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Issue: Donkey and Mule Medicine

Key aspects of donkey and mule reproduction

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Key-points

- Donkeys are non-seasonal, polyestrous, territorial and non-harem breeders.
- The jenny cervix is long and prone to laceration and adhesions after dystocia.
- Jacks have large testicles with a high spermatogenic efficiency.
- Frozen donkey semen has better pregnancy rates in mares than jennies.
- Embryo transfer in donkeys is less efficient than in horses.
- Mules can be used as embryo recipients for horse or donkey embryos
- Mule males are infertile and do not produce sperm

Abstract

Donkeys are non-seasonal, polyestrous, territorial and non-harem breeders. While there are many similarities between horses and donkeys, there are also many reproductive features that differ between species, from the relatively longer cervix in the Jenny cervix to the very high spermatogenic efficiency in the Jack. There are also interesting differences in assisted reproduction between species. Semen freezing of jacks result in high pregnancy rates when used to breed mares, but lower pregnancy rates when used to breed jennies. Cooling and shipping donkey semen can be satisfactorily performed if the semen is centrifuged or if additional source of cholesterol is added. Embryo transfer in donkeys results in poor outcomes when compared to horses. Mules display reproductive cyclic activity but are overall infertile. Rare cases of fertile mules delivering donkeys or horses offspring bred by jacks or stallions have been reported. While there are several similarities between donkeys and horses which allow them to breed and produce hybrids, there are also many unique reproductive features of donkeys and mules. Therefore, the objectives of this manuscript are to review key aspects of donkey and mule reproductive physiology, reproductive medicine, and assisted reproductive techniques. Knowledge of mule and donkey reproduction is useful both for practitioners offering assisted reproductive techniques, and also for practitioners with the occasional client with a reproductive question about these animals. Assisted reproductive techniques can be very useful to enhance the mule production, and to preserve and disseminate desired genetic material.

Keywords: Asinine, assisted reproductive techniques, equid reproduction, reproductive physiology, reproductive pathology, mules*, Equus mulus, Equus asinus*

Introduction

Donkeys (*Equus asinus*) and their hybrids with horses (i.e., *Equus mulus mulus*) have been used for centuries in agriculture, transport and wars across multiple civilizations. Mechanization of agriculture and industrialization in the last century led to a reduction in the importance and number of both donkeys and their hybrids. However, in some African and Asian countries, these animals are still heavily used as pack animals and to pull carts. In the Americas, donkeys are frequently crossed with mares to produce mules. Mules are used in mountain regions for tourism purposes (e.g., Grand Canyon), trail rides, territory defense (e.g., mountains of Argentina) and to herd cattle in large beef operations in Brazil [1]. In North America and Western Europe, donkeys may also be kept as pets (miniature donkeys) or as guardians for livestock [2].

In Western Europe, industrialization almost led to the extinction of many European breeds. However, in recent years, conservation programs led by many countries (in particular Spain, Italy, and Portugal), are rescuing some of the endangered donkey breeds. Recently, the rediscovery of donkey milk for consumption, cheese, and cosmetics has helped some threatened breeds such as the Amiata rebound [2]

In contrast, in desert areas of the United States, some Caribbean Islands (e.g., Saint Kitts and Nevis), and the Northeast of Brazil, overpopulation of small frame donkeys is a major concern. In these countries, feral donkeys have no commercial value, and depend on governmental and non-governmental agencies to develop contraceptives and population control programs. In the United States for instance, the Bureau of Land Management runs a program to control the population of feral donkeys on public lands.

While there are several similarities between donkeys and horses which allow them to breed and produce hybrids, there are also many unique reproductive features of donkeys and mules.

Therefore, the objectives of this manuscript are to review key aspects of donkey and mule reproductive physiology, reproductive medicine, and assisted reproductive techniques. Knowledge of mule and donkey reproduction is useful both for practitioners offering assisted reproductive techniques, and also for practitioners with the occasional client with a reproductive question about these animals. Assisted reproductive techniques can be very useful to enhance mule production, and also to preserve and disseminate desired genetic material.

Overview of the functional female genital anatomy

The internal genitalia of jennies and mules are similar to mares; however, the jenny's internal genitalia appears to be proportionally larger than the mares and mules[3]. The uterus of these females is Y-shaped, has a uterine body of similar length to the uterine horns, with the tips of the uterine horns reaching the fifth lumbar vertebra cranially [3]. The ovaries are located ventrally from the fourth to the fifth lumbar vertebrae, which is more cranial than the ovaries of female horses [3]. Similarly to mares, the mesometrium in the jenny is attached to the dorsal margin of the uterine horns and dorso-laterally to the body of the uterus excluding the cervix; this feature makes the two horns form a cranio-ventral convexity [3]. Jenny and mule ovaries have a similar bean-shape to the mare, with the ovarian cortex located inside the ovary, the medullar region located outside the ovary, and the ovulation fossa located at the free margin of the ovary. Interestingly, the broad ligament appendix and ovarian bursa are much more prominent in the jennies than mares [3].

The caudal reproductive tract (vestibule and vagina) of jennies is slightly more tilted dorsally than other farm animals, and commonly the ventral commissure of the vulva is more concealed ventrally than the dorsal commissure. In normal mares, the vulvar length is about 2/3 below the pelvic brim; however, in normal jennies, the vulvar length is entirely below the pelvic brim

(Figure 1). Subfertile jennies with abnormal vulvar conformation suffering with pneumovagina and pneumouterus may benefit from a Caslick's operation or perineal body reconstruction (Figure 2). Proportionally, jennies appear to have a larger clitoris than mares. The vulva of jennies have larger minor lips (visible inside the vestibule) than mares, and the vulva is slightly tilted ventrally, making contamination of the reproductive tract less likely in this species (Figure 1) [3].

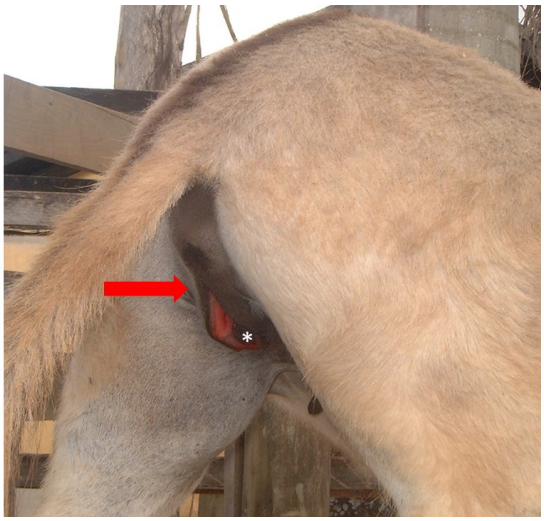


Figure 1. Vulva of a jenny in estrus. The arrow denotes the level of the pelvic brim, and the asterisk denotes the clitoris glans.

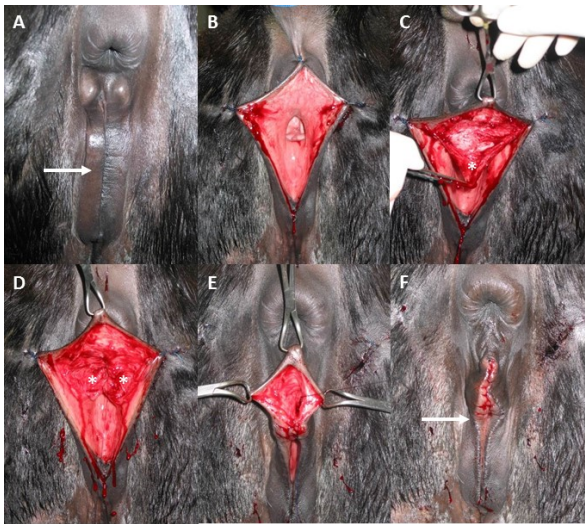


Figure 2. Perineal body reconstruction in a subfertile jenny suffering with pneumovagina and physometra (A) The jenny's vulva is excessively long, the arrow points to the pelvic brim and the two bulging structures show the lidocaine block. (B) Two stay sutures have been applied and dorsal aspect of the vulva exposed; (C) A flap (*) was created by gentle dissection of the mucosa. (D-E) Both sides of the flap (*) were sutured together in a simple continue pattern using absorbable suture (#2) to recreate a "roof" for the vestibule. (F) Finalized surgical reconstruction. After the "roof" was created, the space between the roof and the perineal body was closed with simple interrupted sutures (#2).

The jenny cervix has a narrowed and tortuous lumen and a very prominent vaginal portion (1.5-3cm). The vaginal portion is connected to dorsal and ventral vaginal longitudinal folds that limit its lateral displacement. These features likely make the donkey cervix prone to lacerations during dystocias [3,4]. In addition, the vaginal portion of the cervix may have a straight conformation

or various conformations that resemble the letters “L,” “C” or “V” (Figure 3). The clinical significance for these variations remains to be determined. The narrowed and tortuous lumen represents a challenge for routine intrauterine procedures, particularly in small frame maiden jennies.

In one report involving Poitou donkeys, a large French breed, the cervix varied from 4.5 to 8.0 cm in length, and 2.5-3 cm in width [4]. Cervical dimensions for other asinine breeds remain to be determined. Similarly to the mare, the cervix in jennies becomes longer, thinner, and well-toned during diestrus, and shorter, wider, softer, and relaxed during estrus [4]. Similarly, the uterus becomes well-toned during diestrus and softer and relaxed during estrus [4]. These cervical and uterine changes are less likely to be appreciated by practitioners less experienced with donkeys.

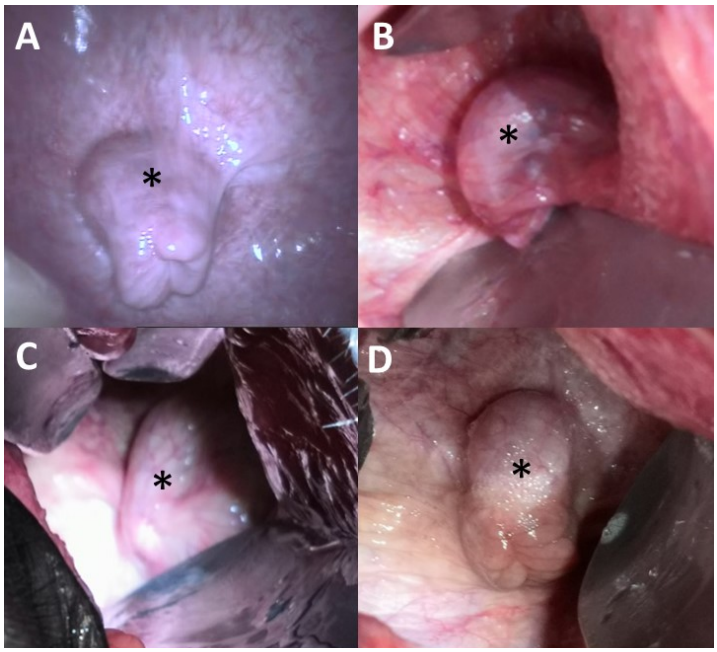


Figure 3. Representative images of the vaginal portion of the jenny cervix (*). (A) Straight type (B) C-like cervix; (C) L-like cervix; (D) V-like cervix. Images B-D kindly provided by Dr. Shenming Zeng, China Agricultural University.

Puberty and seasonality

Puberty in healthy and well-fed jennies is usually reached between the first and second year of life [5,6]. Although no controlled studies have been performed to determine the sexual maturity of jennies, it is recommended to refrain from breeding them before three years of age[5]. Studies performed prior to the widespread use of ultrasonography in animal reproduction described donkeys as a long-day seasonal polyestrous species, particularly in temperate latitudes[7]. Further reports described the jenny as a non-seasonal annual polyestrous female even in temperate areas of the world [8-10]. A recent study out of Portugal showed that young jennies with a poor body condition score (i.e., ≤4 out of 9) were likely to stop cycling during the fall and winter, while jennies with satisfactory scores ≥5 continued cycling throughout the year[11]. This is consistent with a previous report out of Ethiopia showing a positive association between body condition score and ovarian activity[12]. Various authors reported the occurrence of silent estrus, prolonged luteal phase and split estrus, but not in association with the anovulatory season [7,9,13]. Ovarian tumors like granulosa cell tumors and teratomas have been recorded in donkeys [14]

Estrous cycle

The duration of the donkey estrous cycle (~23-27 days) tends to be longer than mares (~21days), whereas mules tend to be intermediate in estrus cycle length (22 days) [7,15,16]. In donkeys, diestrus varies from 15 to 19 days, whereas estrus varies from 4 to 10 days, and ovulation occurs the day before the end of estrus [7,16,17]. In a recent study out of Mexico, the inter-estrus-

147 ovulation interval varied from 21.2 ± 0.3 to 26.2 ± 0.3 days; however, diestrus remained constant
148 at 17 days [16]. There appears to be remarkable variations according to the donkey breed and envi-
149 ronment as summarized in Table 1. Similarly to mares, estrus tends to be shorter during the spring
150 and summer than in the fall and winter [9,18-20].

