

Sexual behavior and ejaculate characteristics in Pêga donkeys (*Equus asinus*) mounting estrous horse mares (*Equus caballus*)

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Abstract

The objectives were to (i) characterize sexual behavior of donkey stallions (jacks; *Equus asinus*) during on-farm semen collection using estrous horse mares (mares; *Equus caballus*); (ii) compare behavior of young (less experienced) versus older (more experienced) jacks; (iii) determine whether semen suitable for artificial insemination (AI) could be collected using mares; and (iv) determine the suitability of using mares in field collection of semen from jacks. Six Pêga jacks (3.5 to 16 yr old), previously conditioned to breed mares, were used. Mount mares were confirmed in estrus by a teaser horse stallion (stallion) and a jack. Semen was collected with an artificial vagina, at intervals of 48 to 72 h (180 collections). The mean \pm SD (young [3.5 yr] vs. old [14 to 16 yr]) were Flehmen response frequency, 7.4 ± 5.8 (8.1 ± 3.0 vs. 7.0 ± 2.0); number of mounts without erection, 1.1 ± 1.3 (2.1 ± 1.4 vs. 1.2 ± 0.4 , $P < 0.05$); latency from first exposure to mare to full erection on the ejaculatory mount, 18.3 ± 17.7 min (25.3 ± 21.3 vs. 12.2 ± 6.2 , $P < 0.05$); latency from erection to insertion, 5.1 ± 3.5 sec (5.3 ± 3.8 vs. 4.8 ± 3.2); and duration of copulation from insertion to dismount after ejaculation, 25.4 ± 7.8 sec (22.1 ± 2.9 vs. 28.1 ± 9.3). In all jacks, sexual behavior was generally normal, with the notable absence of open mouth behavior. Mare estrous behavior was markedly less intense than that in the presence of a stallion and usually absent. Semen characteristics were gel free volume, 47.3 ± 28.7 mL; gel volume, 71.8 ± 54.8 mL; total motility, $84.3 \pm 6.0\%$; progressive motility, $74.3 \pm 74.5\%$; sperm vigor, 3.9 ± 0.5 (scale 1 to 5); sperm concentration, 253×10^6 cells/mL; and total number of sperm, 10.3×10^9 cells. Copulation duration was significantly correlated with gel free volume ($r = 0.9$) and gel volume ($r = 0.7$). We concluded that (i) the sexual behavior of jacks during semen collection using mares was similar to that reported for natural mating to jennies, (ii) precopulatory and copulatory behavior for the young (less experienced) jacks and older (more experienced) jacks were generally similar (except number of mounts without erection and latency to full erection); (iii) semen obtained using mares as stimulus and mount females was similar to that reported with estrous jennies; and (iv) semen collection from previously conditioned jacks, using estrous mares, was appropriate for field collection of semen.

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1. Introduction

Donkeys (*Equus asinus*) are bred in many countries of the world. In some places, there is particular interest in using donkey stallions (jacks) for mule (*Equus mulus*)

production, for both in-hand natural mating and artificial insemination (AI) [1–3]. Mules are in demand in rural areas, due to their physical strength [4]; they are used for herding cattle, general agricultural activities, and pack animals. In Brazil, more than half of the cattle are herded using mules, and they are also used in western sports, dressage, and for pleasure. The Pêga donkey, a large (130 to 145 cm), saddle-type asinine, is a particularly valuable Brazilian breed. These donkeys have been selectively bred and improved for >200 yr [5] and are popular in many states for mule production [2,3,5]. Although this breed was previously considered endangered [5], it has gained substantial popularity in the past 5 to 10 yr.

