Brazilian sheep raising is a sustainable alternative for generation of profits and is one of the primary production activities of rural populations, consolidating itself as an important sector for socio-economic development of the country. The sheep is also a major source of animal protein, and technical support is needed to guide the use of biotechnology to increase the productivity of flocks [1].

Embryo transfer is the most useful technique to accelerate genetic improvement of sheep livestock in Brazil because it provides the opportunity to disseminate the genetics of highly proven elite females and males. However, the scarcity of female recipients for the high number of embryos produced by hormonal stimulation have motivated development of several types of vitrified embryos [2] to avoid wasting excess embryos and retain these high genetic quality embryos under appropriate conditions to be used in future embryo transfer programs [3].

Determination of fetal sex in utero is useful in make management decisions for dairy or meat goats as well as for commercialization of fetuses of a certain sex [4]. The development of integrated reproductive management systems that combine ultrasonography with other reproductive technologies, such as artificial insemination and embryo transfer, will further increase the practical applications of ultrasonography [5].

Previous studies have show that embryo cryopreservation extends the gestation period by at least four days, probably due to a slow down of cryopreserved embryo cellular activity [2, 3, 6]. Preliminary data also showed that the migration period of genital tubercle in Dorper [7] and Morada Nova fetuses [8] derived from natural mating occurs earlier compared with fetuses originating from frozen embryo transfer; for this reason, the authors suggested that these fetuses should be sexed five days later.

Determination of the ideal time to visualize the genital tubercle definitively positioned by ultrasonography in small ruminants is vital in order to obtain higher accuracy rates in fetal sexing [9]. There are several studies defining the period of genital tubercle migration in ewe fetuses derived from natural mating [9, 10] and from frozen embryo transfer [7, 8]; however, this information is still unknown in fetuses derived from fresh and vitrified embryo transfer.

The objective of this study was to identify the migration period of the genital tubercle and the period of visualization of external genital structures.

**Key words:** Genital tubercle, Prepuce, Scrotal bag, Vulva, Ultrasound
genital structures in fetuses of the Dorper breed derived from natural mating and from fresh, frozen and vitrified embryo transfer.

**Material and Methods**

In this study, 130 Dorper fetuses, 75 males and 55 females, from 130 single pregnant ewes were examined and allocated into four treatments (NM, FrE, FE and VE).

In NM, the fetuses (n=33) originated from controlled natural mating (monitoring the female mating day and hour), whereas FrE (n=36), FE (n=33) and VE (n=28) derived from embryo transfer, with the embryos collected 7 days after breeding [11]. Treatment FrE was composed of fresh embryos transferred to recipients immediately after collection. Treatment FE was composed of frozen embryos [12], and Treatment VE was composed of vitrified embryos [3]. The cryopreserved embryos were transferred to recipient ewes [12].

All females were mated only once, and the day of mating during estrus was designated as day 0 of pregnancy. The gestation period begins with natural mating in all females (embryo donors and females from natural mating). After pregnancy diagnosis by transrectal ultrasonography, the fetuses were monitored from the 40th to 60th day of pregnancy at 24 H intervals. Fetuses were diagnosed as males when the genital tubercle was positioned immediately caudal to the umbilical cord and were diagnosed as females when the genital tubercle was positioned below the tail (Fig. 1). Even after genital tubercle migration, fetal monitoring continued until identification of external genital structures, including the vulva in females and the prepuce and scrotum in males.

The same experienced operator performed all ultrasound examinations with the animals restrained in a chute in a standing position. Ultrasound was carried out with an Aquila Pro (Pie Medical, Maastricht, the Netherlands) apparatus equipped with a dual frequency, linear transducer (6.0 and 8.0 MHz) coupled to a PVC support to facilitate manipulation within the rectum of each doe [13]. A Sony printer (Seikosha VP/1200, Tokyo, Japan) was also attached to the ultrasound equipment.

Prior to the exam, the content of the rectum was manually removed, and an ultrasonic coupling gel was applied to the transducer prior to introduction into the rectum. After location of the fetus, a scanning technique for fetal sexing was established using an ultrasonographic longitudinal ventral plane [14].

In the last week of pregnancy, the females were transferred to individual boxes to confirm the sex of fetuses immediately after birth.

The average values for the migration day of the genital tubercle as well as for the visualization day of the scrotum, prepuce and vulva nipples of fetuses from controlled natural mating and fresh, frozen and vitrified embryo transfer were evaluated using analysis of variance and Tukey’s test. The accuracy of fetal sexing was analyzed by the chi-squared test with a 5% level of significance.

**Results**

The migration period of the genital tubercle in NM was earlier (P<0.05) than in FrE, FE and VE; however, no difference (P>0.05) was observed among FrE, FE and VE (Table 1). The periods of visualization of the scrotal bag, prepuce and vulva in NM were earlier (P<0.05) than in FrE, FE and VE; however, no difference (P>0.05) was observed among FrE, FE and VE (Table 1).

No difference (P<0.05) was observed between the periods of visualization of the scrotal bag and prepuce; however, the vulva was visualized earlier (P<0.05) than the scrotal bag and prepuce in FrE, FE and VE (Table 1).

