. (2007), where they used transrectal ultrasound, the optimal time for sex determination was considered to be between 100 and 150 days of gestation.

The evaluation of fetal presentation within the uterus might allow the clinician to predict dystocia ante-partum (Crisci et al., 2014). At the time of parturition the asinine fetus is physiologically in anterior presentation. Although the fetus is often able to change presentation during gestation, this change is no longer possible after the 11$^{th}$ month of pregnancy because of its body size which is later than for horse fetuses, who can only change presentation up to the 9$^{th}$ month (Crisci et al., 2014; Nervo et al.,

2019). This difference in timing is due to the longer gestation length and to the horse fetus typically being relatively larger in size than the donkey fetus (Crisci et al., 2014; Nervo et al., 2019).

### 3.4.6 Placenta

As in all equids, donkey placentation is diffuse, epitheliochorial, and noninvasive (Carluccio et al., 2008; Canisso et al., 2019). The villi are organized in microcotyledons diffusely distributed over the entire allantochorionic surface (Carluccio et al., 2008). At parturition, the placenta weighs approximately 12% of the foal's birthweight which is similar to the 11% reported in the horse (Carluccio et al., 2008; Miragaya et al., 2018; Canisso et al., 2019). Whereas the macroscopic characteristics of the donkey placenta are similar to that of horses, at microscopic level, the surface density of the microcotyledons is significantly higher in donkeys, owing to extensive branching of the villi (Carluccio et al., 2008; Veronesi et al., 2010; Canisso et al., 2019). This results in a higher vascular density, thinner interhaemal membrane, and thus a higher total microscopic area of feto-maternal contact (Carluccio et al., 2008; Veronesi et al., 2010; Canisso et al., 2019).

It has been argued that the complex development of the placental villi in the jenny compared to mares could be the explanation for a longer gestation period (Carluccio et al., 2008). The donkey and horse show relatively similar birth weight, despite the longer duration of gestation in jennies and the more developed placental villi (Carluccio et al., 2008; Miragaya et al., 2018). This suggests a lower placental efficiency in terms of foal birthweight per square meter of feto-maternal contact (Carluccio et al., 2008; Miragaya et al., 2018). Hence, a longer gestation would be needed to allow for the full development of the fetus (Carluccio et al., 2008). The placental effciciency in donkeys lowers with age, similar as in horses (Veronesi et al., 2010).

### 3.4.7 Combined utero-placental thickness (CUPT)

The asinine CUPT grows linearly as from the $6^{th}$ month of pregnancy until foaling, with a substantial increase from the $9^{th}$ to the $12^{th}$ month of pregnancy (Carluccio et al., 2016; Canisso et al., 2019). This finding is different from data reported for mares, where CUPT starts increasing from the $8^{th}$ month of pregnancy onwards, and substantially from the $10^{th}$ to the $12^{th}$ month of gestation (Carluccio et al., 2016; Miragaya et al., 2018). This slight difference could be due to the longer gestation period in donkeys (Miragaya et al., 2018). Further research is needed to define possible breed or body-size CUPT specific differences (Carluccio et al., 2016).

The transrectal CUPT measurement in the second half of gestation to assess an ascending placentitis in mares is also suitable in jennies according to a few studies (Carluccio et al., 2016; Miragaya et al., 2018; Canisso et al., 2019). However in a study by Nervo et al. (2019), CUPT remained virtually unchanged during gestation, even in animals that aborted. Therefore, more studies are needed to define the CUPT values in case of pregnancy disturbances or placental abnormalities.

## 3.5 Parturition

### 3.5.1 Predicting imminent foaling

Prediction of foaling is based on udder development and waxing which seem to reliably start 24 to 48 hours before foaling (Tibary, 2004). In mares, electrolytes and pH of mammary gland secretions are used to predict imminent foaling: the days preceding parturition, calcium, magnesium, and potassium concentrations in the milk increase, whereas sodium and chloride concentrations decrease as well as the pH (Canisso et al., 2019).

Induction of parturition is possible with oxytocin and the criteria for induction are the same as in mares, at a time when the fetus is mature (Oussey, 2002; Tibary, 2004). The criteria usually used for induction of delivery in equids are: (1) presence of good quality colostrum with a calcium concentration > 10 mmol/l; (2) gestation period > 330 days; (3) softening of the cervix (Oussey, 2002).

### 3.5.2 Peri-partum

The partus consists of three stages, prodromi, expulsion of the foal and expulsion of the fetal membranes. The normal duration of each parturition stage has been reported similar to horses, and its knowledge is crucial to improve the chances of optimal foal and mare health (Carluccio et al., 2015; Miragaya et al., 2018).

The first stage of foaling in jennies lasts on average 65 minutes (20-135 minutes) and often goes undetected because jennies, similar to mares, may not show obvious signs (Carluccio et al., 2015; Canisso et al., 2019). Possible signs may include walking, frequent defecation and urination, flank watching, and the Flehmen response (Carluccio et al., 2015; Canisso et al., 2019).

