Eur J Wildl Res (2016) 62:767–770
DOI 10.1007/s10344-016-1040-y

METHODS PAPER

First live offspring of Amazonian brown brocket deer (*Mazama nemorivaga*) born by artificial insemination

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Received: 31 March 2015 / Revised: 14 July 2016 / Accepted: 27 July 2016 / Published online: 26 August 2016

Abstract The Amazonian brown brocket deer (*Mazama nemorivaga*) is an endemic species of the Amazon rainforest region, which has suffered constant threats due to hunting and increasing deforestation. Artificial insemination (AI), combined with genomic banks, is considered an important tool for maintaining conservation programs of endangered species; however, the number of live offspring born from AI in non-domesticated cervids is extremely low. Thus, studies designed to develop, adapt, or enhance AI techniques are of fundamental importance. This report describes a successful transposition of the cervix with semen deposition in the uterine lumen of a *M. nemorivaga* female, based on the transcervical AI technique used in sheep, as well as using specific tools develop for IA in small ruminants, which have resulted in the birth of a healthy male fawn.

Keywords Transcervical insemination · Cervical traction · Neotropical deer

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Introduction

The Amazonian brown brocket deer (*Mazama nemorivaga*) is a small deer that is endemic to the Amazon rainforest region and its ecotones with the "Cerrado" biome (Rossi et al. 2010). Given its apparently wide distribution, the species is classified in the IUCN Red List as least concern (LC); however, in Brazil, it is under threat due to deforestation and hunting (Rossi and Duarte 2008).

Information concerning its reproductive biology is scarce. In Brazil, there are only a few reports of births in captivity, observed at different times of the year (Rossi et al. 2010), which supports the idea that species from equatorial regions tend to present less well defined or asynchronous breeding (Morrow et al. 2009). The lack of knowledge concerning this species is a consequence of the difficulties inherent in accessing their habitat and the small number of deer maintained in captivity. The only confirmed captive population in Brazil (two females and one male) is maintained by the Deer Research and Conservation Center (NUPECCE).

Among assisted reproductive biotechnologies, artificial insemination (AI), coupled with genomic banks, is considered an important tool in the maintenance of in situ and ex situ conservation programs (Holt and Pickard 1999) because it enables the reproduction between valuable, but behaviorally incompatible pairs, thereby eliminating the risks of animal transport (Wildt 1989). Pregnancies resulting from laparoscopic or transvaginal without cervical traction, AI have been reported for only ten species of deer (*Mazama americana*—Zanetti et al. 2009; *Mazama gouazoubira*—Peroni 2013; *Dama dama*, *Axis axis*, *Cervus eldi thamin*, *Cervus nippon*, *Cervus elaphus*, *Elaphurus davidianus*, *Odocoileus virginianus*, and *Rangifer tarandus*—reviewed by Morrow et al. 2009). The authors also mention that the number of live offspring born as a result of AI is extremely low in non-domesticated cervids.

In cervids, laparoscopic intrauterine AI is the most effective method for ensuring semen deposition directly into the uterine horns (Mulley et al. 1988); however, this involves a surgical procedure. In contrast, transvaginal approaches would permit more widespread use of AI by breeders because these methods are less invasive than laparoscopic AI (Aller et al. 2009). However, similar to sheep, the cervix of most species of deer presents tortuosity and reduced dimensions, facts that hinder the transposition of the insemination pipette by transcervical route (Duarte and Garcia 1995; Zanetti et al. 2010). Thus, studies that develop, adapt, or enhance intrauterine insemination techniques by transcervical route are of fundamental importance, since they enable genetic management of populations by minimally invasive methods. The purpose of this study was to report the use of transcervical AI with cervical traction in the species *M. nemorivaga*, which resulted in the unprecedented birth of offspring in captivity.

Materials and methods

An adult female of the species *M. nemorivaga* (5 years old, weighing 14 kg) and an adult male (9 years old, weighing 16 kg) maintained by the NUPECCE were used in this study. The deer were isolated in pens exposed to the natural photoperiod and supplied the same diet and water ad libitum.

For 1 month (August, 2012), a trained examiner manually observed the female, every morning, for manifestations of natural estrus. Eight hours after estrus detection, the doe was physically restrained and anesthetized (7 mg/kg of ketamine hydrochloride + 1 mg/kg of xylazine hydrochloride, i.v.). The deer was maintained in sternal recumbency on an exam table in order to perform an ovarian ultrasound examination using the Aquila equipment (Pie Medical, Netherlands) coupled to a 6-MHz transrectal linear transducer. Transcervical AI was performed soon after.