151 Progesterone concentration remains low up to the day after ovulation, slowly increases
152 until 4-6 d post-ovulation, plateaus until 14-16d post-ovulation and then starts to decline to reach
153 baseline concentrations (<1 ng/mL) in two days [21]. In donkeys, estradiol-17β starts to rise from 10
154 pg/mL during early estrus (5-6 days prior to ovulation). and peaks around 40-60 pg/mL (1-2 days
155 surrounding ovulation) [20]. In donkeys, FSH concentrations remain baseline throughout the estrous
156 cycle, and peak 3 and 9 days post-ovulation [17]. Similar to mares, LH concentrations increase pre-
157 ovulation and peak two days after ovulation, then LH concentrations decrease and remain baseline
158 throughout the remainder of the estrous cycle [17].

159 Follicular deviation in jennies happens 8-9d before ovulation at approximately 19-20 mm
160 follicular diameter [22-24]. In most donkey breeds the dominant follicle grows 2-3 mm/day from
161 deviation to ovulation [13,16], however, in Catalonian donkeys, a large breed, the follicles grow up to
162 ~4 mm/day from follicular deviation to the static follicular growth phase [25]. In jennies, one follic-
163 ular wave was identified during the estrous cycle [26], whereas there were no follicular waves de-
164 tected in mules [15]. The ovulatory diameter varies from 30 to 48 mm (Table 2), with a positive
165 association between body frame and follicular diameter [10,16,27,28]. In mules, the average follicular
166 diameter the day before ovulation was 38.2 ±2.2 mm [15].

167 Once in estrus the jenny will display signs such as standing to be mounted by another
168 female or male, mouth clapping, clitoral winking, urinating and tail raising. Females may display
169 the Flehmen's response after smelling urogenital secretions from estrus jennies [10,29-32] (Figure 4).

Jennies congregate into a sexually active group similar to cows, and this is thought to help jacks identify females ready to be bred from far away [31,32]. Mules show similar estrus signs with a more discreet clap mouth (IFC personal observations). Close to ovulation, the estrus signs will intensify, the ovarian follicle(s) stop(s) growing, become(s) irregular and softer [20,25]. Occurrence of multiple ovulations is extremely variable (0-50%) across breeds [16,18,33,34], with the highest incidence in the Chinese Black donkey and Spanish and Portuguese breeds [13,25,34] (Table 2). In mules, the incidence of double ovulation was reported as 33.33% (15/45 cycles) in one study [15].

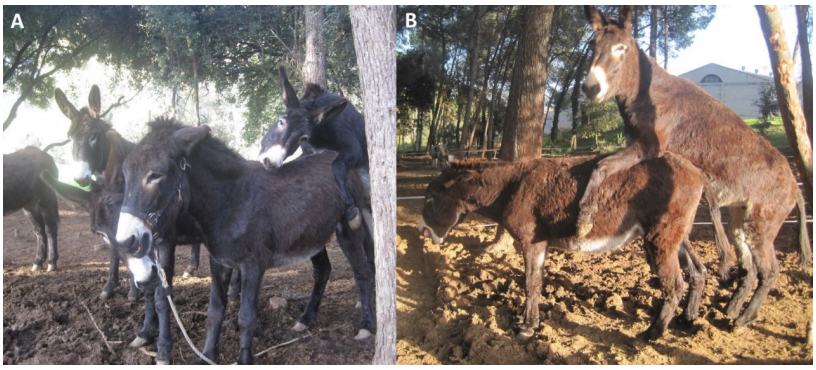


Figure 4. (A) Group of estrus jennies interacting in a sexually active group. One female is being mounted by an estrus jenny and teased by a third jenny. (B) Estrus jenny clap-mouthing and widening the pelvic limbs apart while being mounted by another estrus jenny.

182 Table 1. Breed and regional variations in the donkey estrous cycle.

Breeds	Loca-tion	Estrous cycles (n)	Diestrus (d)	Estrus (d)	IOI (d)	Ovulation till end of estrus (d)
Mammoth [17]	USA	19	19 ± 0.6	6 ± 0.6	25 ± 0.7	
Pega [7]	Brazil	13	18 ± 2.3	8 ± 2.5	26 ± 2.7	
Pega [31]	Brazil	21		6 ± 2.1		0.7 ±0.7
Jegue norderstino [21]	Brazil	13	18 ± 2.0	6 ± 2.2	24 ± 3.2	
Mammoth [18]	USA	33	17 ± 2.6	6 ± 2.1	23 ± 2.6	
Amiata [35]	Italy	4	15 ±2.0	8.5 ± 1.5	24 ±1.8	2.2 ±0.8
Catalonian donkey [10]	Spain	10	20 ± 0.4	5.6 ± 0.2	25 ± 0.3	
Andalusian, Zamorano-Leones, Catalonian [34]	Spain	58		5.5 ±2.1	24 ± 3.5	
Ethiopian cross [36]	Ethiopia	9			24 ± 7.4	
Martina-Franca [20]	Italy	12	17 ± 0.6	6.5 ± 0.6	23 ± 0.8	0.7
Burro Mexicano [16]	Mexico	27			21-24 ± 0.2*	

183 I.O.I. Inter-ovulatory interval IOI)

Table 2. Variation in follicular diameter at ovulation by donkey breed.

Breeds	Location	Estrous cycles (n)	Ovulatory diameter (mm)		Daily follicular growth (mm)
			Mean ± SD	Ranges	
Catalonian donkey [37]	Spain	36	44.9±0.5	35-60	4
Martina-Franca [27]	Italy	20	42.9±2.97		
Martina-Franca [20]	Italy	120	43.7 ± 0.13		2.8
Poitou [38]	France		36.2±3.0		2.0
Poitou [39]	India	9	41.1±1.0		2.7
Mammoth [24]	USA	19	36	30-40	
Pega and crosses [21]	Brazil	20	36.7±3.6	28.5-46	
Miranda [ref 7][40]	Portugal	33	38.40±0.68	30.29–47.86	3.18
Burro Mexicano [16]	Mexico	27	36.9±0.7		2.0

While the reproductive tract of jennies is proportionally larger than mares and mules, assessment of the reproductive tract is challenging in small frame animals. The use of lidocaine mixed with palpation lubricant (20 mL 2% lidocaine in 80 mL carboxymethylcellulose) alone or in a combination with N-butylscopolammonium bromide (10-20 mg/animal, IV) may aid in transrectal examinations of jennies.

The ultrasonographic appearance of the reproductive tract of donkeys resembles the mare and mule, with the exception that uterine edema is less pronounced in donkeys than in mares [15,22]. While ovarian structures (i.e., stroma, antral follicles, corpus luteum, corpus hemorrhagicum, and regressing corpus luteum) are similar in equids, the donkey corpus luteum often has a horizontal hyperechoic band or circular spot [22,41,42]. In some unusual cases, a central hypoechoic lacuna can also be seen [22]. During spontaneous and induced luteolysis, there is a transient increase in the blood flow to the corpus luteum which is associated with a concomitant reduction in progesterone concentrations [41,42].

Functional and reproductive anatomy and physiology of donkey jacks

The jack penis (root, shaft, and glans) and prepuce are anatomically similar to stallions; however, the donkey's penis is longer (Figure 5A), and two nipples can be seen on each side of the sheath (Figure 5B). When erect, the penis doubles in length and the glans increases up to four times in size [30]. Upon penile exposure, the prepuce's internal lamina forms a structure identifiable externally as "preputial ring" although the ring is less pronounced than stallions. While the gross anatomy of the scrotum is similar for all equids, jacks have a very pendulous scrotum, which allows for the determination of scrotum circumference similar to ruminants, although this assessment is not used in clinical practice. The scrotum's skin is soft and covered in sweat glands which plays a role in testicular thermal regulation.

Equids have ovoid testicles, with stallions having smaller testicles that are narrowed laterally, while the jacks' testicles are larger and more globular shaped (Figure 6). Mules have very small testicles with a shape intermediate to the stallion and jack. In the normal donkey, the testicles should be freely movable within the scrotum and may have a horizontal orientation or a slight inclination cranio-dorsally. The combined testicular volume in mature donkeys varies from 250 - 500 cm^3 [43,44]. Testicular parenchyma consists of the seminiferous tubules where spermatogenesis takes place, and the interstitium, constituted primarily of Leydig cells responsible for the production of androgens [45]. Each spermatogenic cycle lasts 10.5 days, and spermatogenesis duration is estimated to be 47.5 days [45].

High spermatogenic efficiency and a relatively short length of spermatogenesis, coupled with large testicles make the jack the most efficient domestic mammal for sperm production [45]. This demands that donkeys have very large epididymides for sperm maturation and storage. The epididymal head is adhered to the cranial testicular pole, while the body is located dorsal-medially

to the testicle. The epididymal tail can be seen bulging from the caudal view of the scrotum (Figure 5C). This anatomical feature can help in differentiating testicular rotation (≤180º, non-pathological, no compromise of the blood flow) from testicular torsion (>180º, pathological, compromised blood flow) (Figure 5E). Despite the anecdotal suggestion that testicular rotation predisposes to testicular torsion, this condition is rare in jacks.

The spermatic cord extends from the abdominal inguinal ring to the testis. It serves as a passageway for the deferent duct, nerves and blood vessels. The cremaster muscle runs laterally alongside the cord. The testicular artery is a very large vessel winding in association with testicular veins to form the pampiniform plexus (Figure 6), which is responsible for maintaining the testis 4-5ºC cooler than body temperature. Donkeys have very prominent spermatic cord (23-28 mm of diameter) [43], with unique histological features (e.g., capsule rich in muscular tissue and veins with a thick muscular layer) which presumably facilitates the venous blood flow from the testis and consequently better testicular efficiency [46]

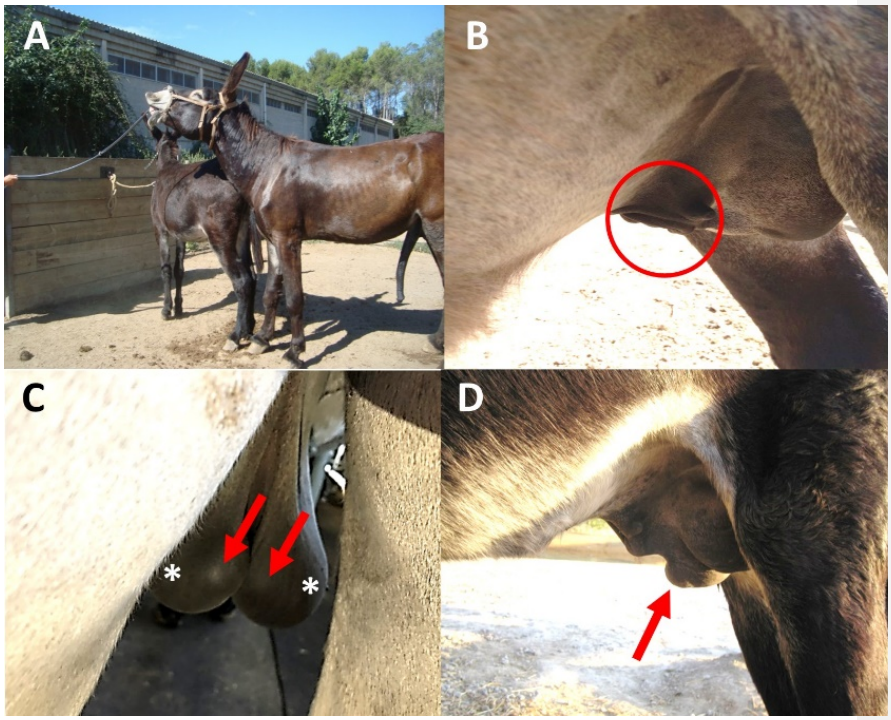
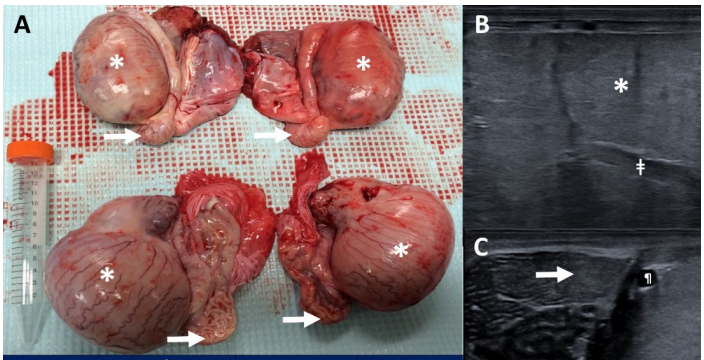


Figure 5. The external genitalia of donkey jacks. (A) Lateral view of a Catalonian donkey with a fully erect penis. (B) Lateral view of the prepuce and scrotum from a mature jack. The circled area shows a prominent nipple; (C) Caudal view of the scrotum from a mature jack, note the typical pendulous scrotum with large testicles (*) and prominent tail of the epididymides (➡); (D) Lateral view of the scrotum from a donkey presenting unilateral rotation of the right testis, the arrow points to the epididymal tail pointing cranially rather than caudally.