The second stage starts with the rupture of the allantochorion and the expulsion of the allantoic fluid. This stage generally lasts 10-30 minutes (Carluccio et al., 2015; Canisso et al., 2019). Foaling managers should remember that dystocia is more likely with jennies than with mares, hence if no signs of a fetus are seen within 20 minutes after allantoic fluid expulsion, the jenny should be evaluated for a dystocia (Hagstrom, 2009; Carluccio et al., 2015; Canisso et al., 2019).

The third stage of parturition is completed in 10-175 minutes after foaling (Carluccio et al., 2015; Canisso et al., 2019). Jennies are, similar to mares, susceptible to retentio secundinarum, which can be worsened by the donkey's predisposition to insulin resistance and metabolic syndrome (Canisso et al., 2019). When faced with retained fetal membranes, similar techniques to those used in horses are proposed (Canisso et al., 2019).

### 3.5.3 Post-partum

The average birth weight of a foal is about 25 kg, but it is very variable depending on the breed (Yilmaz et al., 2012). The umbilical cord ruptures on average within 16 minutes after birth (Carluccio et al., 2015; Miragaya et al., 2018). A healthy foal stands up within 1 hour and suckles colostrum for the first time within 1.5 hours after birth. The elimination of meconium occurs approximately 2 hours after birth (Carluccio et al., 2015; Miragaya et al., 2018). Foals who are unable to stand and suckle within 2 hours after birth are considered potentially abnormal (Carluccio et al., 2015). Foals begin nibbling on vegetation at 5 days of age; however, weaning does not occur naturally until 12-14 months of age and should not be attempted before a foal has reached the age of 6 months (Yilmaz et al., 2012).

After a difficult parturition, one should bear in mind that jennies seem to be more prone to cervical laceration, due to their longer and cone shaped cervix (Tibary, 2004). Cervical lacerations are recognized as having a negatively effect on the future fertility (Tibary, 2004).

### 3.5.4 Foal heat

Careful management of the puerperal period is crucial, since foal heat usually occurs 5-13 days post-partum, with an ovulation as early as 9 days post-partum (Pugh, 2002; Hagstrom, 2009; Renner-Martin et al., 2009; Galisteo and Perez-Marin, 2010; Carluccio et al., 2017; Miragaya et al., 2018; Canisso et al., 2019). The duration of estrus appears to be significantly shorter at foal heat than in subsequent estrus (Galisteo and Perez-Marin, 2010). The suppression of foal heat is associated with environmental factors and foal at foot, and silent post-partum estrus seems to be more common with fall-winter than with spring-summer foalings (Dadarwal et al., 2004; Galisteo and Perez-Marin, 2010; Canisso et al., 2019).

The pregnancy rate is lower with breeding during foal heat, although it seems to increase when ovulation occurs more than 12 days after foaling (Galisteo and Perez-Marin, 2010; Tosi et al., 2013; Miragaya et al., 2018). Parameters for foal heat breeding have not been established for donkeys, but it is advised that jennies should not be bred on foal heat if they: (1) retained fetal membranes more than 3 hours; (2) have poor uterine involution 7 days postpartum; (3) had dystocia; (4) developed metritis; (5) have an urovagina and/or urometra; (6) have bruising in the vagina or vestibule; (7) ovulated less than 10 days after foaling (Canisso et al., 2019).

### 3.5.5 Uterine involution

Complete uterine involution in jennies occurs around 22 days post-partum, which is almost the same time period as reported in mares (Dadarwal et al., 2004; Canisso et al., 2019). However, it is delayed in jennies who are in foal heat within 9 days post-partum (Dadarwal et al., 2004). The pattern and extent of uterine involution appear to have several similarities to that in mares (Dadarwal et al., 2004). The process of uterine involution is most clear at the corporocornual junction of the post-gravid uterine horn (Dadarwal et al., 2004).

## 3.6 Particularities to interspecies breeding

### 3.6.1 Fertility

The horse has 64 chromosomes while the donkey only has 62 and the hybrid offspring of the mating of a horse to a donkey is an animal with 63 chromosomes (Hagstrom, 2009). The mating of a mare to a jack results in a mule (Hagstrom, 2009). The reverse cross, a jenny mated to a stallion, is called a hinny (Hagstrom, 2009). Each of these hybrids come in both sexes but they are generally infertile due to the homologous chromosome pairing failure that occurs during meiosis; however, there have been reports in the literature of fertile female mules and hinnies producing and delivering fertile offspring when bred to stallions or jacks (Neves et al., 2002; Hagstrom, 2009; Canisso et al., 2019). In contrast, the existence of fertile male mules or hinnies has never been reported (Canisso et al., 2019). Usually, spermatogenesis in the male mule and hinny does not advance beyond spermatocytes and thus cannot produce spermatozoa (Neves et al., 2002; Hagstrom et al., 2009). Despite being mostly infertile, the female mule and hinny do cycle, although it is typically extremely variable and erratic; and similarly, the male mule and hinny produce testosterone and thus will display stallion-like behavior (Hagstrom, 2009).

### 3.6.2 Breeding

Breeding efficiency of jacks breeding mares at pasture is low because most mares are not receptive to jacks, particularly without previous exposure (Lodi et al., 1995; Canisso et al., 2019). Hand breeding is the most suitable approach to breed mares with jacks (Canisso et al., 2019). Mares hand breeded by donkeys typically have good pregnancy rates (Canisso et al., 2019).

