Abstract

The objective of this study was to evaluate the reproductive performance of Santa Ines rams subjected to successive semen collections in an Amazonian climate. Four rams were subjected to successive ejaculations during a maximum period of three hours. This procedure was repeated three times at 15-day intervals. Sexual and behavioural (libido) and andrological (testicular and seminal) assessments were performed. A total of 81 ejaculates were collected. Libido and semen vigour, volume, appearance and concentration decreased as the ejaculation frequency increased (P<0.05) and sperm motility showed a decreasing trend (P=0.06). The seminal pH increased over the sequence of collections (P<0.05). The only significant differences observed between individual rams were the variable scrotal circumference and the percentages of live sperm and sperm abnormalities (P<0.05). All the parameters of the first ejaculation were within the normal range for this species, which suggests that the local climatic conditions (high temperature and humidity) did not affect the behavioural, testicular or seminal parameters of experimental rams. Our findings indicate that the reproductive performance of Santa Ines rams could be affected by the intensification of ejaculation frequency; however, individual male variation needs to be taken into consideration.

Introduction

The sheep industry in Brazil benefits from favourable environmental, economic and social conditions. According to the Brazilian Institute of Geography and Statistics (IBGE, 2010), the Brazilian sheep population consists of 16.8 million animals and, of these, approximately 65% belong to the Santa Ines breed. An increase of 16.5% in the Brazilian sheep population was observed from 1998 to 2009. Given this, the reproductive efficiency of herds plays an important role.

Male fertility is much more important in reproductive programmes than female fertility. This is because male ruminants can mate with a large number of females (Katz, 2007). Genetic improvement is best achieved by exploiting the male’s capability for greater selection pressure (Salgueiro and Nunes, 1999).

The reproductive performance of a breeder ram is related to several characteristics: sperm production, viability and fertilising capability of the ejaculated sperm, sexual behaviour and ability to service (Inwalle and Katz, 2004; Jainudeen and Hafez, 2004). Of these, sperm production capacity and libido are the main factors that allow the extensive use of a breeder ram for a larger number of females over a longer period (Kaya et al., 2002). These reproductive characteristics can be influenced by genetic and environmental factors as well as the interactions between them (Jainudeen and Hafez, 2004). Noticeable differences have been observed between species with regard to mating strategies. According to Simitzis et al. (2006), breed and age also influence the level of the ram’s sexual interest. Furthermore, an increase in the intensity of ejaculation can influence seminal characteristics (Aisen and Venturino, 2008). In horses, the ejaculation frequency has been shown to affect only the total number of sperm cells (Gastal et al., 1997). Reduction in ejaculate volume and sperm concentration have been reported in different goat breeds without, however, ejaculation sequence having any effect on sperm motility (Moraes et al., 2003; Ritar et al., 1992). Studies in wool sheep have reported declines in all seminal variables resulting from increases in ejaculation frequency (Kaya et al., 2002; Jennings and McWeeney, 1976); however, there have been no reports of such an increase in Santa Ines sheep.

On the other hand, also climatic factors are closely related to animal reproductive performance. The Amazon region is known for its high temperatures and humidity throughout the year. This combination can cause animals severe discomfort (Costa et al., 2010) because of the intense thermoregulatory activity required to maintain homeostasis (Magalhães et al., 1998). In turn, heat stress reduces the reproductive capacities of males and females, with a resultant negative impact on herd fertility (Garcia, 2006). In males, there is interference in spermatogenesis, reductions in the quantity and quality of semen, and effects on libido (Jainudeen and Hafez, 2004). No studies have been carried out on the sexual activity of rams in an Amazonian climate so it is important to evaluate whether these climatic conditions may limit male performance, particularly under intense reproductive management, such as several successive ejaculations over a short time period.

The objective of this study was to evaluate the reproductive performance of rams subjected to successive semen collections in an Amazonian climate by evaluating sexual behaviour and semen characteristics.

Materials and methods

The present study was performed on the experimental farm of the Federal Rural University of Amazonia in the northern region of Brazil (01°07’44”S and 47°37’12”W) during March and April 2005; this is the normal breeding season on the farms in the region. Monthly climatic data are presented in Table 1; these include data from the pre-study period.
Climatic data were recorded approximately 100 meters from where the animals were kept. At night, the animals were brought inside and put into open pens that allowed a constant exchange of air; microclimatic conditions were the same as those outside. Table 1 also shows the temperature and humidity indices (THI). The effects of these on thermal animal comfort levels were grouped into categories: the absence of stress (THI <70); alert (THI 70-72); critical (THI 73-78); danger (THI 79-82); and emergency (THI ≥83) (Hahn, 1985).