The sperm used for insemination was collected by electroejaculation (Electroejaculator – P-T Electronics®, Boring, OR, USA), following chemical restraint of the male (1 mg/kg of xylazine + 7 mg/kg of ketamine, i.v.) (Duarte and Garcia 1995), 2 h before AI. Following collection, the total semen volume (200 μL) was diluted 1:1 with TRIS-egg yolk extender (Botu-Bov®, Biotec, Brazil) and refrigerated at 4 °C. Just prior to AI, the chilled semen was evaluated under an optical microscope and presented 30 % motility and vigor of 2.

Before initiating the AI procedure, the perivulvar region was cleaned with 1 % chlorhexidine. Next, a number 1 Collins speculum (ABC Stainless, Brazil) was introduced into the vagina, which was previously lubricated with non-spermicidal gel. After locating the cervical ostium with the aid of a flashlight, a gauze moistened with local anesthetic (2 % lidocaine) was placed near the site for 1 min. The region lateral to the cervical ostium was fixed by two Allis type tweezers (26 cm) to perform traction of the cervix toward the vulvar commissure, while simultaneously withdrawing the speculum. The procedure was performed with the utmost caution, respecting the anatomical limits of the female (Fig. 1a). With the cervix tractioned (approx. 5 cm), digital manipulation of the vagina was performed in the laterolateral and dorsoventral directions, to place the semen applicator through the cervical canal until it reached the uterine body. Transposition of the cervical canal was achieved using a short seminal applicator (12 cm) with a joint expander function, which was developed for sheep (Aplicador Expansor Ovino®, Alta Genetics, Brazil). After reaching the lumen of the uterine body, the expanding mandrel was removed and semen straws were placed (Fig. 1b). Two 0.25 ml semen straws were used, with a concentration of 7.5×10^6 sperm. Following semen deposition, all the instruments were withdrawn and the female's clitoris was massaged.

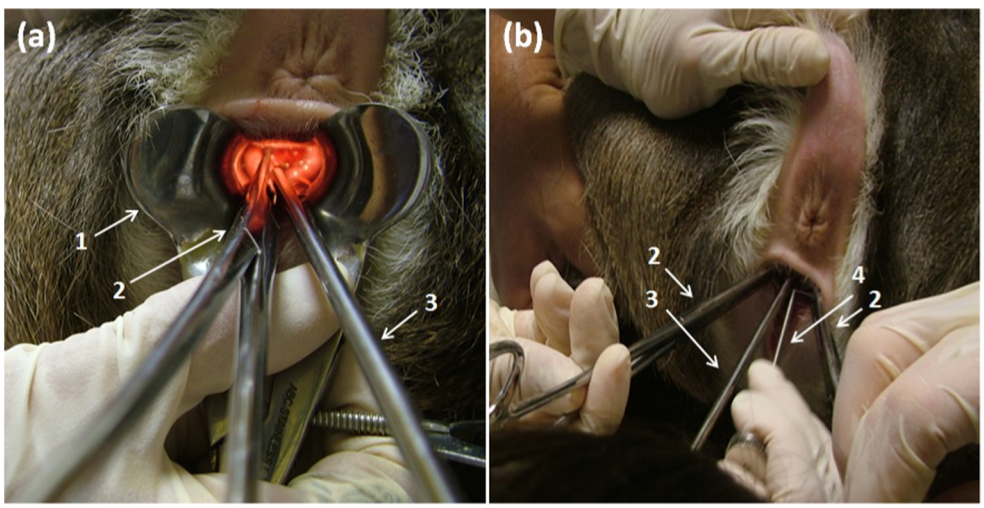

Fig. 1 Images demonstrating the steps of the AI procedure by transcervical technique, using cervix retraction into the vaginal canal in a female *Mazama nemorivaga*: **a** clamping using Allis forceps fixed laterally to the cervical ostium and **b** semen deposition in the uterine lumen; *1* Collins vaginal speculum; *2* Allis forceps; *3* light source; *4* AI pipette (Aplicador Expansor Ovino®)

Ninety days after AI, the doe was again anesthetized (7 mg/kg of ketamine + 1 mg/kg of xylazine hydrochloride, i.v.) prior to performing a transabdominal ultrasound examination to determine pregnancy, using Mindray M7 equipment (Mindray, China) and a 10-MHz linear transducer.