Jacks that have not been raised around horses are generally not interested in breeding mares (Hagstrom, 2009). However, jacks who are brought up in an environment with horses will have a much stronger affinity for mares (Hagstrom, 2009). Sexual behavior shown by jacks breeding mares is similar to the sexual behavior shown by jacks breeding jennies (Lodi et al., 1995). Jacks covering both mares and jennies are very rare; hence, if a jack is to be bred to both species, AI should be considered (Hagstrom, 2009).

It is not natural for a mare to allow a jack to breed her. They will only show mild estrus signs in the presence of a jack and will not participate actively by showing estrus behavior and stimulating the male (Lodi et al., 1995; Canisso et al., 2009b; Hagstrom, 2009). The rejection of jacks by mares in estrus could be related to a lack of familiarization of the mares to jacks; however, it cannot be ruled out that mares are just not naturally attracted by jacks (Lodi et al., 1995). Mares can seriously injure the jack in their attempts to successfully dissuade a jack from mounting and hand breeding is the most suitable approach to breed mares with jacks (Hagstrom, 2009; Canisso et al., 2019). The mare can be physically restrained with a twitch, breeding hobbles, or restraining breeding stocks (Canisso et al., 2019). Chemical restraint may also be necessary to ensure safety of the jack and personnel involved (Canisso et al., 2019). Regardless of restraint, the risks for accidents, such as the mare kicking the donkey or laying down during mounting is still of concern (Canisso et al., 2019).

### 3.6.3 Embryo Transfer

Cyclic or acyclic mules can be excellent embryo recipients for donkey or horse embryos (Canisso et al., 2019). Cyclic mules can be administered PGF2α to induce luteolysis, and then hCG or GnRH agonists to induce ovulation (Canisso et al., 2019). After ET, the pregnancy can be maintained with weekly doses of long-acting progesterone (Canisso et al., 2019). Mules carrying implanted horse or donkey embryos will foal similar to mares, and the placenta is grossly similar to mares and jennies (Canisso et al., 2019).

Donkeys can be used as a horse embryo recipient and appear able to carry horse fetuses to term, perhaps because of normal endometrial cup development and the resulting high secretion rates of

eCG and P4 (Allen et al., 2010). However, horses cannot be used as a donkey embryo recipient due to failure to develop endometrial cups because, differently from horse-to-donkey ET pregnancies, in a vast of majority of donkey-to-horse ET pregnancies, endometrial cup formation and fetus implantation never occurrs and all fetuses are aborted between days 80 and 100 of pregnancy (Allen et al., 2010; Panzani et al., 2018b). In the remaining of donkey-in-horse pregnancies, endometrial cups are formed at a slower rate than in normal pregnancies and half of the pregnancies results in a normal donkey foal, the other half of the pregnancies ends with an abortion during the last 2 months of pregnancy (Panzani et al., 2018b).

### 3.6.4 Conception rates

Although good conception rates are achieved when breeding mares to jacks, there is a higher rate of early fetal loss in the first trimester of pregnancy when compared with mares bred by stallions (Canisso et al., 2019). This is probably a result of lower eCG production, and thus decreasing the formation of secondary corpora lutea (Canisso et al., 2019). Jennies bred to stallions have much lower conception rates than jennies or mares bred to jacks (Canisso et al., 2019). It is currently unknown why there is such a discrepancy between these types of pregnancies (Canisso et al., 2019).

### 3.6.5 Gestation

Gestation length, behavior, and duration of foaling stages of mares carrying mule/hinny pregnancies are similar to mares carrying and delivering horse pregnancies (Canisso et al., 2019). However, mares apparently do not achieve the same degree of uterus tonus when carrying a mule foal (Burnham, 2002).

### 3.6.6 Neonatal isoerythrolysis

Neonatal isoerythrolysis occurs in about 10% of the mule and hinny foals, which is significantly higher than in horse foals (Canisso et al., 2019). Donkeys carry an antigen on their erythrocytes which is also carried by mules and hinnies on their erythrocytes (Canisso et al., 2019). Mares carrying and delivering mule pregnancies develop antibodies against this antigen and during transfer of passive immunity, mule and hinny foals absorb these antibodies and can consequently develop a massive immune-mediated destruction of their erythrocytes (Canisso et al., 2019). It is advisable that mule and hinny foals born from mares who previously carried mule or hinny pregnancies, should be closely monitored for signs of neonatal isoerythrolysis in the first 3 days postpartum (Canisso et al., 2019).

# 4. Discussion and conclusion

Breeding donkeys can be an interesting and exciting endeavor. However, to be successful it is important to be educated about the reproductive idiosyncrasies of donkeys. There is an increasing interest in this species and quite a number of studies have been performed in the recent past. However, more research on donkey reproductive physiology is still needed to fully understand this species and to establish standard reproductive protocols, like estrus synchronization and artificial breeding.

Jennies are similar to mares in most aspects, but there are some important reproductive peculiarities to be kept in mind.