Jacks are naturally reluctant to breed with horse mares (mares) and typically require training [3]. This conditioning of jacks to respond to mares is a vital aspect of mule production [4,6–8]. Consequently, there is a high demand for jacks conditioned to breed mares, especially large-breed jacks (e.g., Pêga), which can command prices four or five times that paid for other breeds [2]. The natural reluctance of jacks to breed mares is probably partially related to differences in estrous behavior of mares and donkey mares (jennies) [9,10]. For example, jennies play a more active role in mating than do mares [11,12]. Jennies frequently approach the jack, vocalize, mouth clap, and congregate into sexually active groups displaying intensive homosexual behavior [10,11]. Additionally, natural reproductive organization differs between donkeys and horses. In that regard, jacks are naturally territorial breeders [11–14] and guard a specific territory rather than specific females (either jennies [11] or mares [4]), whereas stallions are harem breeders [15,16]. Mating behavior also differs. Under natural mating conditions, the jack will approach the jenny as she enters his territory or be sought out by the jenny when she is in estrus. Once interest has been established, teasing the jenny by the jack then follows, including behaviors such as perineal and ground sniffing, open mouth, vocalization, Flehmen response, rolling, and so forth. The jack frequently mounts the female and may then retreat and remain at a distance from the jenny displaying apparent disinterest, during which time masturbation may occur and an erection is achieved. At this stage, the jack will return to the jenny and successfully copulate [11,14,17,18]. Differences between jacks and stallions regarding sexual behavior and interaction with the female may contribute to the reduced sexual interest and response typically shown by jacks for mares, and vice versa [4,12]. In mule production, this presents a particular challenge; there-

fore, several practical strategies of breeding management have been used to overcome these differences [12,13,18–20]. Although many of these management techniques were successful, it would be apparently easier to use AI.

Both AI and breeding soundness examination (BSE) require the collection of semen, which itself might present challenges as it interrupts natural sexual behavior [6,18,21,22]. Traditionally, jennies are used as stimulus and mount females for semen collection from jacks [6,18,21]. However, in practice, many mule stud farms do not keep jennies, as they are considered difficult to maintain due to their migratory behavior, especially during the breeding season [20]. Therefore, this necessitates the use of mares for semen collection and the conditioning of jacks to respond to mares. Apparently, there are only limited reports suggesting that estrous mares can be used with jacks for semen collection [7,8,23].

Because jacks are valuable animals, they are often used for breeding well into old age. Although jacks are reported to be reproductively active well beyond 20 yr of age, the effects of increasing age on their sexual behavior have not been reported. In stallions, age is known to affect libido and semen quality [24]; semen quality increases from puberty to maturity (5 to 6 yr) and then remains relatively constant until the late teen years, at which time semen quality and/or libido may decline [24,25]. However, this decline was not evident in all stallions, as several retained good semen quality, as well as high libido, well into old age [24,26]. There are apparently no corresponding studies in jacks.

Although donkey sexual behavior has been the subject of many studies [10–13,17,18,27], little work has been conducted on behavior during natural mating with mares [4], and apparently none on sexual behavior and semen quality when mares are used for semen collection. If mares are to be advocated for use as mount animals for semen collection on mule stud farms, it is essential to document the expected behavior of jacks and to determine whether ejaculate quality is appropriate for AI.

The objectives of this study were to (i) describe the sexual behavior characteristics of jacks during on-farm semen collection using estrous mares (*Equus caballus*); (ii) compare behavior of young (less experienced) versus old (more experienced) jacks in response to mares; (iii) determine whether semen collected using mares under field conditions met acceptable standards for AI; and (iv) speculate on the potential for the routine use of mares in semen collection from jacks on mule stud farms.

2. Materials and methods

2.1. Animals

Sexual behavior during semen collection using mares was measured in six Pêga donkey jacks from August 2006 to February 2007. The study was carried out under commercial conditions on a private stud farm producing mules and donkeys in Guacariaba, Minas Gerais, Brazil (20°45'20"S, 42°52'40"W). The jacks were 3.5 to 16 yr old, 230 to 330 kg body weight, and 132 to 144 cm in height. There were three older, more experienced breeders (D1, 16 yr; D2, 15 yr; and D3, 14 yr), and three young, less experienced breeders (D4, D5, and D6, all 3.5 yr). All had successfully been conditioned to breed mares by natural service and had been used for at least one breeding season. The young jacks had been kept from weaning to 2 yr of age only with horse fillies, with no contact with jennies. In the older group, D1 was conditioned to breed both mares and jennies, whereas D2 and D3 were conditioned to breed only mares and did not respond successfully to jennies.