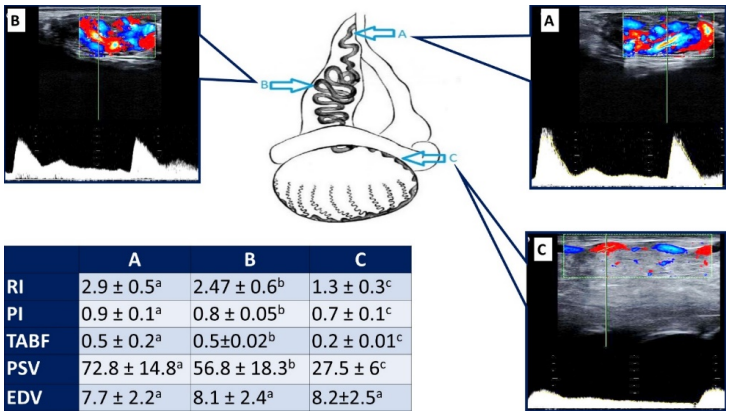
	Catalan	Ragusano	Pega
Length (cm)	10-12	8-11	8.4-11
Height (cm)	7.5-8.5	5.8-8	6.5-8
Width (cm)	6.5-8	5.1-6.5	6.5-7.5

Figure 6. (A) Gross appearance of testicles and epididymides of a 2-yr-old stallion (top-row) and a 2-yr-old donkey (bottom row) immediately after castration (B) B-mode ultrasonography (lateral view) of the testicular parenchyma (*). A very prominent central testicular vein can be seen in this image (‡); (C) B-mode ultrasonography (lateral view) of the scrotum content showing the testicular parenchyma (*), the testicular artery (¶) and epididymal tail (→). The table displays mean testicular measurements for Catalan, Ragusano [44]and Pega [43] jacks.

The ultrasonographic appearance of the jack scrotum content is similar to the stallion. The testicular parenchyma has an echogenic granular homogeneous appearance (Figures 5BC), and a small amount (<5mm) of anechoic fluid can be seen in the vaginal cavity as a normal finding. The central vein, an anechoic duct crossing the middle of the testis, is visibly larger than in the stallion (Figure 5B). Interestingly, the donkey epididymal tail has a heterogeneous ultrasonographic appearance because of the convoluted epididymal duct (Figure 5C).

Pulse Doppler ultrasound is suggested to be a useful tool to diagnosis chronic testicular problems in stallions [47], and while this technology would likely be effective, it has not been used

for this purpose in donkeys. A recent study assessed the testicular artery pampiniform plexus proximally, and the supra-testicular along with the peripheral testicular artery in normal donkeys concluded that donkeys have higher testicular artery flow than horses, and that pulsatility index was negatively correlated with sperm numbers and sperm velocity [48] (Figure 7). It remains to be determined if these findings are useful in clinical practice.



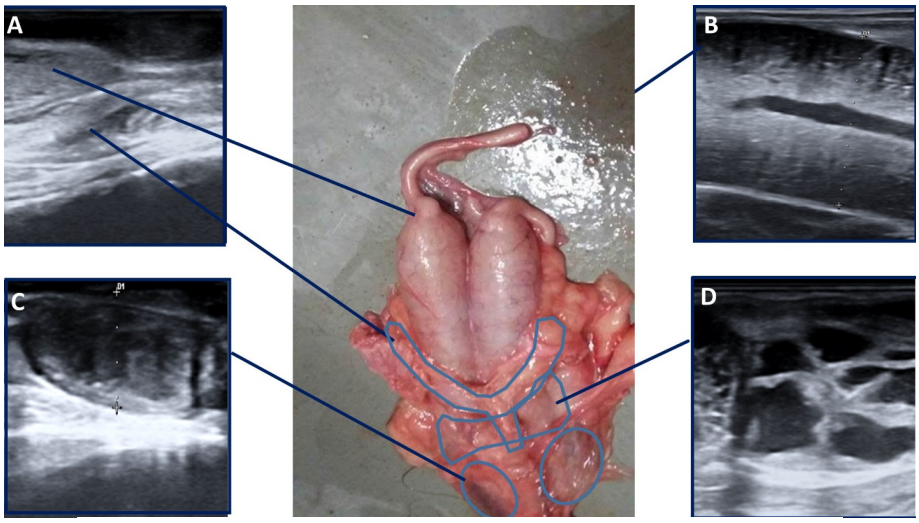
	A	B	C
RI	2.9 ± 0.5^{a}	2.47 ± 0.6^{b}	1.3 ± 0.3^{c}
PI	0.9 ± 0.1^{a}	0.8 ± 0.05^{b}	0.7 ± 0.1^{c}
TABF	0.5 ± 0.2^{a}	0.5±0.02^{b}	0.2 ± 0.01^{c}
PSV	72.8 ± 14.8^{a}	56.8 ± 18.3^{b}	27.5 ± 6^{c}
EDV	7.7 ± 2.2^{a}	8.1 ± 2.4^{a}	8.2±2.5^{a}

Figure 7. Blood flow in donkey testicles (n=6). (A) Pampiniform plexus proximal; (B) Pampiniform plexus supra-testicular; (C) testis periphery. Acronyms RI: resistive index; PI: pulsatility index; TABF: total arterial blood flow; PSV: peak systolic velocity (cm/s); EDV: end diastolic velocity (cm/s). Different superscripts denote statistical differences. The data was adapted from a previous publication [48].

The ductus deferens are muscular tubes connecting the tail of the epididymides to the urethra. At the scrotum level, the ductus deferens is easily palpable through the skin. The end of the duct entering the pelvic cavity, the ampulla, is more muscular and thicker in equids than other

species. The ampulla is remarkably larger in donkeys than horses (25-35 *vs.* 8-15mm, respectively), and the mucosa of the ampulla is more folded and glandular in donkeys[49] than stallions (Figure 8). The ampullae are directed caudally and ventrally to the vesicular glands and the prostate, and dorsal to the neck of the urinary bladder (Figure 8). Stallions are frequently affected with ampullary blockage; however, surprisingly there is only one report of ampullary blockage in a four-year-old jack. This jack was reported to respond to standard stallion treatment with oxytocin and transrectal massage coupled with frequent semen collections [50]

While all four accessory sex glands contribute to the seminal plasma in jacks, their specific contributions remain to be determined. Apart from the vesicular glands, all the other glands are larger in jacks than stallions[30,51]. In donkeys, these glands can be easily evaluated via transrectal palpation and ultrasound. The bulbourethral glands can be felt as a paired oval structure off the midline immediately after passing the entrance of the anus, and the parenchyma has heterogeneous echogenicity on ultrasound (Figure 8). Due to lumen collapse, small size, and proximity to the ampulla, the vesicular gland is the most challenging to appreciate on rectal palpation, but can be easily imaged via transrectal ultrasound, particularly after teasing. The prostate gland can be felt as a bulging structure at midline caudal to the ampulla; it has heterogeneous parenchyma as it has intermingled anechoic areas of glandular tissue even before sexual stimulation (Figure 8).



	BUG (L ×H)	PROST	VG	AMP
Jack	18.5 × 36.7mm	28.3mm	8.5 mm	31.9mm
Stallion	12.2 × 14.3mm	14.1mm	34.7mm	9.1mm

Figure 8. BUG: Bulbourethral gland; PROST: prostate height at center (mm); VG: vesicular gland diameter in mm; AMP: ampullae diameter (mm). Reference for the stallion [51].

Reproductive endocrinology is poorly studied in donkeys. One report out of Italy involving five Amiata donkeys described that the first appearance of sperm in the ejaculate occurred on average at 18.7 months of age [52]. Little is known about the seasonal variations in jack reproductive parameters. One study involving six small-frame donkeys in Brazil concluded that there were no apparent seasonal variation on subjective assessment of semen quality, other than pH, which was lower in the summer [53]. The authors' experience in the Midwest of the United States suggest that there is an increase in semen volume and sperm defects and a reduction in sperm motility in the spring and summer in comparison with the fall (IFC, REE unpublished observations). Studies describing the endocrinology of the subfertile jack are lacking at this point in time.

Semen collection and evaluation

Jacks can be collected similarly to stallions, however, a remarkably longer teasing time should be allowed [32,54]. If stallion standards are applied, this will generate incredible frustration on the practitioner. It should be expected that a semen collection from a jack can take up to 30- 60 minutes, with younger jacks being remarkably slower than mature jacks [54]. Jacks can be trained to collect on a dummy mount, off the back of a jenny, or off a mare (Figure 9). Ground collection can also be attempted in the presence of an estrus jenny or mare, and some jacks may allow multiple collections before losing interest. Consistently collecting semen from jacks can be one of the most daunting experiences under intensive programs. A previously published review provides in-depth information on managing jacks for semen collection and breeding [32,55].

In donkeys, chemical induction of ejaculation was unsuccessful in one study evaluating the use of oral imipramine (3 mg/kg) followed by xylazine (0.66 mg/kg, IV) two hours later, or a combination of a single dose of butorphanol (0.02 mg/kg, IV) and xylazine (0.33 mg/kg, IV). [56] A second study involving 55 donkeys evaluated various protocols combining oral imipramine (2 or 3 mg/kg, PO) followed by various doses of xylazine (0.44 mg/kg, 0.66 mg/kg or 0.7 mg/kg) one or two hours later and concluded that while 74.5% of the animals presented preputial relaxation and erection, and 44.6% were associated with masturbation, only one animal ejaculated after 38 minutes of xylazine injection [57]. Additional studies are needed to optimize dosages and protocols for donkeys.

Figure 9. (A) Mammoth jack collected with a Missouri artificial vagina mounting an estrus mare; (B) Catalan jack collected with a Hannover artificial vagina mounting an estrus jenny; (C) Mammoth jack fully erected having its penis washed before semen collection; (D) Jack collected with a Missouri artificial vagina while mounting the phantom.

The authors have used PGF2α as an ancillary method to promote erection if thorough teasing strategies[32] fail to stimulate a jack to mount and obtain semen. The authors typically use a single dose of dinoprost (2.5-5 mg/IM) or sodium cloprostenol (125-250 mcg/IM). Donkeys may show some side effects like sweating and leg trembling after PGF2α administration which does not appear to affect the donkey's balance, ability to expose the penis, achieve an erection, and then mount the female or the phantom. The authors are currently performing a controlled prospective

study to establish normal responses across breeds, but it seems that the benefits of PGF2α are typically seen within 10 minutes after injection.

Donkey jacks can be collected with any type of horse artificial vagina. The authors typically use the Missouri type and rigid types such as the Hannover, Colorado or Botucatu (Figures 9), and it does not appear that donkeys prefer one type versus another. Regardless of the type of artificial vagina, it should be lubricated with a non-spermicidal commercial lubricant (e.g., carboxymethylcellulosis based). Missouri, Hannover, and Botucatu artificial vaginas are typically loaded with warm water at 51-55ºC, whereas Colorado should be filled at 55-60ºC. Despite donkeys having relatively longer penis than stallions, most donkeys can be collected with artificial vagina of medium "regular" length (e.g., 45-50 cm, 18-22 inches). Miniature donkeys may need shorter artificial vaginas of ~30cm (13 inches). Similar to stallions, filters can be coupled with the collection of bottles, or semen filtration can be performed after collection, especially with animals producing large amounts of gel. A clean gauze can also be used to filter semen in donkeys producing excessive amounts of gel. Interestingly, the gel fraction appears to be present in a smaller proportion of donkeys than stallions [32,53,54]

Penis washing is a common practice in stallion reproduction as a method to decrease contaminants such as debris and bacteria in semen (Figure 9C). The authors strongly recommend that all jacks are washed before semen collection and natural cover. For shy donkeys that continually lose an erection when washing is attempted, we may elect to let the donkey mount the dummy mount or female and deviate the penis for washing while the donkey is still mounted and erect. For shy donkeys or while training, it is best to wash the penis with wet cotton and avoid the use of a cup or hose, as water splashing will make the donkey lose interest in the collection. Occasionally, despite washing before collection, semen may still look grossly contaminated with dirt (Figure

10A). Normal gross appearance of donkey semen varies from slightly grey (Figure 10B), to yellowish (Figure 10C) to whitish (Figure 10D), and the slightly yellow-tinged semen is a normal variation not to be confused with urospermia (Figure 10C). If the semen is heavily contaminated, the first sample should be discarded, the jack washed again, and collected in one or two hours if the donkey has a good libido, or the next day when possible.