- Their genital organs resembles those of mares, with the exception that they are proportionally larger. The jenny's cervix protrudes into the vagina, which makes them predisposed for cervical lacerations after a difficult parturition.
- The estrus cycle in the jenny appears to be longer, with a range of 20-40 days and an average of 24 days.
- A jenny in estrus shows some typical estrus behavior like mouth clapping: she opens and closes the mouth with the ears pressed against the extended neck accompanied by an excessive saliva flow.
- On transrectal ultrasound uterine edema is less pronounced in jennies and thus cannot be used as a predictor for an imminent ovulation. The best predictors for an imminent ovulation in jennies are estrus behavior, follicle size and follicular texture.
- There is still controversy about the seasonality of the jenny but there is a consensus that donkeys show less seasonality than horses.
- A healthy jenny endometrium has more neutrophils, eosinophils, and highly branched uterine glands.

There are numerous reliable studies available on the reproductive anatomy of jennies of different breeds. However, research on the reproductive physiology of the jenny is rather scarce and most studies only include a small sample size consisting of only one single breed. Therefore, most observations resulting from these studies provide a broad range of outcomes with a lot of variabilities. Hence, it is important to invest in more qualitative research to determine better references, e.g. for the estrus length.

Like the jenny, most reproductive characteristics of the jack are in accordance with the stallion, apart from a few exceptions.

- The penis, testes and accessory sex glands are relatively larger.
- The jack has a nipple on each side of the preputial sheath.
- Castration of jacks is associated with a higher incidence of hemorrhage complications because of larger supplying blood vessels.
- Prostaglandin metabolites (PGFM), testosterone, LH, estrone sulfate and cortisol play a role in the endocrinology of the stallion, but the last three hormones are not as important in jacks.
- Sexual behavior in jacks differs markedly from stallions; they show a period of disinterest and inactivity after multiple mounts, called male isolation.
- Jacks take longer to achieve erection and to ejaculate, which should be kept in mind when trying to collect semen.
- The semen volume and the total number of spermatozoa are relatively higher than in stallions, but both are highly variable due to the influence od the accessory sex glands. The percentage of viability of spermatozoa is much higher in jacks than in stallions. The total motility of spermatozoa in jacks is higher, whereas the progressive motility in jacks is similar to stallions.

As in the jenny, the reproductive anatomy of the jack is mostly well understood while there is still a lot unknown about the reproductive endocrinology of the jack. Studies including a larger number of males should be performed to assess standardized references for the seminal parameters in jacks.

Reproductive biotechnologies may contribute to the preservation of donkey breeds in danger of extinction. Nonetheless, the direct transfer of technologies developed for the horse to donkeys, yields poor results. More studies on donkey reproductive physiology of the jenny as well as the jack are needed to establish standard protocols. Although not widely accepted by owners at this time, artificial insemination can be performed successfully in donkeys, with pregnancy rates varying from 40% to 80%. Standard equine equipment can be employed to collect, evaluate, transport, and inseminate semen in donkeys. The technique of artificial insemination is similar in all equine species. Experience with cooling and freezing sperm is still limited in donkeys but jacks have excellent semen quality and the semen can be cooled in a manner similar to that of the stallion, using the same extenders and storage techniques. The number of studies comparing semen extenders in donkeys is limited, but in most studies INRA82-Y ensured better sperm motility characteristics during cooled storage than other extenders. Protocols used to process donkey semen for freezing have been adapted from stallions. To date, various authors have reported low fertility rates varying from 6% to 60% with donkey cryopreserved semen using different extenders and amounts of cryoprotectant. Improvement of cryopreservation in donkeys has been achieved in three ways: (1) by reducing the amount of glycerol; (2) by the addition of glutamine and replacing hen egg yolk with quail egg yolk; (3) by adding autologous seminal plasma to frozen-thawed semen.

The technique for ET in donkeys was initially derived from equine consolidated protocols with disappointing results, and ET still remains a challenge to this day. ET results have improved over the last years, but pregnancy rates need to improve to become an effective reproductive technique. Surgical and nonsurgical ET in the donkey results nowadays in pregnancy rates varying from 17% to 23%. The technique for nonsurgical embryo flushing and for transferring the embryo in a recipient are both similar to the current technique used in horses. Transcervical catheterization in jennies requires more manipulation, which is however not responsible for the low pregnancy rates. Embryos from each species need specific washing and holding media because of their specific characteristics and it seems that Ringer lactate and Emcare Holding Solution is a better choice for donkey embryos than PBS.

Pregnancy in donkeys differs in many aspects form horses, with gestation length being the most important difference. Gestation in donkeys lasts approximately 12 months, but there are no references reporting a range of acceptable variation, as there are available for mares, which makes it hard for the practitioner to differentiate a physiological from a pathological gestation. The reason for the longer gestation is probably the lower placental efficiency in donkeys. Donkeys have a higher frequency of multiple ovulations in donkeys, making an early pregnancy diagnosis is advisable to prevent twinning. The CUPT values are slightly different in donkeys due to the longer gestation. Further research is needed to define possible breed or body size CUPT specific differences, and to define the CUPT values in case of pregnancy disturbances or placental abnormalities.

Parturition in donkeys is very similar to horses, except for the fact that dystocia is more likely with jennies. After a difficult parturition, jennies are more prone to cervical laceration, affecting the future fertility negatively. Jennies and mares are equally susceptible to retention of fetal membranes, but the donkey is predisposed to insulin resistance and metabolic syndrome which can worsen this problem.