Before the start of this study, each jack underwent a breeding soundness evaluation, based on the recommendations for breeding soundness of stallions, as reported by Kenney et al. [28] and the Brazilian College of Animal Reproduction [29]. All jacks met criteria for classification as Satisfactory Prospective Breeders. Furthermore, 2 mo before the start of this study, these jacks were familiarized with the procedures of semen collection and, in an attempt to standardize extragonadal sperm reserves, they all underwent a daily single semen collection or natural in-hand breeding of an estrous mare from 7 to 14 d before the start of the study (Day 0), followed by a 7-d period of sexual rest. The jacks were exposed to natural photoperiod and were maintained in individual stalls in male-only yard, with free access to water, trace mineralized salt, and hay (*Cynodon dactylon*). In addition, twice a day, they were offered 20 kg of freshly cut grass (*Pennisetum purpureum*). On a daily basis, jacks were turned out individually into a paddock for 2 to 3 h (during daylight). In the stalls, jacks had visual and auditory access, but no physical contact, to mares in paddocks 200 to 500 m away.

2.2. Semen collection

At the time of use, mares were confirmed to be in estrus (natural estrus, not hormonally induced) during teasing exposure to a stallion and by acceptance of the

approaches of a jack. Good acceptance of the jack by the mare was characterized by showing estrous signs in the presence of the jack and by not kicking or excessively moving when the jack teased or mounted her, as described by Canisso et al. [23]. If the mare resisted the approaches of the jack, an alternate mare that demonstrated good acceptance of the jack was used.

Semen collections were done in an isolated area of the stud, away from sight or sound of other donkeys (400 m). A Brazilian artificial vagina (AV; Botucatu model) filled with water at 51 °C was used by the same veterinarian throughout the study. The jack was allowed to interact with the mare, as for natural mating. As the jack mounted, the penis was deflected toward the AV. Three semen collections were taken per week at intervals of 48 to 72 h. A total of 180 semen collections were carried out, with 17 to 40 per jack (40, 17, 40, 28, 26, and 29 for D1 to D6, respectively), according to the demand for that jack's semen. During the study, jacks were not permitted to retreat away from the semen collection site, as is their natural tendency [11]. Mounting without an erection was permitted as a normal behavior and as an encouragement to achieve an erection.

2.3. Sexual behavior characteristics

For semen collection, two assistants were positioned 3 m from the mare, one on the right side to record the behavior of the jack, and the other on the left side to control the jack (via the halter rope). The following behaviors were recorded: (i) Flehmen response frequency (FLEH): the number of Flehmen responses in the presence of the estrous mare at each semen collection. (ii) Mounts without erection (MWE): the number of mounts without erection, during semen collection; as soon as the jack showed the intention of mounting the mare, the halter rope was released to allow as natural behavior as possible to be expressed. (iii) Erection latency (EL): the interval between the first exposure of the jack to the mare to achieving a complete erection for the ejaculatory mount. (iv) Latency from erection to insertion (LEI): the interval between complete erection and initial introduction of the penis into the AV; if during the erection, the jack did not mount the mare, the time was added into the EL and the timing for LEI restarted. (v) Copulation duration (COP): the interval from introduction of the penis into the AV to dismount and retraction of the penis from the AV. (vi) General clinical observations of

characteristic courtship behaviors of both jacks and mares were also recorded and described. The maximum time allowed per jack per semen collection procedure was 90 min. If semen had not been successfully collected within this interval, the jack was removed and another attempt made 1 h later. If this second attempt was unsuccessful after 90 min, another attempt was made the following day. The additional day for semen collection was only necessary in the adaptation stage (pre-experiment) for which data were not included, and predominately in young jacks.