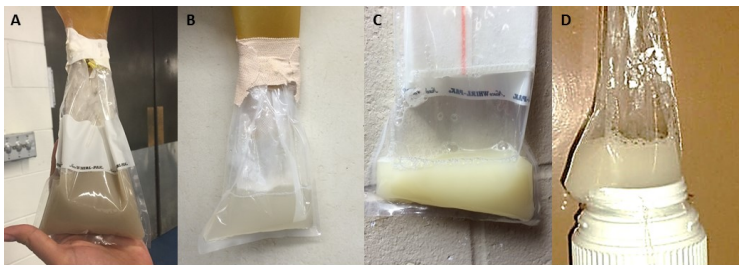


Figure 10. Gross appearance of donkey semen. (A) Grossly contaminated semen with dirt, despite extensive washing before collection in a sexually rested jack; (B-D) normal appearance of donkey semen.

Once the semen is collected, and the gel fraction is separated if present, the gel-free semen fraction can be processed similarly to stallions. Gel-free semen volume can be assessed by direct measurement, or more accurately by weighting the semen (1ml~1 g). Sperm concentration can be assessed with a hemocytometer, spectrophotometer, or Nucleocounter using horse settings. In donkeys, the sperm concentration and the total number of sperm cells ejaculated are remarkably higher than horses [54]. Younger donkeys tend to produce ejaculates with lower volume and higher concentrations than older jacks [54]. Sperm motility parameters can be subjectively assessed with a standard optical microscope or with a computer assisted-sperm analyzer, and donkey sperm motility param-

eters including velocity and progressive motility are also higher than stallions [58,59]. It is not uncommon for jacks to have ejaculates containing 300-400 million sperm/mL, with 80-90% of total and progressive sperm motility. Young donkeys tend to produce gel-free ejaculates of ~30-50 mL and older donkeys tend to produce gel-free ejaculates of ~60-90 mL. There is no evidence to indicate that donkey semen is more or less tolerant to cold shock to the stallion.

Sperm morphology is not typically assessed in donkeys throughout the breeding season, but as part of the pre-purchase examination and as part of infertility/subfertility evaluation [58]. Overall donkeys tend to have more morphologically normal sperm than stallions, and a review of various studies using different donkey breeds showed that most donkeys have ≤15% morphologic defects [58]. Sperm morphology can be performed with a wet-mount preparation with semen fixed in temperature matched buffered formalin, or dry-mount preparation where smears are stained with eosin-nigrosin, Karras, or Romanoswsky [58,60-62]. Additionally, a new system (Trumoph Proiser, Valencia, Spain) was recently introduced to evaluate sperm morphology. This system heats sperm to 41°C, the spermatozoa becomes immotile, and with gentle pressure sperm morphology assessed without staining with the use of a software (ISAS™) (Figure 11). While this software is not readily available to field practitioners, it has potential to become a popular tool in referral centers.

Sperm morphology has rarely been linked with infertility in donkeys [58], and one infertile mix-breed donkey was described to have olizoospermia, and asthenozoospermia with most sperm having proximal droplets had microtubules in disarray [60]. Currently, the authors' preference is to describe the sperm morphological defects according to the type and region of the sperm using wet-mount preparations and a differential interference contrast microscope; however, there are many ways to classify sperm morphologic abnormalities (e.g., primary and secondary defects, major and minor defects, compensatory and non-compensatory) [58,61]. Donkeys with poor sperm motility and

morphology can be processed with single-layer gradient centrifugation to select morphologically normal sperm with superior sperm kinematic parameters [62].

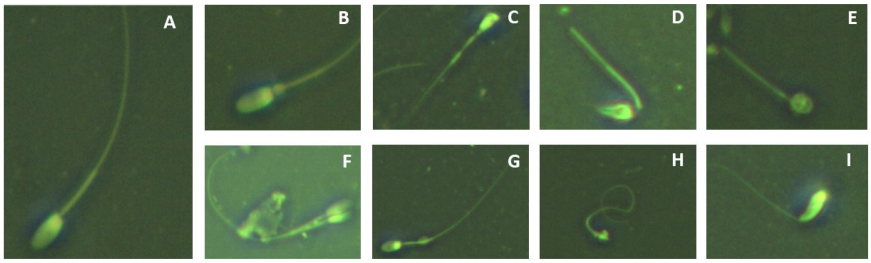


Figure 11. Donkey sperm morphology. (A) Normal; (B) Proximal protoplasmatic droplet; (C-E) Abnormal sperm heads; (F) Double sperm head; (G) Distal protoplasmatic droplet; (H and I) Malformed sperm heads.

Semen cooling and shipping

Donkey semen can be cooled and shipped in passive cooling semen containers such as the Equitainer, styrofoam boxes of various types, and other devices (e.g., Botubox, Botutainer). Equitainers and similar devices are preferred over styrofoam boxes, particularly in regions with extreme weather conditions, as they provide good insolation and proper cooling. Many equine extenders successfully preserve donkey semen at 4°C *in vitro* and for artificial insemination, including ultra-high temperature pasteurized skimmed milk [63], Kenney's extender [64,65], INRA82 alone [63,66], or with 2% of egg yolk added [66], INRA96 [66-68], Botusemen [69], Baken (3% egg yolk) and modified Baken extender (10% egg yolk) [65](Table 3). While milk-based extenders are most commonly used to cool stallion semen, donkeys benefit from the addition of egg yolk to these extenders for cooling to 4°C [65,66]. Removal of seminal plasma by centrifugation can increase the longevity of donkey semen upon cooled storage [70], however, adding 2% egg yolk to milk-based or to milk-protein based

418 extenders eliminates the need to centrifuge the semen. It has been thought that donkey seminal
419 plasma contains proteins that remove cholesterol from the plasma membrane and thus reduce
420 sperm longevity during cooling.

421 While the optimal extension practices have been well studied in stallions (i.e., 25-50 mil-
422 lion sperm/mL and at least one-part semen to three parts of extender), such studies are lacking for
423 donkey semen. Thus, until studies are performed the authors elect to use horse guidelines while
424 preparing donkey semen for shipment. While various donkey studies having used different breed-
425 ing doses and volumes (Table 3), it appears that better conception rates can be obtained when
426 jennies are bred with 1 billion progressive motile sperm rather than the 500 million progressively
427 motile sperm recommended for horses [69]. Artificial insemination of mares with fresh jack semen
428 results in conception rates varying from 40-80% [63,69,71]

429 Currently, the authors extend jack semen in INRA 96 containing 2% of egg yolk to a con-
430 centration of 25-50 million/mL, and ship jack semen in an Equitainer or commercial styrofoam
431 equine semen container. Alternatively, semen be extended (1:1 to 1:5 v:v) with a skim milk based
432 extender (e.g., Kenney or INRA 96), and then centrifuged with a traditional method (600 g x 15
433 min) or with cushion centrifugation (1000 g x 20 min with 1 mL of cushion solution) and then
434 supernant is discarded and the pellet is resuspended in the same extender (e.g., INRA 96 and Ken-
435 ney) at 50-100 million/mL. In addition, we recommend breeding mares or jennies with 2 billion
436 progressive motile sperm. It is also recommended to flush the mare or jenny uterus 6 hours post
437 breeding to maximize conception rate and treat with ecbolics to aid uterine evacuation [72]. Barren
438 animals or females with signs of endometritis (e.g., intrauterine fluid accumulation or infertility
439 despite optimal breeding management with fertile semen) require a detailed breeding soundness
440 evaluation and treatment if necessary.

Table 3. Pregnancy rates in jennies inseminated every 48 hours until ovulation with fresh or cooled donkey semen.

Semen type	Breeding dose (million/mL)	Vol. (mL)	Extenders	Fertility
Fresh [63]	400†	10	Skimmed milk	6/7 (86%)
Cooled [63]	200†	10		9/20 (45%)
	460†	4		7/9 (78%)
	460†	4	INRA82+ 2% egg yolk	5/8 (64%)
Fresh [73]	500*	Extended 1:2	INRA96	30/60 (50%)
Fresh[69]	500†	15		6/15 (40%
	1000†	15	Botusemen	11/15 (73%)
Fresh [68]	800*	15	INRA96	25/31 (81%)

†Denotes total sperm regardless of sperm motility; *Denotes progressive motile sperm.

Semen freezing

The protocols used to process donkey semen for freezing have been adapted from stallions. Centrifugation is deemed necessary to remove seminal plasma and to concentrate sperm before the freezing extender can be added. Egg-yolk based extenders such as the Lactose-EDTA, Saccharose-yolk, and most recently Botucrio have been most widely used to freeze to donkey semen [61,69,74]. All these extenders have 10% egg-yolk (Botucrio) or 20% for the other two extenders. Others have used milk-based or sodium caseinate milk-based extenders with some apparent success [63,75].

For the University of Illinois Urbana-Champaign's donkey semen freezing program for jacks (i.e., American Mammoths, Gaited Jacks and Spotted Jacks) used to breed mares, semen is collected and initially processed as described above. The gel-free semen fraction is extended (semen: extender) to 1:1 when raw concentration is ≤200 million sperm/mL, 1:3 when raw semen concentration is >200 and ≤ 300 million sperm/mL, or 1:5 when sperm concentration is above 500 million sperm/mL. The extenders used for centrifugation are either Kenney type extenders, INRA 96, or Botugold, or Equi-Pro Cool Guard. Raw semen is extended at 37°C (98.6F) and then allow

to cool down to room temperature at 20-23ºC(68-73.8F) Preference is given to lightly colored extenders (e.g., Botugold and Equi-Pro Cool Guard), thus allowing for easy identification of the sperm pellet. After extension, the semen is cushion-centrifuged (1 mL of cushion {iodixanol} in 50 mL conical tube 1000 g for 20 min). However, traditional centrifugation (i.e., 600 g for 15 min) can also be successfully used with expected sperm recovery of ~75% of sperm and cushion centrifugation has an expected recovery of ~95% sperm. Donkeys with poor post-thaw motility can have semen extended at 50 million/mL and then centrifuged at 600g or 1000 g (personal observations IFC). After centrifugation, the supernatant is discarded, the cushion solution is discarded, and the sperm pellet is resuspended in Botucrio. Sperm concentration is determined with a Nucleocounter and then adjusted to 200 million sperm/mL. The extended semen is automatically loaded and sealed in 0.5 mL straws. Straws are cooled at 5ºC for 20 minutes, before being placed 4-6 cm above liquid nitrogen for 15 minutes or inserted into an automated freezing machine. Since optimal cooling and freezing curves have not been critically assessed for donkey semen, the authors use horse cooling and freezing curves (i.e., 0.6-0.8ºC/min and -30ºC/min, respectively). Semen is thawed at 37ºC for 30-60 seconds, or 42ºC for 7 seconds followed by 20-30 seconds at 37ºC. A cooling curve of 0.25 ºC/min is successfully used by one of the authors at the Autonomous University of Barcelona (JM).

Endometrial culture, cytology, and biopsy in jennies

Endometrial culture, cytology, and biopsy can be performed as pre- and post-breeding screening tools, or as part of the workup following a pregnancy loss. Similarly to horses, endometrial culture in jennies should be performed in association with endometrial cytology. Cytological findings may rule out false-positive or -negative results or may indicate the presence and type of

microorganisms involved (e.g., morphology of bacteria, presence of yeast, or hyphae). Culture and cytology can be performed with cotton-tip double guarded swabs, with cytobrushes, by small volume uterine lavage, or by using endometrial biopsy for tissue-imprint culture and histological evaluation. As aforementioned, jennies have very narrowed, tortuous, and folded cervical lumen that can make the passage of gynecological instruments difficult, particularly in small frame jennies.