Donkeys are frequently crossed with mares to produce mules. The difficulties associated with interspecies breeding emphasize how different donkeys are from horses. These hybrids come in both sexes but they are generally infertile due to the homologous chromosome pairing failure that occurs during meiosis. Mules are mostly used as a companion or labor animal but they can also serve as an excellent embryo recipient for donkey or horse embryos. Hand breeding is the most suitable approach to breed horses with donkeys. However, they should be previously exposed to the other species because otherwise they will not be interested. When breeding mules and hinnies, the most important thing to keep in mind is that neonatal isoerythrolysis occurs in about 10%, which is significantly higher

than in horse foals. Thus, it is advisable that mule foals born from multiparous mares should be closely monitored.

In conclusion, there are similarities between donkeys and horses, which allow them to breed and produce hybrids, but there are also many unique reproductive features specific for the donkey. These idiosyncrasies are crucial to know when aiming to treat the donkey and advice the owner correctly. Published data on the reproductive characteristics of donkey are still scarce, and the available literature mostly consists of small sample size studies with one specific breed in a specific climate. Techniques for artificial breeding in donkeys should not just be derived from the current techniques used in horses, but should be adapted so better results can be achieved. Hence, more intensive and qualitative research should be performed to understand and treat this species better so we can treat the individual donkey better and save endangered breeds from extinction.

# 5. References


Abd-Elnaeim, M.M., Zayed, A.E., Leiser, R., 2001. The blood vasculature as the forming element of the uterus of the estrous donkey (*Equus asinus*). Italian Journal of Anatomy and Embryology 106, 307–315.

Ali, M., Baber, T., Hussain, T., Awan, F., Nadeem, A., 2014. The contribution of donkeys to human health. Equine Veterinary Journal 46, 766-767.

Allen, W.R., Kydd, J., Boyle, M.S., Antczak, D.F., 2010. Between-species transfer of horse and donkey embryos: A valuable research tool. Equine Veterinary Journal 17, 53–62.

Bansal, R.S., Pal, Y., Pareek, P.K., Purohit, G.N, Legha, R.A., Gupta, A.K., 2006. Progesterone profile during oestrus cycle, pregnancy and post-foaling in donkey mares. Veterinary Practitioner Vol. 7 No. 1, 5-6.

Blanchard, T.L., Taylor, T.S., Love, C.L., 1999. Estrous cycle characteristics and response to estrus synchronization in Mammoth asses (*Equus asinus americanus*). Theriogenology 52, 827-834.

Burden, F., Thiemann, A., 2015. Donkeys are different. Journal of Equine Veterinary Science 35, 376–382.

Burnham, S.L., 2002. Anatomical Differences of the Donkey and Mule. Proceedings Annual Convention of the AAEP 2002 48, 102–109.

Camillo, F., Panzani, D., Scollo, C., Rota, A., Crisci, A., Vannozzi, I., Balbo, S., 2010. Embryo recovery rate and recipients' pregnancy rate after nonsurgical embryo transfer in donkeys. Theriogenology 73, 959–965.

Camillo, F., Vannozzi, I., Tesi, M., Sabatini, C., Rota, A., Paciolla, E., Dang-Nguyen, I., Panzani, D., 2014. Induction of ovulation with buserelin in jennies: In search of the minimum effective dose. Animal Reproduction Science 151, 56–60.

Camillo, F., Rota, A., Biagini, L., Tesi, M., Fanelli, D., Panzani, D., 2018. The Current Situation and Trend of Donkey Industry in Europe. Journal of Equine Veterinary Science 65, 44–49.

Canisso, I.F., Davies Morel, M.C.G., McDonnell, S., 2009a. Strategies for the management of donkey jacks in intensive breeding systems. Equine Veterinary Education 21, 652–659.

Canisso, I.F., Coutinho da Silva, M.A., Davies Morel, M.C.G., 2009b. How to manage jacks to breed mares. Proceedings Annual convention of the AAEP 55, 342–348.

Canisso, I.F., Panzani, D., Miró, J., Ellerbrock, R.E., 2019. Key Aspects of Donkey and Mule Reproduction. Veterinary Clinics of North America: Equine Practice 35, 607–642.

Carder, G., Ingasia, O., Ngenoh, E., Theuri, S., Rono, D., Langat, P., 2019. The Emerging Trade in Donkey Hide: An Opportunity or a Threat for Communities in Kenya? Agricultural Sciences 10, 1152–1177.

Carluccio, A., Pau, S., Picci, G., Tosi, U., De Amicis, I., Zedda, M.T., Scialandrone, M. and De Fanti, C., 2003. Il ciclo estrale nell'asina di Martina Franca. Veterinaria Pratica Equina, 3, 35–41.

Carluccio, A., Tosi, U., Contri, A., De Amicis, I., Veronesi, M.C., 2006. Corpus luteum sensitivity to PGF2α administration in the Martina Franca Jenny. Veterinar Research Communications 30, 171–173.

Carluccio, A., Panzani, S., Tosi, U., Faustini, M., De Amicis, I., Veronesi, M.C., 2007. Efficacy of hCG and GnRH for inducing ovulation in the jenny. Theriogenology 68, 914–919.