This study was conducted on 4 Santa Ines rams aged between 12 and 18 months, weighing 48.2±4.5 kg, and considered sexually mature after breeding soundness evaluation. Animals exhibited normal characteristics for the breed. The animals underwent a period of semen collection prior to this study to stabilise the extra-gonadal spermatozoa reserve. The animals were managed under a semi-intensive system and released for grazing on Brachiaria humidicola grass in the morning, where they remained throughout the day. At the end of the day, once the animals had been brought inside and put into pens, they were fed a diet supplemented with Pennisetum purpureum grass and a concentrate diet with 16% crude protein (400 g/head/day), which is used for animal maintenance. Water and mineral salt mix were provided _ad libitum_.

The experiment consisted of three semen collection series at 15-day intervals. In each series (replication), the males were subjected to successive semen collections over a maximum period of three hours. Behavioural (libido) and andrological assessments (testicular and semen evaluations) were also performed.

Semen collections were performed with a short artificial vagina warmed to 42-45°C; all the males were conditioned to the method at the beginning of the experiment. Sexual stimulation was achieved using a female in artificially-induced estrous by the administration of 10 mg of estradiol benzoate (RIC-BE® Syntex S.A., Argentina), intramuscularly, twice a week.

Semen was collected as follows: Animal I was subjected to collection starting at 9 am on the first day, Animal II at 5 pm on the first day, Animal III at 9 am on the second day, and Animal IV at 5 pm on the second day. All semen collections were performed indoors.

Libido was evaluated during semen collection. Sexual interest was measured using the following behavioural characteristics: identification of the female in heat, olfaction, lip curl (Flehmen reflex), courtship, pelvic movements, and exposure of glans with dripping semen or seminal plasma. The time between recognition of the estrus ewe and the first mount, and the time between successive ejaculations were recorded. These data provide a basis for the following classification of libido: excellent (0-59 seconds), good (60-120 seconds) and regular (>120 seconds), in accordance with Freitas and Nunes (1992).

Collections were interrupted on two occasions: 1) when libido was not sufficient for the next ejaculation (>1 h); and 2) when the maximum period of three hours was reached.

The following testicular physical characteristics were evaluated at the beginning of each step: shape, symmetry, mobility, normal position, touch sensibility, consistency and biometry. Consistency was classified on a scale of 1 to 5: 1, flaccid; 2, decreased firmness and elasticity; 3, firm and elastic; 4, increased firmness and elasticity; and 5, hardened (fibrosis). Scrotal circumference was measured with a flexible measuring tape across the widest region of the scrotum while holding the gonads ventro-caudally.

Semen was evaluated immediately after collection; samples were maintained at 37°C. The following physical parameters were assessed: volume (mL); appearance (1, translucent; 2, opalescent; 3, milky; 4, creamy); pH (0-14, using a specific pH meter); sperm mass motility (a scale of 0-5); progressive sperm motility (%), and sperm vigor (0-5; CBRA, 1998). The last three characteristics were assessed under a microscope with magnifications of 40X, 200X and 200X, respectively.

Sperm concentration was determined by spectrophotometry. For this, 0.02 mL of semen was diluted into 8 mL of a 3% sodium citrate solution. Results were determined by reading the absorbance value at a wavelength of 535 nm. The value obtained was converted to a final value according to:

\[ y = 0.4539 + 11.6062x \]

A supravital staining technique (eosin-nigrosin) was used to determine the percentage of live spermatozoa. A smear of semen diluted in sodium citrate was stained using the panoptic staining method for the morphological evaluation of sperm cells. The morphological classification recommended by CBRA (1998) was used. Sperm abnormalities were classified into major, minor and total defects according to Blom (1973).

The experimental design was completely randomised and SAS™ software was used for data analysis (SAS, 2004). Means of the number of ejaculations and the scrotal circumference were compared between animals and between series of ejaculation repetitions. Data of seminal characteristics were compared between animals and sequences of ejaculations. All the variables showed a normal distribution. Data were analysed using ANOVA (multivariate), and their mean values were compared using Kruskall-Wallis (seminal scores) and Friedman’s (percentages expressed as means) tests. Statistical significance was considered as _P_<0.05 and tendency as _P_>0.05 and _<0.10_.