In mares, small volume uterine lavage is suggested to be the more representative of the endometrium [76]. A study in jennies comparing double guarded swab and small volume uterine lavage suggested that these techniques were equivalent [77]. Cytology of the healthy jenny endometrium collected during estrus has a large amount of debris and the occasional presence of inflammatory cells (Figure 12). After insemination, jennies have a physiological post-breeding inflammatory response marked by massive infiltration of neutrophils and eosinophils in the uterine lumen (Figure 12), particularly with frozen semen [75,77]. Presumably persistently high inflammatory cell counts in the uterine cytology are associated with endometritis, however, in jennies the cutoffs for physiological versus persistent (pathological) post-breeding inflammatory response has not been determined. In mares, the suggested cutoffs for physiological inflammation are 48-96 hours [72]. Interestingly, while eosinophils are rarely seen in endometrial cytology of mares, their presence is a common finding in jennies [77]. The role of eosinophils in jenny inflammatory response remains to be determined.

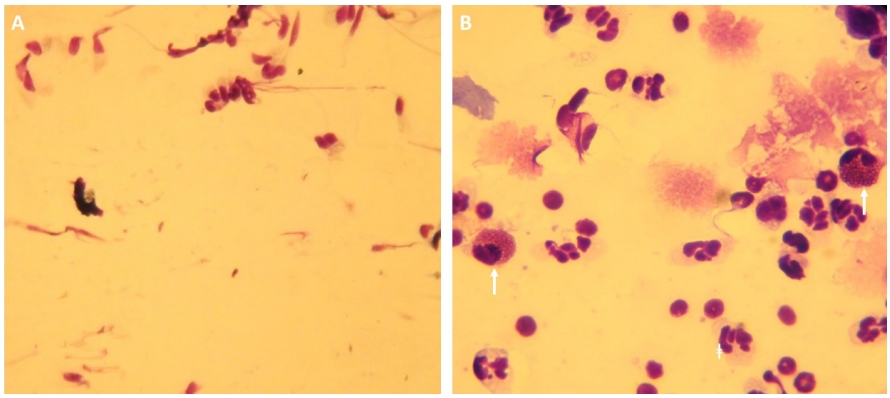


Figure 12. Endometrium cytology of a jenny in estrus before (A) and after artificial insemination (B). The arrows denote the eosinophils, and the symbol (ɬ) a neutrophil.

A recent study using 16S sequencing samples collected from the clitoral fossa, vestibule, vagina, and uterus of fertile and subfertile jennies showed that bacterial families were similar between the different segments sampled, however, fertile animals had few bacterial counts in the vagina and uterus, and subfertile animals had richer diversity and counts of *Enterobacteriaceae* in the uterus and vagina [78]. Additionally, subfertile animals also had anatomical abnormalities of the reproductive tract such as poor vulvar conformation and cervical damage.

Endometrium biopsy can be used to assess inflammation and degenerative changes (e.g., periglandular fibrosis). The same technique used in mares can be used in jennies, however, due to narrowed, tortuous and folded lumen, the passage of the biopsy forceps can be challenging particularly in small frame jennies in diestrus. A seminal work classified the mare's endometrium in four categories: I (normal), II-A (minor changes), II-B (moderate changes) and III (drastic changes) [79]. In jennies, there is a scarcity of literature on endometrial histological changes and

comprehensive studies involving the association between endometrial culture, cytology, biopsies, and fertility are lacking in jennies.

One study carried out with slaughterhouse specimens demonstrated that all jennies graded as category I had negative aerobic cultures, and jennies in the remaining categories had positive cultures with bacterial agents (Table 4) [80] mostly similar to mares[81]. As Enterobacteriaceae, the main bacterial group identified in the 16S sequencing study[78],was underrepresented and Staphylococcus species were overrepresented in the slaughterhouse study, it is possible that contamination happened during sampling. Staphylococcus is a rare cause of chronic endometritis in mares [81]. In mares, *Streptococcus zooepidemicus* the most common cause of endometritis[81], and was the second most prevalent group isolated from donkeys [80](Table 4). *Candida albicans* was isolated in jenny specimens categorized as III.

The healthy donkey endometrium normally appears to have more neutrophils and eosinophils and highly branched uterine glands [82] in comparison to mares. Since these features by default render a higher category on the Kenney & Doig's (1986) classification, it has been advocated for adjustments in the Kenney & Doig scale to better reflect jenny's endometrium [77](Table 5). Biopsies with many degenerative changes (19-26%) had a high percent of PMNs compared to healthy jennies in estrus (1.5%), but a lower % PMNs than jennies immediately after breeding (87.7%). Eosinophils increase during endometritis in jennies, and remain high in inflammatory and degenerative processes, but the role of eosinophils is unknown. [83]

Table 4. Aerobic culture of slaughterhouse specimens (n=110) according to the endometrial classification and frequency of isolated infectious agents [80].

Isolates		Endometrial categories vs. #isolates			
Types	n	I	IIA	IIB	III
Staphylococcus aureus	23	0	11	7	5
Coagulase-negative staphylococci	19	0	8	5	6
Streptococcus zooepidemicus	33	0	5	11	17
Non-hemolytic streptococci	4	0	3	1	-
Corynebacterium sp	20	0	7	5	8
Micrococus sp.	5	0	3	1	1
Proteus sp.	1	0	-	1	-
Escherichia coli	2	0	-	-	2
Candida albicans	3	0	-	1	2

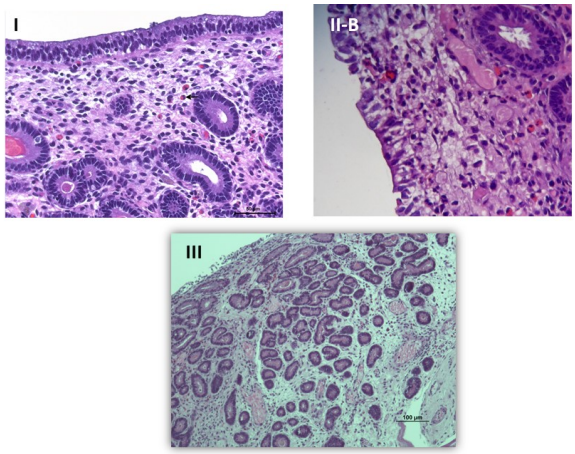


Figure 13. Endometrium biopsy in jennies.

Table 5. Kenney & Doig (1986) biopsy categories adapted to the histopathological particularities of the jenny [84].

Category	Description
I	Healthy endometrium

IIA	Inflammation (↑PMNs-including Eosinophils) ↓fibrosis. (<2 glandular nests). Normal stratum compactum
IIB	Moderate inflammation and fibrosis (2-4 glandular nests, ↓peri-glandular fibroblasts layers). Moderate endothelium changes. Reduced stratum compactum.
III	↑Fibrosis (>4 glandular nests, ↑peri-glandular fibroblasts layers). Significant endometrium damage. Absence or damaged stratum compactum.

In mares, endometrial degenerative changes are characterized by an increase in total collagen and collagen type 1 and a reduction in collagen type 3. In donkeys, such association does not appear to exist. In aged jennies with severely degenerated endometrium, the collagen remains under the basement membrane and surrounding endometrium glands [84]. The maintenance of collagen type 3 in aged jennies despite large numbers of PMN infiltrating the endometrium may explain why aged jennies are still able to conceive and carry pregnancies to term.

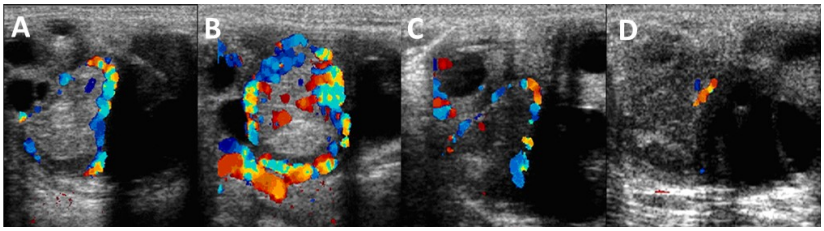
Hormonal manipulation of the estrous cycle

Prostaglandin F2alpha (PGF2α) and its analogs can be used to induce luteolysis and bring jennies or mules back into estrus. There are minor to no adverse reactions (e.g., colic-like, sweating, and loose manure) associated with dinoprost (5 mg), cloprostenol (0.075 mg), alphaprostol (3mg), or luprostiol (7.5 mg). Dinoprost should be reduced to 2.5 mg when administered to very small jennies, as side effects have been noted in these animals if larger doses are used. Most studies reported an average time from PGF2α administration to return to estrus of about four days (Table 6).

Since progesterone concentration is correlated with the luteal blood flow, color Doppler ultrasound can be used to assess luteolysis in response to PGF2α administration in jennies (Figure 14). However, controversy exists regarding the earliest time-point that jennies can respond to a

565 single PGF2α post-ovulation. Two studies reported that most jennies responded to cloprostenol
566 administration three days post-ovulation by returning to estrus in four to five days [41,85,86] (Table
567 6). Clinical work in practice appears to contradict these findings. In addition, in a preliminary
568 study, 1 out of 6 jennies responded to alphaprostol administration three days post-ovulation [87]. It
569 is worth noting that these studies involved small numbers of jennies and that different types of
570 PGF2α were used. To date, there are no studies assessing the interactions between doses of PGF2α
571 and time post-ovulation. In horses, the induction of luteolysis is not conducted before 5 days post-
572 ovulation, and all jennies responded to a standard horse dose of PGF2α administered 5 or 10 days
573 post-ovulation [41].

574

575 

576 Figure 14. Color-Doppler of a 10d-old donkey corpus luteum immediately before (A) and 1 h (B),
577 7 h (C) and 24 h (D) after administration of 5 mg of dinoprost.

578

579 Table 6. Induction of luteolysis in jennies using prostaglandin F2α analogs

Drug	Dose (IM)	Cycles (n)	Days after ovulation at PGF2α	Estrus	Interval to estrus (d) or P4 decline
Dinoprost [18]	5 mg	58	undefined	44/58 (76%)	4.4±1.6
Cloprostenol [85]	0.075 mg	10	3	10/10 (100%)	3.7 ± 1.2
		6	5	6/6 (100%)	3.5 ± 0.5

		5	7	5/5 (100%)	4.2 ± 1.3
		5	9	5/5 (100%)	4.6 ± 1.5
Cloprostenol [86]	0.075 mg	22	3	21/22 (96%)	2.9 ± 0.5
Luprostiol [88]	7.5 mg	169	Diestrus	NS	5 ca.
Alfaprostol [42]	3 mg	6	3	1/6 (17%)	2*
		6	6	6/6 (100%)	4*

NS Not specified. * Progesterone decline to <1 ng/mL.

As aforementioned, the duration of estrus is highly variable across jennies depending on time of the year, body condition score, and age, and thus induction of ovulation may be useful as a tool to narrow the ovulation window and facilitate breeding management or to prepare recipients for embryo transfer. Similarly to mares, human chorionic gonadotropin (hCG) and different types of GnRH agonists have been used to hasten ovulation in jennies [27,28,89] (Table 7). Jennies with periovulatory follicles (36-40 mm) receiving either hCG or lecirelin also ovulated additional smaller follicles (30-35mm) [27]. In another study, buserelin was administered to estrus jennies with periovulatory follicle averaging ~33 mm, and only a small percentage of jennies ovulated [28].

The ideal follicular size for induction of ovulation follows a trend with body size, with small frame jennies tending to ovulate small follicles (28-32 mm), and larger frame jennies like American Mammoth ovulating 40-44 mm follicles[18]. While endometrial edema in the presence of at least one periovulatory follicle is a common criterion to induce ovulation in mares, endometrial edema is less pronounced in jennies. If the practitioner waits for a similar endometrial edema pattern in jennies, ovulations and cycles can be missed. Thus, whenever possible, teasing can be vital to determining when a jenny should be induced and bred. If no jack is available, jennies will show estrus to other jennies, horses, or male mules. Practical experiences suggest that large donkeys should be induced with follicles ranging from 35-40 mm in the presence of mild endometrial edema and positive teasing. Small frame (i.e., small standard for the American classification) jennies will respond well under similar circumstances with follicles ranging from 32-35 mm. Very

small (between mini- and small standard) jennies will respond when follicles are 28-32 mm in diameter.

Table 7. Induction of ovulation in jennies using hCG and GnRH agonists.