Carluccio, A., Panzani, S., Contri, A., Tosi, U., De Amicis, I., Veronesi, M.C., 2008. Luteal function in jennies following PGF2α treatment 3 days after ovulation. Theriogenology 70, 121–125.

Carluccio, A., Panzani, S., Contri, A., Bronzo, V., Robbe, D., Veronesi, M.C., 2013a. Influence of season on testicular morphometry and semen characteristics in Martina Franca jackasses. Theriogenology 79, 502–507.

Carluccio, A., Contri, A., Amendola, S., De Angelis, E., De Amicis, I., Mazzatenta, A., 2013b. Male isolation: A behavioral representation of the pheromonal "female effect" in donkey (*Equus asinus*). Physiology & Behavior 118, 1–7.

Carluccio, A., Gloria, A., Veronesi, M.C., De Amicis, I., Noto, F., Contri, A., 2015. Factors affecting pregnancy length and phases of parturition in Martina Franca jennies. Theriogenology 84, 650–655.

Carluccio, A., Noto, F., Parrillo, S., Contri, A., De Amicis, I., Gloria, A., Robbe, D., Veronesi, M.C., 2016. Transrectal ultrasonographic evaluation of combined utero-placental thickness during the last half of pregnancy in Martina Franca donkeys. Theriogenology 86, 2296–2301.

Carluccio, A., Gloria, A., Robbe, D., Veronesi, M.C., De Amicis, I., Cairoli, F., Contri, A., 2017. Reproductive characteristics of foal heat in female donkeys. Animal 11, 461–465.

Contri, A., Tosi, U., De Amicis, I., Veronesi, M.C., Panzani, S., Carluccio, A., 2008. Ultrasonographic evaluation of sexual glands before and after ejaculation in the jackass. Veterinary Research Communications 32, 135–137.

Contri A, De Amicis I, Veronesi MC, Faustini M, Robbe D, Carluccio A, 2010. Efficiency of different extenders on cooled semen collected during long and short day length seasons in Martina Franca donkey. Animal Reproduction Science 120, 136–141.

Contri, A., Robbe, D., Gloria, A., De Amicis, I., Veronesi, M.C., Carluccio, A., 2014. Effect of the season on some aspects of the estrous cycle in Martina Franca donkey. Theriogenology 81, 657–661.

Crisci, A., Rota, A., Panzani, D., Sgorbini, M., Ousey, J.C., Camillo, F., 2014. Clinical, ultrasonographic, and endocrinological studies on donkey pregnancy. Theriogenology 81, 275–283.

Dadarwal, D., Tandon, S.N., Purohit, G.N., Pareek, P.K., 2004. Ultrasonographic evaluation of uterine involution and postpartum follicular dynamics in French Jennies (*Equus asinus*). Theriogenology 62, 257–264.

Deleuze, S., Douet, C., Couty, I., Moros-Nicolás, C., Barrière, P., Blard, T., Reigner, F., Magistrini, M., Goudet, G., 2018. Ovum Pick Up and In Vitro Maturation of Jennies Oocytes Toward the Setting Up of Efficient In Vitro Fertilization and In Vitro Embryos Culture Procedures in Donkey (Equus asinus). Journal of Equine Veterinary Science. 65, 111–117.

Dorado, J., Acha, D., Ortiz, I., Gálvez, M.J., Carrasco, J.J., Díaz, B., Gómez-Arrones, V., Calero-Carretero, R., Hidalgo, M., 2013a. Relationship between conventional semen characteristics, sperm motility patterns and fertility of Andalusian donkeys (*Equus asinus*). Animal Reproduction Science 143, 64–71.

Dorado, J., Acha, D., Gálvez, M.J., Ortiz, I., Carrasco, J.J., Díaz, B., Gómez-Arrones, V., Calero-Carretero, R., Hidalgo, M., 2013b. Sperm motility patterns in Andalusian donkey (*Equus asinus*) semen: Effects of body weight, age, and semen quality. Theriogenology 79, 1100–1109.

Fanelli, D., Tesi, M., Bagnato, G., Salari, F., Martini, M., Panzani, D., Camillo, F., Rota, A., 2019. New simplified protocols for timed artificial insemination (TAI) in milk-producing donkeys. Theriogenology 139, 126–131.

Fielding, D., 1988. Reproductive characteristics of the jenny donkey - *Equus asinus*: A review. Tropical Animal Health and Production 20, 161–166.

Galisteo, J., Perez-Marin, C.C., 2010. Factors affecting gestation length and estrus cycle characteristics in Spanish donkey breeds reared in southern Spain. Theriogenology 74, 443–450.

Gastal, E.L., Santos, G.F., Henry, M., Piedade, H.M., 1993. Embryonic and early foetal development in donkeys. Equine Veterinary Journal 15, 10-13.

Gastal, M.O., Henry, M., Beker, A.R., Gastal, E.L., GonÇalves, A., 1996. Sexual behavior of donkey jacks: Influence of ejaculatory frequency and season. Theriogenology 46, 593-603.

Hagstrom, D.J., 2009. Donkeys are Different : An Overview of Reproductive Variations from Horses.