2.4. Semen evaluation

All ejaculates were evaluated within the constraints of commercial field conditions to determine whether the minimum requirement for use with AI was met. Immediately after each collection, semen was filtered to remove the gel free fraction. Immediately after separation, aliquots (5.0 μ L) of raw semen were evaluated using phase-contrast light microscopy (magnification, \times 200) with a heated microscope stage (37 °C). Semen characteristics were evaluated and measured according to Kenney et al. [28] and the Brazilian College of Animal Reproduction [29] and included gel free volume, gel volume, total motility (0 to 100%, in increments of 10%); progressive motility (0 to 100%, in increments of 10%), sperm vigor (scale of 1 to 5), and sperm concentration (\times 10⁶ sperm/mL) using a hemocytometer, from which total number of sperm ejaculated (\times 10⁹ cells) was calculated.

2.5. Statistical analysis

The mean \pm SD for each behavior characteristic measured for each jack, each group (young and old jacks), and for all jacks were calculated. Differences among individual jacks and between age groups were analyzed using a Kruskal-Wallis one-way test. Pearson correlation coefficients were calculated for all pairwise combinations of the five behavior variables and seven semen characteristics. All analyses were performed with SAEG software (Statistics and Genetic Analyses Systems, ver. 9.1; SAEG/UFV, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil).

3. Results

3.1. Sexual behavior

The behavior of all six jacks individually and within their two age groups is summarized in Table 1. Although there were significant differences between individual jacks for FLEH, MWE, EL, and COP, no single jack was consistently different from all the others. There were differences between the two age groups for MWE and EL. Based on general clinical observations, all jacks had normal sexual behavior, such as rolling in soil, vocalization (when the jack was introduced to the mare), and sniffing the mare's perineal area, inguinal region, hind legs, ventral abdomen, and ground. However, the characteristic open mouth behavior was not demonstrated by any of the jacks. All mares used to collect semen demonstrated

Table 1

Analysis of sexual behavior in six Pêga jacks used for semen collection with mares. Jacks D1 to D3 were older (more experienced), whereas jacks D4 to D6 were young (less experienced).

Jack	Number of semen collections	FLEH	MWE*	EL* (min)	LEI (sec)	COP (sec)
Older jacks						
D1	40	9.2 \pm 5.1 ^a	0.4 \pm 0.8 ^a	11.6 \pm 10.0 ^{ab}	6.0 \pm 4.4	35.9 \pm 8.5 ^a
D2	17	6.1 \pm 1.9 ^a	1.0 \pm 0.6 ^a	7.5 \pm 8.7 ^b	4.0 \pm 1.7	27.8 \pm 5.4 ^{ab}
D3	40	3.7 \pm 4.5 ^b	0.7 \pm 0.5 ^a	14.7 \pm 11.3 ^a	3.9 \pm 1.3	20.4 \pm 2.6 ^b
Mean	32.3	7.0 \pm 2.0	1.2 \pm 0.4	12.2 \pm 6.2	4.8 \pm 3.2	28.1 \pm 9.3
Young jacks						
D4	28	10.3 \pm 6.4 ^a	0.8 \pm 1.1 ^a	18.3 \pm 11.4 ^c	6.3 \pm 4.8	21.2 \pm 2.4 ^b
D5	26	9.0 \pm 6.7 ^a	2.4 \pm 1.9 ^b	21.5 \pm 24.2 ^{cd}	5.4 \pm 4.3	22.3 \pm 3.0 ^b
D6	29	4.8 \pm 4.5 ^b	1.5 \pm 1.1 ^b	35.6 \pm 22.4 ^d	4.3 \pm 1.3	22.8 \pm 3.5 ^b
Mean	27.8	8.1 \pm 3.0	2.1 \pm 1.4	25.3 \pm 21.3	5.3 \pm 3.8	22.1 \pm 2.9
All jacks	180	7.4 \pm 5.7	1.1 \pm 1.2	18.2 \pm 17.6	5.0 \pm 3.5	25.3 \pm 7.7

FLEH, Flehmen response (number per collection); MWE, mount without erection (number per collection); EL, erection latency (time from first exposure to the mare to full erection ready for ejaculatory mount); LEI, latency from erection to insertion (time from complete erection to insertion into the artificial vagina); COP: copulation duration (time from insertion into AV to dismount after ejaculation).