Hormones	Number of cycles	Dose(route)e	Ovulation rates at 48h post induction	Induction-ovulation interval (hours)
hCG [27]	27	2500 IU (IV)	23/25 (92%)	42.4 ± 13.0
Lecirelin [27]	43	100 µg(IV)	29/43 (67.4%)	42.8 ± 14.0
Control [27]	66	Control	6/66 (9.1%)	Not described
Buserelin [28]	103	3.3-0.4 mg (SC)	72/103 (69.9%)	49.1 ± 25.9
Control [28]	14	Control	2/14 (14.3%)	83.6 ± 31.9
Deslorelin [89]		0.8 mg (IM)	100% (30/30)	37.7 ± 2.3

Estrus synchronization protocols

In jennies, estrus synchronization can be performed with PGF2α and its analogues alone [18,90], in combination with sex steroid hormones [18,91], and/or with GnRH [92] (Figure 15, Table 8). These synchronization protocols are ideal for situations in which the practitioner is unable to follow a jenny or group of jennies very closely with frequent palpations and ultrasounds. Synchronization can reduce the number of reproductive examinations before breeding. In addition, in a small frame jenny and a practitioner with large arms, it is in the best to reduce the frequency of palpation due to inherent risks for rectal tears.

The rationale for combining two injections of PGF2α is that jennies with a mature CL should respond to the first PGF2α, return to estrus, ovulate and by 16-17 days later have another CL that is mature and responsive to the second PGF2α injection (Figure 15, Table 8). If the jenny does not have a CL mature enough to respond to the first PGF2α injection, in 16-17 days she should have a CL that will be responsive to the second PGF2α, thus allowing her to return to estrus (Figure 15, Table 8). Further modification of this protocol would be to administer GnRH seven

618 days after the first PGF2α to ensure ovulation, and then to administer a second PGF2α injection 7
619 days later to induce luteolysis [92] (Figure 15, Table 8).

620 The combination of progesterone (injectable or intravaginal releasing devices, e.g., CIDR
621 and PRID) and PGF2α attempts to mimic a luteal phase followed by luteolysis; this assumes that
622 progesterone has a suppressive effect on LH secretions in donkeys as it does in cows (Figure 15,
623 Table 8). In mares, progesterone alone does not inhibit LH and follicular growth, thus, in mares
624 progesterone is frequently used in combination with estrogen and administered for 10 days to sup-
625 press follicular growth and provide better synchrony. This protocol has been tried in jennies, but
626 resulted in apparent inferior results to double PGF2α injections 16 days apart [18]. It is thus uncertain
627 how the sex steroid hormones affect gonadotropin secretions and subsequent follicular develop-
628 ment in jennies. One study obtained satisfactory estrus synchronization with PRID, however, there
629 presence of vaginitis and intrauterine fluid accumulation resulted in poor fertility [93]

630 Administration of PGF2α, followed by an injection of GnRH two days later, and a second
631 PGF2α 7 days after GnRH presumes that the first injection of PGF2α will bring the jenny back
632 into estrus, and then the jenny will presumably have a periovulatory follicle seven days later which
633 will respond to the GnRH by ovulating in two days. Thereafter, the second injection of PGF2α
634 would then bring the jenny back into estrus as the presumed CL would be about 5 days old, and
635 thus able to respond to PGF2α [92] (Figure 15, Table 8). Random administration of GnRH followed
636 by a dose of PGF2α, presumes that the jenny would have a follicle in the ovaries able to respond
637 to the GnRH, regardless, if there is a CL present, and then the injection of PGF2α 7 days later,
638 would induce luteolysis in the jenny if she ovulated after GnRH administration or if she already
639 had a CL at the onset of the protocol [92].

Table 8. Summary of estrus synchronization protocols in jennies

Protocol	Days to estrus	Induction of ovulation	Jennies in estrus (%)	Pregnancy rate (%)
PGF2α + PGF2α 16ds apart [18]	4.5 ± 0.9	NP-	10/10 (100%)	-
P4+E2 daily x 10 days & PGF2α at day 10 [18]	9.0 ± 0.9	NP	8/11 (73%)	-
PGF2α + PGF2α 17d apart [90]	5	-	56/81 (69%)	-
PGF2α: day 0, 1, 17, 18 [90]	5	Ø≥30 mm*	21/74 (28%)	-
Intravaginal P_4 device for 10 days and PGF at day 8 [91]	12-16	Ø≥30 mm**	31/40 (77%)	-
PGF2α (day 0) + PGF2α (day 14) [92]	21	No CL and Ø≥28 mm**	6/9 (67%)	1/6 (17%)
PGF2α (day 0) + GnRH (day 7) + PGF2α (day 14) [92]			8/9 (89%)	5/8 (62%)
GnRH (day 0) +PGF2α (day 8) [92]			5/9 (55%)	3/5 (60%)

*Denotes daily reproductive ultrasound after the last PGF2α; ** Denotes daily reproductive ultrasound starting two days after intravaginal progesterone-releasing device removal; ***Denotes administration of buserelin acetate (0.4 mg/jenny, SC), seven days the second PGF2α injection and then AI with fresh extended semen.

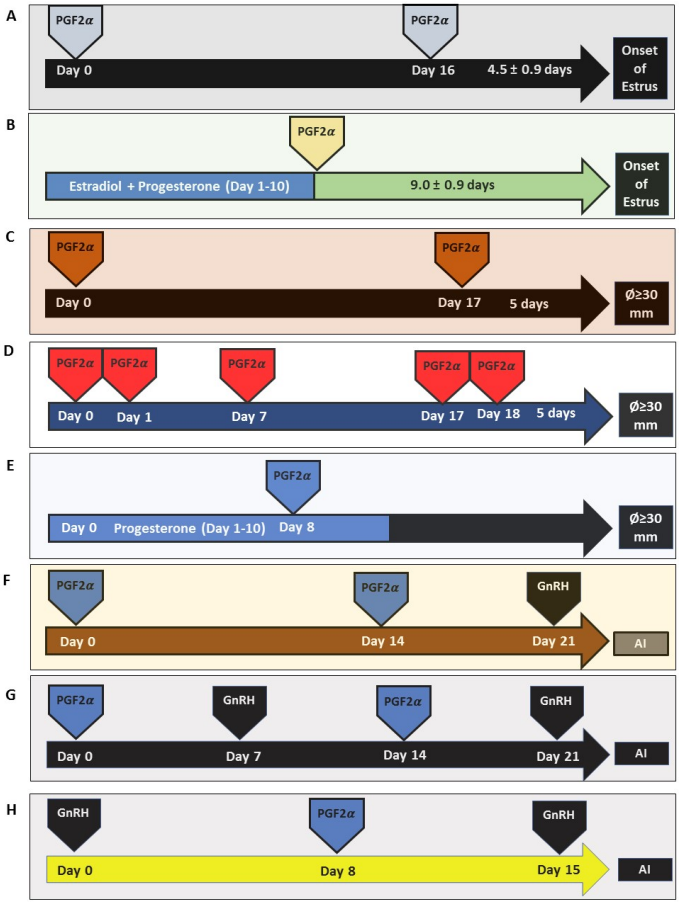


Figure 15. Estrous synchronization protocols for jennies. (A) Double PGF2α 16 days apart [18]; (B) Progesterone, estradiol- PGF2α [18]; (C) Double PGF2α 17 days apart [18]; (D) Multiple PGF2α administrations [90]; (E) Progesterone-PGF2α [90]; (F) Double PGF2α-GnRH timed artificial insemination (AI) [92]; (G) PGF2α-GnRH-PGF2α-GnRH [92]; (H) GnRH-PGF2α-GnRH [92].

652 Natural cover

653 Despite the popularity of artificial insemination, natural cover is still frequently used by
654 the donkey industry to breed mares and jennies with jacks. Pasture breeding of mares with jacks
655 is often unsuccessful. While jacks can identify all mares in estrus, most mares are not receptive to
656 jacks, particularly if they have not been previously exposed to them [32,55,94]. Mares mated by don-
657 keys tend to increase the interest and tolerance to jacks when they are in heat. Additionally, some
658 jacks may completely lose interest in mares after repeatedly being repelled or kicked by mares[55].
659 While breeding jennies to stallions to make hinnies is less commonly done, stallions are less inter-
660 ested in mating to jennies than jacks are interested in mares. Additionally the fertility in jennies
661 bred with stallion semen is very low.

662 In-hand-mating is the most suitable approach to breed mares with jacks. The mare can be
663 physically restrained with a twitch, breeding hobbles, or restraining breeding stocks [32,54,55]. Chem-
664 ical restraint with alpha-2-agonists and opioids may also be used in combination with physical
665 restraint to ensure safety of the jack and personnel involved. Regardless of restraint, the risks for
666 accidents like the mare kicking the donkey's genitals or laying down during mounting is still of
667 concern. Mares in-hand mated by donkeys typically have good fertility and may benefit from
668 standard pre-breeding and post-breeding management with ecbolics (e.g., oxytocin) and uterine
669 lavage with crystalloids starting 6 h post-breeding to reduce endometrium inflammation [72]. In the
670 United States, it is not uncommon for veterinarians to perform pre- and post-breeding management
671 of mares mated by donkeys managed by owners.

672 During pasture breeding, donkeys are non-harem territorial breeders, which is the opposite
673 of stallions [1,32]. The jack delimitates an area in the paddock, typically close to water source and
674 shade and food [32]. Jennies attracted to the jack's territory in pursuit for these essential elements

675 are mated by the jack [32]. As estrus jennies are receptive to jacks, and less violent than mares,
676 breeding accidents are extremely rare. Estrus jennies require minimal physical restraint during in-
677 hand mating, or while being used as a mount during jack semen collection. Interestingly, jacks
678 used to breed mares exclusively may refuse to breed jennies and may require training to breed
679 jennies.

680

681 Artificial insemination

682 Artificial insemination of jennies and mares with raw donkey semen can result in satisfac-
683 tory fertility if the semen is obtained cleanly and is used immediately after collection. It is advisa-
684 ble to extend the semen to have the benefits of extenders (e.g., antibiotics and protection against
685 cold shock). In comparison to stallions, jacks typically have excellent semen quality. For instance,
686 motility of fresh extended semen kept at room temperature (19-24°C) will only decrease ~10%
687 over 24 hours [64]. Semen cooled at 4°C for 48 hours can have satisfactory fertility for up to 48 hours
688 after insemination [64,95] However, by 72-96 hours, even cooled extended semen motility will de-
689 crease to ~10% [64].

690 Table 9. Pregnancy rates in jennies after insemination with fresh or cooled donkey semen. For all
691 studies included, artificial insemination was performed every 48 hours until ovulation.

Type	Breeding dose (million/sperm)	Volume (mL)	Extenders	Pregnancy rates (%)
Fresh [63]	400	10		6/7 (86%)
	200	10	Skimmed milk	9/20 (45%)
Cooled [63]	460	4		7/9 (78%)
	460	4	INRA82 + 2% egg yolk	5/8 (64%)
Fresh [96]	500*	Extended 1:2	INRA96	30/60 (50%)
Fresh [69]	500	15	Botusemen	6/15 (40%
	1 billion	15	Botusemen	11/15 (73%)
Fresh [68]	800*	15	INRA96	25/31 (81%)

692 *Progressive motile sperm.

693 Cryopreserved donkey semen has good post-thaw viability and motility [61,75,97]. Fro-
694 zen/thawed donkey spermatozoa can penetrate zona pellucida-free bovine oocytes matured *in vitro*
695 [98], and achieve satisfactory conception of ~50% when used to inseminate mares for mule produc-
696 tion [61,71]. However, if the same donkey semen is used to inseminate jennies, conception rates are
697 very poor, ranging from 0 to 28% [61,63,71,99,100]. Authors have attempted to mitigate the deleterious
698 effects of glycerol on donkey semen quality and jenny endometrial inflammatory response by re-
699 diluting the semen after thawing with extender containing no glycerol, or by adding other cryo-
700 protectants (glutamine, DMSO, dimethylformamide, and dimethylacetamide), however, concep-
701 tion rates remained poor in jennies even after these interventions [71,100].

702 While it is unknown why donkey frozen semen results in poor fertility when used to breed
703 jennies, it has been suggested that the acute endometrial inflammatory response after natural cover
704 or artificial insemination may be responsible for poor conception rates [75,77]. The post-breeding
705 inflammatory response is thought to be remarkably more pronounced in jennies than in the mare.
706 In both species, neutrophils are present in the endometrium by 6 hours post-breeding [72,75,77].
707 However, jennies always also have eosinophils in the uterine cytological smears. Post-breeding
708 histological evaluation of jenny endometrium showed diffuse infiltration of neutrophils in the lu-
709 minal epithelium and stratum compactum, along with eosinophils in the stratum compactum and
710 stratum spongiosum surrounding the endometrial glands [77]. In mares, eosinophils are released into
711 the endometrium in response to fungal endometritis, pneumo-uterus, or during anaphylactic re-
712 sponses, but are rarely seen post breeding [101,102]. The role of eosinophils in the jenny uterus, and
713 reasons for the jenny's pronounced post-breeding response to frozen semen remains unknown.