Henry M., Figueiredo A.E.F., Palhares M.S., Coryn M., 1987. Clinical and endocrine aspects of oestrous cycle in donkeys (Equus asinus). Journal of Reproduction and Fertility 35, 297–303.

Hughes J.P., Stabenfeldt G.H., Evans J.W., 1975. The estrus cycle of the mare. Journal of Reproduction and Fertility 23, 161–166.

Kebede, H., Lemma, A., Negussie, H., 2012. Ultrasonographic studies on ovarian dynamics and associated estrus manifestations of jennies under controlled management, Ethiopia. Tropical Animal Health and Production 44, 1965–1970.

Kenney, R.M., Doig, P.A., 1986. Equine endometrial biopsy. In: Morrow, D.A., Ed., Current Therapy in Theriogenology, W.B. Saunders, Philadelphia, 723-729.

Köhle, N., 2018. Feasting on donkey skin. ANU Press Chapter Title : Arguing With Robots. Chapter Author(s): Lorand Laskai Book Title : Prosperity. Book Editor(s): Jane Golley , Linda Jaivin. Published by : ANU Press.

Lemma, A., Schwartz, H.J., Bekana, M., 2006. Application of ultrasonography in the study of the reproductive system of tropical jennies (*Equus asinus*). Tropical Animal Health and Production 38, 267–274.

Lodi, L.D., Henry, M., Paranhos da Costa, M.J.R., 1995. Behavior of Donkey Jacks (*Equus asinus*) Breeding Horse Mares (*Equus caballus*) at Pasture. Biology of Reproduction 52, 591–598.

Mai, H.M., 2014. Gross Anatomical Study of the Urogenital System of the Indigenous Nigerian Male Donkey (*Equus africanus africanus*) in Comparison with the Stallion. Anatomy & Physiology 4, 145.

Mancuso R., Scollo C., Torrisi C., Catone G., 2007. La determinazione del sesso fetale nell'asina [Fetal sex determination in donkey mare]. Proceedings Annual meeting of the Italian association of equine veterinarians, 173-174.

Meira, C., Ferreira, J.C.P., Papa, F.O., Tornero, M.T.T., Bicudo, S.D., 1995. Study of the Estrous Cycle in Donkeys (*Equus asinus*) Using Ultrasonography and Plasma Progesterone Concentrations. Biology of Reproduction 52, 403–410.

Meira, C., Ferreira, J.C.P., Papa, F.O., Henry, M., 1998. Ovarian activity and plasma concentrations of progesterone and estradiol during pregnancy in jennies. Theriogenology 49, 1465–1473.

Miragaya, M.H., Neild, D.M., Alonso, A.E., 2018. A Review of Reproductive Biology and Biotechnologies in Donkeys. Journal of Equine Veterinary Science 65, 55–61.

Miró, J., Lobo, V., Quintero-Moreno, A., Medrano, A., Peña, A., Rigau, T., 2005. Sperm motility patterns and metabolism in Catalonian donkey semen. Theriogenology 63, 1706–1716.

Miró, J., Vilés, K., Anglada, O., Marín, H., Jordana, J., Crisci, A., 2015. Color Doppler provides a reliable and rapid means of monitoring luteolysis in female donkeys. Theriogenology 83, 485–490.

Neves, E.S., Chiarini-Garcia, H., França, L.R., 2002. Comparative testis morphometry and seminiferous epithelium cycle length in donkeys and mules. Biology of Reproduction 67, 247–255.

Nervo, T., Bertero, A., Poletto, M., Pregel, P., Leone, R., Toffoli, V., Vincenti, L., 2019. Field ultrasound evaluation of some gestational parameters in jennies. Theriogenology 126, 95–105.

Nipken, C., Wrobel, K.H., 1997. A quantitative morphological study of age-related changes in the donkey testis in the period between puberty and senium. Andrologia 29, 149–161.

Oliveira, J.V. de, Oliveira, P.V. de L.F., Melo e Oña, C.M., Guasti, P.N., Monteiro, G.A., Sancler da Silva, Y.F.R., Papa, P. de M., Alvarenga, M.A., Dell'Aqua Junior, J.A., Papa, F.O., 2016. Strategies to improve the fertility of fresh and frozen donkey semen. Theriogenology 85, 1267–1273.

Ousey, J., 2002. Induction of parturition in the healthy mare. Equine veterinary Education Manual 5, 83-87.

Panzani, D., Rota, A., Crisci, A., Kindahl, H., Govoni, N., Camillo, F., 2012. Embryo quality and transcervical technique are not the limiting factors in donkey embryo transfer outcome. Theriogenology 77, 563–569.

Panzani, D., Tardella, M., Govoni, N., Tesi, M., Fanelli, D., Rota, A., Vannozzi, I., Camillo, F., 2018a. Effect of the administration of alfaprostol 3 or 6 days after ovulation in jennies: ultrasonographic characteristic of corpora lutea and serum progesterone concentration. Theriogenology 121, 175–180.

Panzani, D., Rota, A., Tesi, M., Fanelli, D., Camillo, F., 2018b. Update on Donkey Embryo Transfer and Cryopreservation. Journal of Equine Veterinary Science 65, 50–54.

Pugh, D.G., 2002. Donkey reproduction. Proceedings Annual Convention of the AAEP 48, 113–114.