*There were differences ($P < 0.05$) between the older and young jacks for MWE and EL.

^{a-d}Within a column, values from individual jacks without a common superscript differ ($P < 0.05$).

Table 2
Characteristics of repeated ejaculates collected from six Pêga jacks used for semen collection with mares.

Characteristic	Mean \pm SD
Gel free semen volume (mL)	47.2 \pm 28.6
Gel volume (mL)	71.7 \pm 54.8
Total motility (%)	84.2 \pm 6.0
Progressive motility (%)	74.4 \pm 7.0
Sperm vigor (scale 1 to 5)	3.8 \pm 0.5
Sperm concentration (sperm cells/mL)	253 \pm 91.2 ($\times 10^6$)
Total number of sperm	10.3 \pm 3.4 ($\times 10^9$)

acceptance of the jack's attentions by immobility and the absence of active rejection (e.g., kicking and biting). A few mares displayed additional signs of estrus, such as exposure of the clitoral area, although in many, this was discreet. In the minority of mares, this was accompanied by passing of a small volume of urine.

3.2. Semen evaluation

Semen characteristics are summarized in Table 2. A gel fraction was present in half (90 of 180) of ejaculates; D1 produced gel in 97.5% (39 of 40) of his ejaculates, whereas D4 and D5 did not produce any gel.

3.3. Correlation between sexual behavior and semen characteristics

The only significant correlations between a semen characteristic and sexual behavior were between COP and gel free volume ($r = 0.9$) and gel volume ($r = 0.7$).

4. Discussion

4.1. Sexual behavior

Based on clinical observations, all jacks demonstrated normal sexual behaviors, apart from an open-mouth, prior to, during, and after semen collection. After ejaculation, all jacks were permitted to dismount at will, and all showed normal postejaculatory behavior, including sniffing vaginal secretions on the ground, sniffing the mare's perineal area, postcopulatory Flehmen, and so forth. These behaviors were in general agreement with those evident in wild asses [14], natural mating to jennies [11,13], semen collection using jennies [18], and natural mating of jacks with mares on pasture [4], as well as domestic and wild horses during mating [15].

Behaviors such as vocalization and perineal sniffing appear to encourage estrous behavior in jennies. However, such behavior, although still shown by jacks in the presence of an estrous mare, did not seem to have the same effect of encouraging estrous behavior in mares bred to jacks either at pasture [4] or in-hand [20]. Estrous behavior of the mares in response to these jacks was markedly reduced in intensity compared with that typically displayed in the presence of a stallion. This failure to encourage estrous behavior was also evident in mares in this study, whose reaction varied from a minimal display of estrus (e.g., immobility), to more demonstrative estrous behavior such as urination and clitoral exposure. This lack of response by mares may in part account for the relatively long erection latency relative to that reported for jacks breeding jennies [11,13]. Although Lodi et al. [4] reported that the reaction of estrous mares varied among jacks, this was not observed in the current study. However, as previously stated, mares were used only if they accepted the jack, and as such, differences in mare estrous behavior were unlikely to have affected sexual behavior of individual jacks. Selection of suitable receptive mares is known to be extremely important and a major determinant of success [20].