714 Dexamethasone is commonly used to modulate the post-breeding inflammatory response
715 of susceptible mares in combination with uterine lavage [72,103]. As corticoids can induce laminitis

and many jennies are insulin-resistant and susceptible to laminitis, clinical use of dexamethasone in jennies is discouraged. Ketoprofen, a non-steroidal selective COX-2 inhibitor, has also been administered to jennies in an attempt to modulate the endometrial inflammatory response with similarly poor results [77]. Despite a downregulation in COX-2, there was no decrease in PMN post-breeding in jennies treated with ketoprofen [77]. In contrast, in mares, ketoprofen, in combination with oxytocin, is reported to downregulate COX-2 and PMN infiltration [104]

Removal of seminal plasma by centrifugation results in superior donkey semen quality for cooling and is required for semen freezing [70]. However, donkey seminal plasma reduces the inflammatory response of PMN after insemination by suppressing the PMN-sperm bound attachment *in vitro*[70]. Similar numbers of PMN were seen in uterine lavage samples of jennies at 6 and 10 hours after insemination, and no difference was seen in PMN proportion when post-thaw semen was resuspended in seminal plasma for artificial insemination [75]. Post-thaw semen resuspension in seminal plasma, however, tended to increase pregnancy rates in comparison to the other groups where seminal plasma was not added post-thaw [75]. The presence of seminal plasma does not seem to affect PMN migration, but does decrease eosinophil numbers [105]. Seminal plasma appears to modulate the inflammatory response by inhibiting COX-2 gene expression in both the luminal epithelium and stratum compactum. Further exploring the mechanisms of the effects of seminal plasma on the endometrial inflammatory response in jennies might help improve fertility when frozen-thawed semen is used.

In summary, to maximize pregnancy rates it advised to perform deep horn inseminations with 1 billion total motile sperm [106], or to resuspend frozen-thawed semen in seminal plasma to a final volume of 10 mL at time of post thaw, and to flush the jenny's uterus by 6 hours after breeding [75]. Deep horn inseminations are thought to be advantageous for small breeding volumes of 2-4 mL.

Larger volumes should be deposited into the uterine body. Increasing the breeding dose perhaps saturates PMN and allow for more free sperm to enter the uterine tube and fertilize the oocyte. In addition, deep horn insemination may decrease the extent of PMN exposure *in utero*. However, these hypotheses have not been critically assessed.

Pregnancy physiology and diagnosis

Gestation length is approximately 12 months, with a range between 331-421 days [5,34,35]. As in all equids, the donkey placentation is diffuse, epitheliochorial, and non-invasive (Figure 16). However, the donkey chorioallantois has a higher concentration of microcotyledons per area when compared to the mare due to extensive branching of the villi [107]. This feature makes the donkey placenta more efficient and may partially explain reason why it is relatively more common for jennies to delivery live twins than mares (Figure 17).

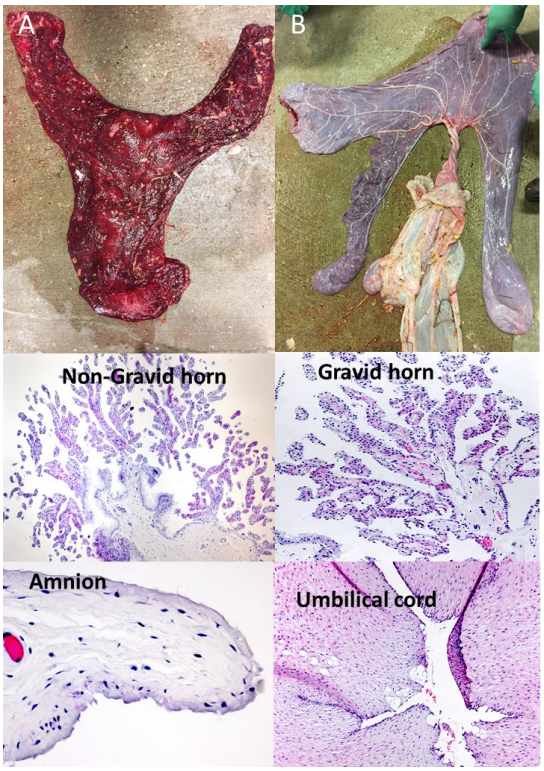


Figure 16. Donkey fetal membranes. A: Chorionic surface. B: Allantoic surface of the chorioallantois.

Pregnancy diagnosis may be possible by transrectal ultrasonography starting the 9th day after ovulation, although the chance of detecting a pregnancy so early is just 9-33%. In clinical practice is advised to perform the first pregnancy diagnosis by 12-15 days after ovulation. Embryonic vesicle diameters on Day 10, 11 and 12 were measured as 3-4 mm, 2.5-7 mm, and 3-7 mm, respectively [108,109], and may be missed by the busy practitioner. Maternal recognition of pregnancy in donkeys seems to be similar to horses, with the embryonic vesicle mobile in the uterine lumen

until day 16 post-ovulation, at which point the embryonic vesicle fixes itself at the base of an uterine horn [109-111].

The embryonic vesicle starts losing its spherical shape around the 16-18th day, and the embryo proper appears at the ventral pole of the vesicle around the 19-21st day (Table 10). Transrectal ultrasound for the first day of detection, diameter at first detection, and embryo features are summarized in Table 10 for eight jenny conceptuses from pregnancy diagnosis until 110th day of pregnancy.

Figure 18. Live twins without any complications in a Chinese Black jenny (A) and a Catalonian jenny (B).

Table 10. First day of detection and diameter at first detection in eight jenny conceptuses. Adapted from: [108,111-113].

Features	Days of gestation (range)	Diameter at first detection(mm)
Detection of embryonic vesicle	10-14	4-8
Fixation of embryonic vesicle	13-21	16-29
Loss of spherical shape	17-23	26-31
Detection of embryo	18-24	3.5-5.5
Detection of heartbeat	20-26	
Detection of allantoic sac	19-28	
Detection of umbilical chord	31-47	

Embryo at dorsal pole	21-44	
Beginning descent of the embryo	31-42	
Concluding descent of fetus	35-53	
Detection of chest	54-64	14-20
Detection of stomach	60-71	
Detection of eyeball	71-96	5.5-8
Detection of aorta	79-109	2.7-4.3

The embryonic vesicle grows approximately 3 mm/day from Day 10 to Day 18, then decreases to 0.5-0.7 mm/day between Days 17 and 31, and thereafter increases again to a rate of 1.6-2 mm/day up to Day 60 [108,109,111] (Figure 19). The increase in diameter of the embryonic vesicle is expected be to linear at 3.3 mm/day from Day 10 to Day 16, decreased to 0.7 mm/day between Days 17 and 31 and thereafter increased again to a rate of 2 mm/day up to Day 50. This appearance of a growth plateau between 20 and 30 days after ovulation was due to the developmental changes in the embryo itself, and in its extraembryonic membranes during this period. A similar growth pattern in the horse has been attributed to an increasing uterine tone providing resistance to expansion of the vesicle in the cross-sectional plane [114,115]. Crown-rump growth of the embryo/fetus seems linear from its first appearance (3.5-5 mm) to the 90th day (120 mm; last day when it was possible to be measured) [108,111]. Chest, eye and aorta diameters grow linearly until term, while the heart beat and umbilical arteries RI and PI linearly decrease [111,116] .

Fetal heart rate may have a tendency to increase the week before parturition [111]. Assessment of the caudal placental pole (e.g., edema, intracervical fluid accumulation and combined thickness of uterus and placenta {CTUP}) has been used to assess equine pregnancy for signs of ascending placentitis [117] (Figure 20). In the healthy pregnancy, the asinine CTUP seems to grow linearly from the from the sixth month of pregnancy until foaling, with a substantial increase from the ninth to the 12th month of pregnancy [118]. The reported CTUP for normal jennies varied in average 8 mm

from 6months of gestation to 12.6 mm by 12 months of gestation [118] (Table 11) Currently, parameters for assessment of high-risk donkey pregnancies are nonexistent.

Table 11. Monthly combined thickness of uterus and placenta in 17 Martina-Franca jennies.

Months	Mean	Ranges
6	8 ± 0.3	7.4-8
7	8.5 ± 0.3	7.9-8.9
8	8.9 ± 0.4	8.2-9.6
9	9.4 ± 0.4	8.8 -10.1
10	10.3 ± 0.4	9.7-11
11	11 ± 0.4	10.5 -11.9
12	11.7 ± 0.5	11-12.6

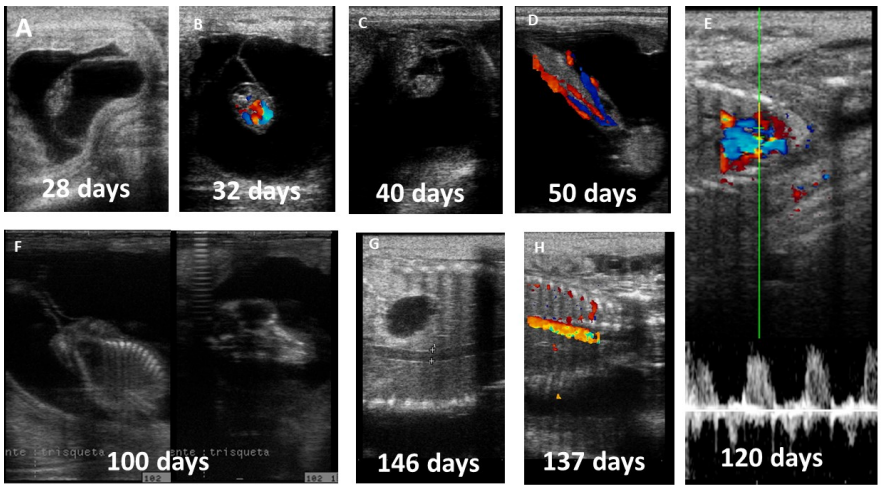


Figure 19. Transrectal ultrasonographic images of jennies.

Endocrinology of pregnancy:

In the jenny, plasma progesterone concentrations increase between days 0 and 10 (from 0.9 to 19.9 ng/mL [29], gradually decrease to day 30 (12-35 ng/mL [29,119]), increase between days 30 and 40, and then remain relatively constant (from 17 to 110 ng/mL [29,119]) until a gradual decline from days 110 to 160. Secretion of donkey chorionic gonadotrophin (dCG) begins around the 40th day of pregnancy [120], leading to secondary corpora lutea formation. From day 165 gestation until 15 days before parturition, mean progesterone concentrations range from approximately 4 to 7 ng/mL [29,111], with an increase a few days prior to parturition [29]. It remains to be determined if the progesterone results obtained during the second and third trimesters of pregnancy are truly progesterone or progestogens, as most of the immunoassays used to test progesterone concentrations cross-react with progestogens.

Plasma estradiol concentrations are higher than 100 ng/mL from day 90 of gestation until the month before parturition [29], and concentrations are over 1000 ng/mg between weeks 21 and 33 of pregnancy [111]. Estrogens are excreted in high concentrations in the urine, and the cuboni reaction, which is a fluorescent chemical reaction with estrogens in the urine, has been successfully used for pregnancy diagnosis in the jenny [121]. The reaction resulted in 83.87% true positives and 16.13% false negatives in pregnant jennies, and 86.96% true negatives and 13.04% false positives in non-pregnant jennies. While the barium chloride test has also been used for pregnancy diagnosis in jennies, it is less reliable, and is highly influenced by season [121].

Progesterone and estradiol assays have been used as ancillary diagnostic tools to assess pregnancy well-being in mares [117], however, in jennies such studies are lacking. Equine herpesvirus, donkey herpes virus, equine virus arteritis, Pseudomonas, Salmonella all have been described as cause of abortions in donkeys [14]. While jennies are affected by similar horse pathogens, the true incidence of pregnancy loss in this species is currently unknown. Equid herpesvirus type 7 (asinine

822 herpesvirus type) has been isolated in an abortion case in a mini donkey [122]. A large field study is
823 being conducted in commercial farms in China to elucidate the causes of pregnancy loss in don-
824 keys.

825 Breeding mares to jacks results in good fertility results. However, there is a higher rate of
826 early fetal loss in the first trimester of pregnancy when compared to mares bred by stallions. It has
827 been suggested that this is due to lower eCG production by mule pregnancies, thus decreasing the
828 formation of accessory and secondary corpora lutea and boost in progesterone. However, breeding
829 jennies to fertile stallions to produce hinnies result in a much lower fertility than the breeding
830 jennies to jacks or mares with jacks. It is unknown why there is such a discrepancy between these
831 types of pregnancies.