Purdy, S.R., 2005. Artificial Insemination for Miniature Donkeys. In: Matthews NS, Taylor TS, editors. Veterinary care of donkeys. Ithaca, NY: International Veterinary Information Service; 2005. A2924.0405.

Purdy, S.R., 2010. Reproduction in donkeys. Proceedings of the North American Veterinary Conference 249–252.

Quaresma, M., Payan-Carreira, R., 2015. Characterization of the estrous cycle of Asinina de Miranda jennies (*Equus asinus*). Theriogenology 83, 616–624.

Quartuccio, M., Marino, G., Zanghì, A., Garufi, G., Cristarella, S., 2011. Testicular Volume and Daily Sperm Output in Ragusano Donkeys. Journal of Equine Veterinary Science 31, 143–146.

Renner-Martin, T.F.P., Forstenpointner, G., Weissengruber, G.E., Eberhardt, L., 2009. Gross anatomy of the female genital organs of the domestic donkey (*Equus asinus* Linné, 1758). Journal of Veterinary Medicine Anatomy Histologia Embryologia 38, 133–138.

Rota, A., Magelli, C., Panzani, D., Camillo, F., 2008. Effect of extender, centrifugation and removal of seminal plasma on cooled-preserved Amiata donkey spermatozoa. Theriogenology 69, 176–185.

Rota, A., Panzani, D., Sabatini, C., Camillo, F., 2012. Donkey jack (*Equus asinus*) semen cryopreservation: Studies of seminal parameters, post breeding inflammatory response, and fertility in donkey jennies. Theriogenology 78, 1846–1854.

Rota, A., Puddu, B., Sabatini, C., Panzani, D., Lainé, A.L., Camillo, F., 2018a. Reproductive parameters of donkey jacks undergoing puberty. Animal Reproduction Science 192, 119–125.

Rota, A., Sgorbini, M., Panzani, D., Bonelli, F., Baragli, P., Ille, N., Gatta, D., Sighieri, C., Casini, L., Maggiorelli, M.M., Aurich, C., Camillo, F., 2018b. Effect of housing system on reproductive behaviour and on some endocrinological and seminal parameters of donkey stallions. Reproduction in Domestic Animals 53, 40–47.

Sabatini, C., Mari, G., Mislei, B., Love, C., Panzani, D., Camillo, F., Rota, A., 2014. Effect of post-thaw addition of seminal plasma on motility, viability and chromatin integrity of cryopreserved donkey jack (Equus asinus) spermatozoa. Reproduction in Domestic Animals 49, 989–994.

Serres, C., Rodriguez, A., Alvarez, A.L., Santiago, I., Gabriel, J., Gomez-Cuetara, C., et al., 2002. Effect of centrifugation and temperature on motility and plasma membrane integrity of Zamorano-Leone´s donkey semen. Theriogenology 58, 329–32.

Taberner, E., Medrano, A., Peña, A., Rigau, T., Miró, J., 2008. Oestrus cycle characteristics and prediction of ovulation in Catalonian jennies. Theriogenology 70, 1489–1497.

The Donkey Sanctuary, 2019. Under the Skin.

Tibary, A., 2004. Reproductive patterns in donkeys and miniature horses. Proceedings of The North American Veterinary Conference, 231-233

Tosi, U., Bernabò, N., Verni, F., Valbonetti, L., Muttini, A., Mattioli, M., Barboni, B., 2013. Postpartum reproductive activities and gestation length in Martina Franca jennies, an endangered Italian donkey breed. Theriogenology 80, 120–124.

Trimeche, A., Renard, P., Tainturier, D., 1998. A procedure for Poitou jackass sperm cryopreservation. Theriogenology 50, 793–806.

Vandeplassche, G.M., Wesson, J.A., Ginther, O.J., 1981. Behavioral, follicular and gonadotropin changes during the estrous cycle in donkeys. Theriogenology 16, 239–249.

Veronesi, M.C., Villani, M., Wilsher, S., Contri, A., Carluccio, A., 2010. A comparative stereological study of the term placenta in the donkey, pony and Thoroughbred. Theriogenology 74, 627–631.

Veronesi, M.C., De Amicis, I., Panzani, S., Kindahl, H., Govoni, N., Probo, M., Carluccio, A., 2011. PGF2α, LH, testosterone, oestrone sulphate, and cortisol plasma concentrations around sexual stimulation in jackass. Theriogenology 75, 1489–1498.

Yilmaz, O., Boztepe, S., Ertugrul, M., 2012. The Domesticated Donkey : III – Economic Importance , Uncommon Usages , Reproduction Traits , Genetics , Nutrition and Health Care . Canadian Journal of Applied Sciences 3, 320–338.

Zakari, F.O., Ayo, J.O., Kawu, M.U., Rekwot, I., 2015. The effect of season and meteorological stress factors on behavioural responses and activities of donkeys (Equus Asinus) - A review. Annals of Animal Science 15, 307–321.

Zhao, G., Wu, K., Cui, L., Zhao, L., Liu, Y., Tan, X., Zhou, H., 2011. In vitro maturation and artificial activation of donkey oocytes. Theriogenology 76, 700–704.