### Table 1. Mean monthly temperatures, humidity, rainfall, and the temperature and humidity index in northern Brazil. Meteorological data provided by the National Institute of Meteorology, 2nd District of Meteorology/PA, Brazil.

| Month   | _T_ max °C | _T_ min °C | _T_ mean °C | Humidity, % | Rainfall, mm³ | THI max | THI mean |
|---------|------------|------------|-------------|-------------|---------------|---------|---------|
| Dec. 2004 | 35.3       | 21         | 26.9        | 77          | 71            | 90.7    | 77.5    |
| Jan. 2005 | 35.6       | 21.5       | 26.6        | 81          | 292.8         | 92.1    | 77.6    |
| Feb. 2005 | 33.5       | 22.3       | 26          | 86          | 307.4         | 89.6    | 77.2    |
| March 2005 | 34.3      | 22         | 25.8        | 88          | 308           | 91.4    | 77.1    |
| April 2005 | 33.8      | 22.3       | 25.7        | 88          | 311.4         | 90.5    | 76.9    |
| Mean     | 34.5       | 21.8       | 26.2        | 84.0        | 258.1         | 90.9    | 77.3    |

_T_ max, maximum temperature; _T_ min, minimum temperature; _T_ mean, mean temperature; THI is 0.8 * _T_ + (humidity/100) * (_T-14.4) + 46.4, where _T_ is temperature, according to Thom (1958).
Results and discussion

A total of 81 ejaculates were collected. The mean successive ejaculation numbers per animal were 2.3±1.5, 5.3±2.5, 10.3±5.8 and 9.0±1.7 for Animals I, II, III and IV, respectively, showing a trend toward significance (P=0.07). According to Houp (2010), a ram is capable of something under 20 ejaculations daily, and the latency to ejaculation increases with the number of observed ejaculations during an observation period, but this is subject to large individual variation. So, the number of ejaculations that occur when a ram is presented with a large number of estrous ewes is similar to the number that occurs in response to an electroejaculator, indicating that physical capability rather than libido is the limiting factor.

The frequency with which semen can be collected depends on the animal’s age, condition and temperament (Simitzis et al., 2006). In the present study, the animals were of similar ages, exhibited similar temperaments, and were subject to the same collection conditions and procedures. Therefore, the number of successive ejaculations per animal appears to be more related to the animal’s libido. For the first, second and third series, the mean ejaculator, indicating that physical capability rather than libido is the limiting factor. Therefore, the libido assessed before the first ejaculation was not necessarily the same as the libido demonstrated when successive collections were performed. According to Imwalle and Katz (2004), a breeder cannot be judged only by this characteristic at one time point because although the animal may show excellent libido with respect to the first ejaculation, the same pattern may not be maintained in subsequent collections. Therefore, libido determines the reproductive performance of males towards a group of females. In this study, out of a total of 12 collection series (4 animals and 3 replicates), eleven (91.7%) were interrupted because of insufficient libido and one (8.3%) because it exceeded the pre-determined period of three hours. According to Ax et al. (2004), although some rams show satisfactory sperm quality, they might show low fertility rates because of their inability to mate with a sufficient number of females. The cause of this deficiency may be low or even absent libido, among other factors, such as repeat breeding to especially persistent ewes. Additionally, the number of successive ejaculations by a ram can be used to evaluate libido (Stellflug et al., 2008). Therefore, the sexual performance of a breeder should be evaluated while considering the conditions of the mating programme to which it will be subjected to ensure that the reproductive efficiency of the herd will not be compromised.

In addition, the gonadic sperm reserve is perhaps one of the most important parameters among male reproductive characteristics. It reflects the breeding potential the ram can achieve during the breeding season, being able to establish a male/female relationship without any decrease in reproductive efficiency at the end of the breeding season (Macmillan and Hafs, 1968; Weisgold and Almquist, 1979; Guimarães, 1997). In small ruminants, the epididymal sperm reserves available each day for ejaculation are readily depleted, with approximately 62% of the potential daily sperm collection being obtained in the first two ejaculates. Epididymal replenishment is fairly rapid, and in well-managed facilities sperm has been collected twice a day at 24-h intervals (Eaton and Simmons, 1952; Ott and Memon, 1980).