Figure 2 shows the high variation in the fetal sexing period based on the final position of the genital tubercle and/or on the presence of external genitalia according to the different treatments.

Considering all fetuses that were born, the accuracy of diagnosis was 100% for all treatments in the experiment.

**Discussion**

In the present study, genital tubercle migration and visualization of external genitalia occurred later in fetuses derived from the
transfer of fresh, frozen and vitrified embryos than in fetuses produced by natural mating. It has previously been observed that the genital tubercle migration [7, 8] of Dorper and Morada Nova fetuses derived from frozen embryo transfer is delayed compared with fetuses originating from natural mating [2, 3, 6]. The authors [2, 3, 6] also reported that the pregnancy period was four days longer in ewes transferred cryopreserved embryos. In regard to equine species, the development of fetuses derived from unfrozen embryo transfer in mares during the first 30 days of pregnancy was slower than that of fetuses derived from natural mating [15].

Considering the information conveyed above, low cellular activity is induced by frozen-thawed embryos [2, 3, 6] and results in a delay in genital tubercle migration due to handling of the frozen embryos [7, 8]. The results of the present study indicate that the outcome was influenced by embryo manipulation because no difference was noticed between the fresh and cryopreserved embryos. Also, it has been suggested that embryo handling outside the uterine environment influences the latency period of the embryos [15].

Regardless of whether the fetus is derived from natural mating, fresh or cryopreserved embryo transfer, fetal sexing performed only based on the final position of the genital tubercle and/or visualization of the external genitalia requires good ultrasound scanning equipment and a skilled and experienced operator who knows the exact migration period for each species and breed. This recommendation is particularly important in the case of female fetuses because the genital tubercle migration from its initial position to its final position is shorter than in male fetuses.

In the present study, the early visualization of the vulva compared with the scrotum might be associated with the smaller

### Table 1.

Mean and standard deviation of the day of genital tubercle migration and identification of the external genital structures of Dorper fetuses derived from natural mating (NM) and fresh (FrE), frozen (FE) and vitrified (VE) embryo transfer

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Genital tubercle</th>
<th>Scrotal bag</th>
<th>Prepuce</th>
<th>Vulva</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$x \pm s$ (day)</td>
<td>$x \pm s$ (day)</td>
<td>$x \pm s$ (day)</td>
<td>$x \pm s$ (day)</td>
</tr>
<tr>
<td>NM</td>
<td>42.10 $^a$ ± 2.86</td>
<td>45.22 $^a$ ± 1.25</td>
<td>45.95 $^a$ ± 1.53</td>
<td>45.01 $^a$ ± 3.10</td>
</tr>
<tr>
<td>FrE</td>
<td>43.98 $^b$ ± 3.00</td>
<td>48.91 $^{bA}$ ± 1.92</td>
<td>48.52 $^{bA}$ ± 1.41</td>
<td>47.41 $^{bA}$ ± 1.41</td>
</tr>
<tr>
<td>FE</td>
<td>44.97 $^{bA}$ ± 1.83</td>
<td>49.97 $^{bA}$ ± 1.08</td>
<td>49.18 $^{bA}$ ± 2.00</td>
<td>47.64 $^{bA}$ ± 1.82</td>
</tr>
<tr>
<td>VE</td>
<td>44.58 $^{bA}$ ± 1.97</td>
<td>50.12 $^{bA}$ ± 1.66</td>
<td>49.27 $^{bA}$ ± 1.61</td>
<td>47.93 $^{bA}$ ± 1.92</td>
</tr>
</tbody>
</table>

Different superscripts indicate significant differences (P<0.05) in the same column (ab) or line (AB).

Fig. 2. Variation in the number of days in which fetal sexing became possible. The sexes of fetuses derived from natural mating (NM) and fresh (FrE), frozen (FE) and vitrified (VE) embryo transfer were determined by transrectal ultrasound taking into consideration the final position of the genital tubercle and/or visualization of the external genitalia.
distance covered by the genital tubercle of the female prior to reaching its final position under the tail when it transforms into this component of the external genital structure.

Although sexing of fetuses produced by natural mating is possible between the 38th and 48th days of pregnancy, it has been suggested that the ultrasonographic exam in ewes be performed from the 50th day onwards [9]. However, this assumption is not always correct for fetuses derived from transfer of cryopreserved embryos since in some fetuses this migration can be delayed up to the 50th day of pregnancy due to species-specific and individual variations. However, the best period to carry out fetal sexing would be from day 60 onwards as migration of the genital tubercle has surely occurred by that time, even in embryos with delayed movement of positioning. Another factor that must be considered is that by 60 days, differentiation of the genital tubercle into external genital structures has already occurred, reducing the probability of mistakes in identification of fetal sex.

The results obtained in the present study suggest that transrectal ultrasonography is a reliable technique for fetal sexing in ewes when ultrasound imaging is properly timed within a specific period of pregnancy and accurately performed with proper equipment and by experienced operators. The results allow one to conclude that genital tubercle migration and posterior differentiation into external genital structures differs between fetuses derived from natural mating and embryo transfer, as well as that fetal sexing can be carefully done from the 55th day of pregnancy onward, especially in fetuses derived from embryo transfer; however, in fetuses produced by natural mating, fetal sexing can be performed from the 50th day onward.

References