832 Embryonic vesicles (~5 mm) were first detected 8 days post-ovulation in 37% of mares
833 carrying mule pregnancies [123]. At ten days post-ovulation, 75% of embryonic vesicles could be
834 detected. Embryonic fixation happened 17 days post-ovulation, with a mean embryonic vesicle
835 diameter of 25 mm [123]. The mule embryo grows on average 4 mm/day from 11 to 16 days post-
836 ovulation, and ~0.4 mm from 16-28 days post-ovulation and 2 mm/day from days 28-45 post-
837 ovulation [123].

838 Interestingly, mules are generally infertile despite some being cyclic [15]. While most female
839 mules are infertile, there have been numerous cases in the literature of fertile mules producing and
840 delivering fertile offspring when bred to stallions or jacks, respectively resulting in donkeys or
841 horses [124]. In contrast, there have been no reports of fertile male mules.

842 Cyclic or acyclic mules can be used as excellent embryo recipients for donkey or horse
843 embryos [125]. The authors preferred protocol is to administer two doses of prostaglandin if the mule

844 is cycling, followed by three to four consecutive days of estradiol-17β, and then 1,500-3,000 of
845 long-acting progesterone. After the embryo transfer, the pregnancy can be maintained with weekly
846 doses of long-acting progesterone. Cyclic mules can be administered prostaglandin F 2alpha sim-
847 ilar to mares and jennies, and then have ovulation induced with hCG or GnRH agonists and then
848 be used as embryo recipients.

849 Mules carrying horse or donkey embryos will foal similar to mares. While their maternal
850 behavior has not been well-studied, they seem to display very affectionate maternal behavior to-
851 wards their foals and produce adequate milk. The placenta is grossly similar to mares and jennies.
852 In Brazil, dozens of mules are used as embryo recipients for horse embryos with satisfactory suc-
853 cess.

854 Parturition

855 Signs seen during first stage of labor may include walking, frequent defecation and urina-
856 tion, flank watching, and the Flehmen response. The first stage of foaling in jennies lasts between
857 20 and 135 minutes, and often goes undetected. The second stage (expulsion) starts with the allan-
858 tochorion rupture and allantoic fluid expulsion, ends with foal expulsion, and generally lasts be-
859 tween 10 and 30 minutes. If no signs of a fetus are seen within 20 minutes of initiation of stage
860 two labor, the jenny should be evaluated for a dystocia. Donkeys are affected by similar fetal and
861 maternal causes observed in horses including lateral deviation of head and neck (Figure 20), fetal
862 monsters, and fetal malpostures [14], however, the true incidence of the various causes of dystocias
863 in donkeys is lacking. The general principals used to manage horse dystocias are applicable to
864 donkeys. The authors have used assisted vaginal delivery, controlled-vaginal delivery, fetotomy
865 and c-section as methods to correct dystocia in donkeys.

Donkeys appear to be particular prone to developing necrotic vaginitis after prolonged dystocias. Small amounts of topical ointment such as Quadritop (Nystatin-neomycin sulfate, thiostrepton-triamcinolone acetonide ointment) applied to the cervix and vagina appears to be beneficial to prevent cervical and vaginal adhesions.

The jenny usually passes the placenta 10 and 175 minutes after the foal [126]. Jennies are susceptible to the retained fetal membranes-metritis-laminitis complex similar to mares. In jennies the condition can be potentiated by genes suffering with insulin resistance/metabolic syndrome. While retained fetal membranes are not as common in donkeys as in mares (10-50%), the true incidence in donkeys is unknown. When faced with retained fetal membranes in donkeys the authors use similar principles and techniques to those used in horses [127], including hydro-cannulation of the umbilical cord vessels (Figure 21) , the Burns technique, and repeated doses of oxytocin. Uterine lavage can be performed with large volume of tap water added of betadine and salt [127]. The inclusion of betadine appears beneficial to prevent and treat metritis in donkeys. Ecbolics such as oxytocin (5-10 units, q 4-6h, IM) and cloprostenol (125-250ug q 12h, IM) are also recommended as part of the treatment of retained placenta and prevention and treatment of metritis. Treatment should also include broad-spectrum antibiotics (e.g., penicillin and gentamicin with metronidazole, or sulfamethoxazole and trimethoprim) to control bacterial infection, and flunixin meglumine to prevent endotoxemia.

In the mare, mammary gland electrolytes and pH are used to predict imminent foaling [128]. In the days preceding parturition there is an increase concentration in calcium, magnesium, potassium, and reduction in sodium and chloride [128], whereas three mammary gland pH profiles have been identified in mares [128]. In the jenny, calcium concentrations in mammary secretions increased starting 10 days before foaling, and reaching 10.3 ±0.65 mmol/l the day before foaling [129]

Figure 20. Assisted parturition in a jenny. (A) Mammary gland development in pre-partum; (B) Swollen vulva immediately before delivery; (C-G) Stage II of parturition. (H and I) Maternal-foal bounding.

Figure 21. Dystocia and retained placenta.

Post-partum ovarian activity:

Complete uterine involution in the jenny has been described around the 20th day post-partum [39,68]. The first ovulation post-partum has been diagnosed as early as day 9, with an average of 13 ± 2.5 days after parturition [34,39,130]. In Spanish jennies, up to 45.6% of jennies are acyclic during the first 20 days postpartum, probably due to foal heat suppression by unspecified environmental factors and by the foal at foot [34]. Silent post-partum estrus have been also described in jennies [39]. It appears that silent postpartum estrus is more common during the fall-winter foalings than spring-summer foalings (personal observations JM). Similar to mares, the first post-partum estrus resulted in lower pregnancy rates (45-57%) compared with subsequent cycles (66-81%)[34]. While minimum parameters for foal-heat breeding have not been established for donkeys, the authors advise to follow horse guidelines, such that jennies should not be bred on foal-heat if: 1) fetal membranes were retained for more than 3 hours; 2) they present poor uterine involution by 7 days post-partum; 3) had dystocia, 4) have signs of metritis; 5) urovagina/urometra; 6) remarkable vaginal bruising in the vagina and vestibule; 7) ovulate before 10 days after foaling.

Assisted Reproductive Techniques

Embryo transfer

Initial results involving donkey embryo transfer were disappointing when compared with mare. Despite satisfactory recovery rates of 64% after insemination with fresh semen [33,131]. Pregnancy rates by 15 days after transfer were extremely low varying from 16.7% (3/18, with surgical transfer) [131] to 22.4% (13/58, non-surgical transfer) [33]

916 Studies exploring extra-specific pregnancies in the equid species by surgical transfers of
917 horse embryos to donkey recipients and *vice versa* demonstrated that 63% and 67% were pregnant
918 40 days after embryo transfer, which were significantly better than what was previously reported
919 for intraspecific ETs. However, it is worth noting that a remarkable pregnancy loss occurs when
920 donkey embryos are transferred to mares [132,133].

921 The embryo recovery and the ET techniques employed in the second study [33] were analo-
922 gous to common practice in the horse [134-136]. Non-surgical embryo flushing in donkeys is similar
923 to the current standard applied to horses. The jenny uterus can be flushed with 0.5 (maiden) to 1
924 L (broodmare) of flushing media (e.g., Lactated-Ringer's Solution, commercial proprietary fluids)
925 at least three times for maximum embryo recovery[33,73,96,135]. While Day 7 donkey embryos are
926 comparable to day 6 horse embryos in diameter, interestingly day 8 donkey embryos are compa-
927 rable in diameter to day 8 horse embryos [73,135]

928 One main difference in the transfer technique is that the vaginal part of the cervix was
929 grabbed with three fingers and pulled backward, the tip of the gun blindly inserted in the cervical
930 *os*, the sanitary sheath then broken, and the cervix manipulated to aid the transferring gun insertion
931 and progression[33]. This maneuver is not necessarily needed in the mare. In addition, it has been
932 suggested that administration of acepromazine (3.3 mg/100 kg/iv) to recipients, 5-10 minutes
933 before embryo transfer appears to aid on cervical relaxation [33]. Interesting while not statistically
934 significant, embryos recovered with Lactated Ringer's Solution and flushed on equine holding
935 media (EM Care Bodinco, The Netherlands) had pregnancy rates of 27.2% at 14 days, while em-
936 bryos recovered on Dulbecco's Phosphate Solution resulted in only the 7% of pregnancies after
937 embryo transfer.

938 Transcervical embryo transfer is associated with PGF2α release, which in turn may affect
939 luteal function marked by a slow or abrupt reduction in progesterone concentration and may affect
940 embryonic survival after transfer in large mammals. Transcervical manipulation of the cervix dur-
941 ing embryo transfer is of particular relevance in donkeys as the jenny has a longer, smaller, and
942 tighter cervix when compared to mares [3,4]. This prostaglandin release may be followed by a de-
943 crease in progesterone plasma concentration [137], and was hypothesized to be the cause of poor
944 pregnancy rates for jennies subjected to transcervical embryo transfer [33]. When four jennies were
945 submitted to a sham transcervical embryo transfer 5-8 days after ovulation, and PGF2α metabolite
946 13,14-dihydro-15-keto-PGF2α (PGFM) and progesterone plasma concentrations were evaluated,
947 cervical stimulation caused a transient PGF2α release in two of four jennies, but no significant
948 decrease in progesterone plasma concentration[96].

949 Concluding this "quest" investigating donkey embryo viability, embryos (d 8 horse, d 8
950 or d 9 donkey), were non-surgically transferred into synchronized recipients [96]. All five horse em-
951 bryos (5/5) transferred into donkey recipients resulted in pregnancies at 15 and 25 days. Fifty
952 percent of both the donkey-in-horse (3/6) and donkey-in- donkey (6/12) embryo transfers resulted
953 in pregnancies at 14 and 25 days, a higher pregnancy rate than previously reported after donkey-
954 in-donkey [33] and comparable to donkey-in-horse [133] and horse-in-horse embryo transfer [135]. These
955 results suggest that trans-cervical technique for embryo transfer was not the reason for the low
956 pregnancy rates previously described in donkey recipients [33,131] and that nonsurgical embryo trans-
957 fer in donkeys can produce acceptable results. These findings were confirmed by others[138] who
958 obtained a 45.4% pregnancy rate (5/11) transferring fresh 9-day-old donkey embryos to synchro-
959 nized Pega donkey recipients in Brazil.

960 Embryo cooling and shipment has been performed commercially with mule and donkey
961 embryos using similar horse technology (e.g., holding media and passive cooling device such as
962 Equitainer), however, to date control studies are lacking. Similarly, there are limited studies in-
963 volving donkey or mule embryo cryopreservation. To date similar approaches used by the horse
964 industry are adapted to donkeys and mules.

965 **Nuclear transfer**

966 The first equid to be cloned was the first of the only three mules ever cloned [139-142]. Clones
967 were created using fibroblasts of a 45 day old mule fetus and a mare's oocytes collected right
968 before or after ovulation by ovary excision, transvaginal ultrasound guided aspiration or oviduct
969 excision and flushing[139]. Oocytes were maturated in M-199 containing 10% FBS, 0.05 U/ml pFSH,
970 0.05 U/ml pLH, 10 U/ml penicillin and 10 mg/ml streptomycin. Oocytes were matured for 12
971 hours (collected prior to the ovulation) or 6 hours (recovered after oviductal flushes) prior to the
972 nuclear transfer. MII oocytes were denuded from the cumulus cells by hyaluronidase, the first
973 polar body and metaphase plate were aspirated by an enucleation pipette, and then a disaggregated
974 donor cell was aspirated and placed in the perivitelline space. Fusion of ooplast (enucleated oocyte)
975 and the donor cell (NT couples) was induced by a single 15 ms, 2.2 kV/cm dc pulse in a 3.5 mm
976 fusion chamber. Fusion medium was 3.5 M D-mannitol containing 0.5 mM HEPES, and 0.05%
977 fatty acid-free BSA. Given that extracellular concentration of calcium is higher in the horse than
978 in humans, fused NT couples were divided in four different activation media containing ionomy-
979 cin: Control (1X; n=142), 1X/3X (n=42), 1X/10X (n=30) and 3X/6X (n=120) (X= standard cal-
980 cium concentration) [139,140,142]. Of 334 manipulated oocytes, 305 were transferred to recipient
981 mares, resulting in 21 (6.9%) 14-day pregnancies. Only the embryos maintained and activated in

the 3X/6X medium established pregnancies (5/113, 4.4%), only 3 of which resulted in the birth of live mules [139,140,142].

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