Specific sexual behaviors did not consistently vary among jacks, although some differences between the two age groups were evident. The Flehmen response, a common sexual behavior observed in several species [30], was shown by all jacks, and to a similar extent by both the older, more experienced jacks and the younger, less experienced jacks. This behavior was commonly observed in stallions [31], in jennies and jacks under free-range conditions [11], and in jacks during semen collection using jennies [17,18,32]. The number of Flehmen responses per collection in this study was similar to that reported by Gastal et al. [18] (6.6 to 4.6), when using jennies for semen collection and during natural mating of jennies at pasture [11]. Mounting without erection is commonly observed in wild and domestic jacks [10,13], as well as stallions [19]. However, from observations on jack sexual behavior in this study and elsewhere [11–13], it is evident that mounting without erection plays a different role in the jack to that in the stallion. In jacks, it is an essential element of courtship that triggers sexual stimulation, resulting in erection; in contrast, in stallions it appears to play less of a role within courtship. However, allowing young stallions and those with poor libido to mount in the absence of an erection has been reported as a means of promoting mating behavior and hastening breeding [19,33]. The number of mounts and time to

ejaculation has been related to poor semen quality; stallions that mounted more than three times prior to ejaculation produced an increased volume of ejaculate, but had reduced sperm concentration, sperm motility (both fresh and postthaw), and sperm membrane integrity [34]. However, no such association between mounts without erection and any semen characteristics was evident in the jacks in the current study. Although mounting without erection appeared to play a much more central role in donkey courtship than it does in stallions, the number of mounts without erection may be linked to libido. In the current study, the young jacks had significantly more mounts without erection than older stallions, perhaps due to their inexperience and, therefore, relative lack of confidence.

The average erection latency in this study, though variable, compared well with that reported by Costa [27] (1 to 45 min) and Veronesi et al. [35] (14.3 ± 9.3 min). The significant variation observed in the current study may well in part be due to the particularly sensitive nature of jacks to environmental disturbances such as noise, weather, general management, and so forth [11]. In the current study, it appeared that reaction times were greater when environmental conditions were extreme, especially hot, cold, or rainy weather. Erection latency included the time spent mounting without erection; therefore, as mounting without an erection was significantly more common in the young jacks, it is not surprising that erection latency was similarly greater. Similarly, Sieme et al. [34] reported that stallions that took longer to collect semen had more mounts per ejaculation. Latency from erection to insertion varied little among jacks in this study. Long latency from erection to insertion has been reported to be a particular problem when using mares for semen collection or during in-hand breeding of jacks with mares [6,17,20]. However, this study did not support this concern, nor did it agree with Silva Filho et al. [36], who reported that latency from erection to insertion was significantly correlated with gel volume. The only behavior significantly correlated with gel volume was copulation duration ($r = 0.7$), which was also correlated with gel free volume ($r = 0.9$); presumably, the longer time spent copulating was linked to the greater total volume of semen. Copulation duration, like several other characteristics, had some significant variation among jacks but in general was similar to that reported by Henry et al. [11] (25 to 30 sec) for natural mating jacks, in stallions as reported by Silva Filho et al. [36] (35 sec), and in stallions as reported by Pickett et al. [37] (30 sec), with no effect due to stallion age.

4.2. Semen evaluation

In this study, semen was simply evaluated under field conditions to give an indication of its quality and hence its appropriateness for use with AI. It was noteworthy that all samples met the minimum requirements for AI [35,38], and in particular those for Pêga jacks [27,39,40]. Therefore, collection of semen from jacks using mares to encourage ejaculation was not detrimental to semen quality, which was suitable for AI.

In conclusion, sexual behavior shown by jacks during semen collection using mares was similar to that reported for natural mating to jennies (except that no open mouth behavior was observed). These behavioral characteristics were largely similar for young, less experienced versus older, more experienced jacks, although the number of mounts without erection and the erection latency were both significantly greater in the young jacks compared with that in the older adults, perhaps due to their relative lack of experience and confidence. All ejaculates met the minimum requirements for use with AI; therefore, the use of mares, rather than jennies, is adequate to obtain semen for AI or to obtain semen samples for breeding soundness examination of jacks. Sexual behavior was not significantly correlated with semen characteristics, except copulation duration was significantly correlated with gel free volume of semen and gel volume. We inferred that, in spite of differences in sexual behavior between mares and jennies, using estrous mares with jacks, previously conditioned to mount mares, was an efficient method of collecting semen for AI and therefore can be advocated for use in stud farm mule production.

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