Testicular characteristics such as shape, symmetry, mobility, position and sensibility

| Interval° | Animal I | Animal II | Animal III | Animal IV |
|-----------|----------|-----------|------------|-----------|
| 0-1       | 10:34    | 14:36     | 1:40       |           |
| 1-2       | 53:00    |           | 42:20      |           |
| 2-3       | 25:05    |           |            |           |
| 3-4       |          |           |            |           |
| 4-5       |          |           |            |           |
| 5-6       |          |           |            |           |
| 6-7       |          |           |            |           |
| 7-8       |          |           |            |           |
| 8-9       |          |           |            |           |
| 9-10      |          |           |            |           |
| 10-11     |          |           |            |           |
| 11-12     |          |           |            |           |
| 12-13     |          |           |            |           |
| 13-14     |          |           |            |           |
| 14-15     |          |           |            |           |
| 15-16     |          |           |            |           |
| 16-17     |          |           |            |           |

°Intervals between ejaculations. Animal I, subjected to collection starting at 9 am on the first day; Animal II, at 5 pm on the first day; Animal III, at 9 am on the second day; Animal IV, at 5 pm on the second day.
were found to be normal for all the animals studied according to normal value for the species (CBRA, 1998). There was a significant difference in mean scrotal circumference between animals (Animal I, 28.2±0.3; Animal II, 30.0±0.0; Animal III, 30.3±0.3; and Animal IV, 27.2±0.8 cm; P<0.0001) showing the individual character of this parameter (CBRA, 1998). In the present study, the mean scrotal circumference was 28.9±1.4 cm (range 26.5-30.5 cm). Similar values were reported by Pacheco et al. (2009) who found a mean value of 29.7 cm for rams of the same breed and age. Our experimental animals were born and raised in the Amazon region. The similarity between the values found in this study and those of animals from other regions suggests that all rams bred in regions with an Amazonian climate present the same degree of sexual development. In addition, no significant variation was observed between successive semen collections (first series, 28.6±1.7; second series, 28.9±1.6; third series, 29.2±1.2 cm). This confirms that the animals did not show any significant environmental effects (e.g. from nutritional, climatic and other factors) between replicates. Similarly, no difference in testicular consistency was observed; all the animals were given a testicular consistency score of 3.

Data regarding the seminal characteristics evaluated according to the sequence of ejaculation are presented in Figure 1. Mean semen volume varied between the animals (1.1±0.2*, 0.6±0.2**, 0.6±0.4*, and 0.8±0.3** mL for Animals I, II, III and IV, respectively; P<0.05). The higher mean semen volume observed for Animal I may be related to its lower number of successive ejaculations. Over the course of the sequence of ejaculations, a decrease was observed in the ejaculated semen volume. According to Ax et al. (2004) and Moraes et al. (2003), the frequency of successive semen collections also resulted in a progressive reduction in ejaculated semen volume. In unusual cases, for example, when an animal is explored over the advised limit (sperm exhaustion), semen volume can be reduced to nearly zero (Ax et al., 2004). This phenomenon was observed in Animal III between collections 13 and 16. However, the increase in the volume of the 17th collection might be associated with the longer recovery period before this collection (37 min). The recovery interval between two ejaculations appears to be necessary in order to produce adequate amounts of seminal plasma. Rodrigues (1997) reported that variations in ejaculate volume are affected by several factors, with the secretory activity of accessory sexual tissues appearing to have the strongest effect. The reduction in the concentrations of some electrolytes (calcium and magnesium ions) and decreases in the enzymatic activity of the seminal plasma in response to an increase in the frequency of semen collection may be the result of a reduction in the concentration of accessory gland fluids. So, when the ejaculate volume increases or decreases, the modifications are mainly due to changes in quantities of fluids secreted by the accessory glands and the epididymis which are androgen dependent (Kaya et al., 2002).

Semen appearance was of gradually poorer quality as the sequence of ejaculations progressed (P<0.05), and this trend was observed in all animals. Semen appearance is closely related to sperm concentration which showed a similar decrease. For the first ejaculate, the mean sperm concentration was 4.0×10⁹ sperm cells/mL of semen. Normal sperm concentration values for rams vary from 3.0×10⁹ to 7.0×10⁹ spermatozoa/mL (Aisen and Venturino, 2008). Therefore, the concentrations obtained in this study are within the established pattern for this species. A decline in these values along an ejaculation sequence were also observed by Moraes et al. (2003) and Ritar et al. (1992) for different goat breeds.

The mean seminal pH determined for the first ejaculation (6.8±0.2) is consistent with the range (6.2-7.3) established by Aisen and Venturino (2008). No differences were observed between animals; however, an increase was observed according to the collection sequence (P<0.05). According to Nunes (1982), the survival capacity of sperm cells is impaired beyond the normal range. In the present study, the survival of sperm cells did not seem to be affected by the sequence of ejaculations. In other words, the percentage of live sperm cells did not vary over the sequence of ejaculations. The mean value of live sperm cells was 85.1±6.3%; differences were observed between animals (88.8±2.9*, 81.9±9.3**, 83.5±4.6** and 88.8±4.9** for Animals I, II, III and IV, respectively; P<0.05).

Figure 1. Mean values (±SD) of semen volume (mL), semen appearance (1-4), pH, sperm mass motility (0-5), progressive sperm motility (%), live sperm cells (%), sperm vigor (0-5), sperm concentration (x 10⁹ cells/mL), and percentages of sperm abnormalities (major, minor and total defects) according to ejaculation sequences in Santa Inês rams; abc, P<0.05.
The observed percentage of live spermatozoa was similar to that reported by Moraes et al. (2003). Therefore, the integrity of the plasma membranes of the sperm cells, which is indicated by this characteristic, does not seem to have suffered from ejaculation repetition, but is rather due to factors relating to the individual animal. There was no difference in sperm mass motility between the animals or among the successive ejaculations. The mean value of the first ejaculation was 3.57±0.2, similar to the 3.72 reported by Salgueiro and Nunes (1999). For progressive sperm motility, the decrease observed in the sequence of ejaculations showed a trend toward significance (P=0.06); likewise, there was no difference in this parameter between animals. The observed absence of any variation in the progressive sperm motility and mass motility was also reported by Moraes et al. (2003) from successive semen collections in goats. The mean progressive sperm motility of the first ejaculation (71.66%) in the present study was also within the pattern observed for this species (Barbas et al., 2001). There was a reduction, however, in spermatic vigor score over subsequent repetitions (P=0.05). In horses, there is no difference in spermatic vigor score over the sequence of ejaculations (Gastal et al., 1997).

Differences in sperm abnormalities were observed. Percentages of major defects were 8.7±5.7a, 2.9±1.0b, 6.0±4.5a and 2.4±1.3b; of minor defects 16.7±16.7a, 3.3±2.7b, 6.3±4.0b and 3.23±1.9b; and for the sum of total defects 25.7±16.3a, 6.3±3.6b, 12.4±7.1b and 5.1±2.1b for Animals I, II, III and IV, respectively. (*Different superscript lowercase letters indicate significant differences for each variable using Friedman’s test; P<0.05). Values oscillated over the ejaculation repetitions; however, no significant variation was observed. All the samples showed a sperm morphology percentage below the maximum limit of 30% recommended by the CBRA (1998) for the use of semen in assisted reproduction programmes. Contrary to our results, Kaya et al. (2002) observed that the percentage of morphological abnormalities in sperm cells increased in line with the intensity of semen collection. In boars, studies have reported that the frequency of ejaculation reduces the osmotic resistance of the acrosomal membrane in sperm cells because of insufficient maturation in the epididymis (Schilling and Vengust, 1987). However, it is believed that the causes of the abnormal sperm morphology observed in this experiment may be due to other individual factors of each ram that were not evaluated (e.g. frequency of semen collection). The mean number of total defects observed in this study (10.2±8.3%) was less than that observed by Pacheco et al. (2009; 37.5%), also in Santa Ines rams.

In summary, all the seminal parameters of the first ejaculation were within the established pattern for this species (CBRA, 1998). This indicates that these animals may be adapted to the climate of the Amazon region and corroborates the findings of Costa et al. (2010). The high temperature and relative humidity of the region caused great animal discomfort (THeatmax classified as critical and emergency levels of discomfort, respectively; Table 1); however, it seems that climatic characteristics did not affect seminal quality. This suggests that these animals have their own mechanisms to control corporal and testicular homeostasis. However, seminal quality was affected by the sequence of ejaculations. This is important when defining the male:female ratio of a breeding population, especially in estrous synchronisation programmes in which the females exhibit short periods of estrus, requiring the males to exhibit high sexual performance. Furthermore, if the intervals between the ejaculations are not carefully monitored, the efficiency (seminal quality) may be compromised.

**Conclusions**

This study shows that intensification of ejaculation frequency can affect the reproductive performance of Santa Ines rams and, therefore, influence behavioural and seminal characteristics, such as libido and semen volume, appearance, pH, spermatic vigor and sperm concentration. However, the climatic conditions of the Amazon region (high temperature and humidity), although causing discomfort to the animals, do not affect the behavioural, testicular or seminal parameters of Santa Ines rams. In addition, characteristics such as scrotal circumference and percentages of live sperm and sperm abnormality present individual variation.

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