Results and discussion

Estrus in the female *M. nemorivaga* was confirmed through the manifestation of behaviors and signs, including diminished reactivity to the examiner's presence, positive stop reflex (Krepschi et al. 2013), and translucent mucus secretion from the vagina. The lordosis response to handlers has been documented for red deer, Pere David's deer, Eld's deer, reindeer (reviewed by Morrow et al. 2009), red brocket deer (Krepschi et al. 2013), and brown brocket deer (Zanetti et al. 2010).

Insemination was performed 8 h after estrus detection. At this time, the female still allowed the examiner to approach, but did not remain motionless following manual pressure on its back, indicating the end of behavioral estrus. There are no reports concerning the duration of estrus and ovulatory moment for *M. nemorivaga*, but according to Drion et al. (2003), estrus duration in cervids is short (12 to 24 h) and in Neotropical species, it has been shown to vary from 18 to 48 h (Pereira et al. 2010). Determining the duration of estrus was not one of the study's objectives; thus, we decided to inseminate the doe as soon as the end of receptivity behavior was detected. The choice was based on studies involving *D. dama*, where it has been demonstrated that ovulation occurs approximately 24 h after the onset of estrus (Asher et al. 1990).

Just prior to AI, ultrasound examination verified that the largest follicle had a mean diameter of 2.6 mm. However, the lack of information on this species regarding follicular dimensions for specific species events (recruitment, divergence, and ovulation) prevented us from defining or predicting the exact time of ovulation. We assumed that ovulatory follicular diameter is slightly larger than that observed at the time of insemination, because larger ovulatory follicles (3.75 and 5.58 mm) have been observed in a Neotropical species of a similar size by laparoscopy (*M. gouazoubira*—Zanetti et al. 2010).

In brocket deer, the small opening of the cervix, its length and the large number of cervical rings make transcervical intrauterine semen deposition practically impossible (Duarte and Garcia 1995). Our group had made three previous attempts to perform AI in *M. nemorivaga* without success, because cervical traction was not possible due to the inadequacy of the instruments used in relation to the anatomical characteristics of these deer (unpublished data), the semen was deposited at the back of the vagina and did not result in pregnancy. This difficulty has also been reported for sheep due to the anatomical similarities of the cervix (Oliveira and Fonseca 2013). The use of specific instruments for AI in conjunction with cervical traction has not been registered in deer, and this is the main purpose of this report. The technique enabled a complete transposition of the cervical canal using the applicator and semen deposition in the lumen of the uterine body. Due to the anatomical features mentioned above, the use of a number "0" or "1" Collins speculum is indicated, as for nulliparous and primiparous ewes (Fonseca et al. 2016, Oliveira and Fonseca 2013) and the Allis forceps must be at least 26-cm long to ensure they grasp the region lateral to the cervical ostium and allow traction of the cervix.

During the procedure, the length of the vagina of *M. nemorivaga* was determined; it is apparently longer than that of female sheep and the diameter of the vaginal canal corresponds to the dimensions of small, nulliparous ewes (Oliveira and Fonseca 2013). Although these comparisons are subjective, they were performed by a technician who specializes in reproduction of small domestic ruminants. This finding is important because we believe that the development of instruments specific to cervids, in accordance with their anatomical characteristics, may be useful in making the technique simple to perform and efficient in other deer species.

Cervical traction was conducted with the utmost caution, fully conscious of the tension of the uterine ligaments. The middle portion of the vaginal canal was the maximum reached during the procedure. The reduced exposure of the cervix attained for the female *M. nemorivaga*, in relation to sheep, limited digital manipulation to smaller movements, which consequently required greater movement of the semen applicator to bridge the cervical canal. Thus, in addition to greater technical ability, performing this technique in deer seems to require instrument adaptation or the development of longer instruments, particularly the semen applicator.

Despite the difficulties outlined above, the time required to complete the entire procedure of AI in the female *M. nemorivaga*—from positioning the female and placement of the speculum to semen deposition in the uterine body and removal of the instruments—was about 20 min. This minimized the time the female/doe was exposed to anesthesia compared with other AI techniques, such as video laparoscopy, which lasts about 60 min in *M. gouazoubira* (Peroni 2013).

Ninety days after AI, we were able to confirm the pregnancy by ultrasound, in which the fetus (Fig. 2a) and its heart rate were clearly visible, indicating the viability of the pregnancy. Exactly 210 days after AI, the doe gave birth to a male fawn (Fig. 2b). The gestation was similar to that previously described for other species of *Mazama* (Pereira et al. 2010). The delivery went smoothly, without the need for veterinary intervention.

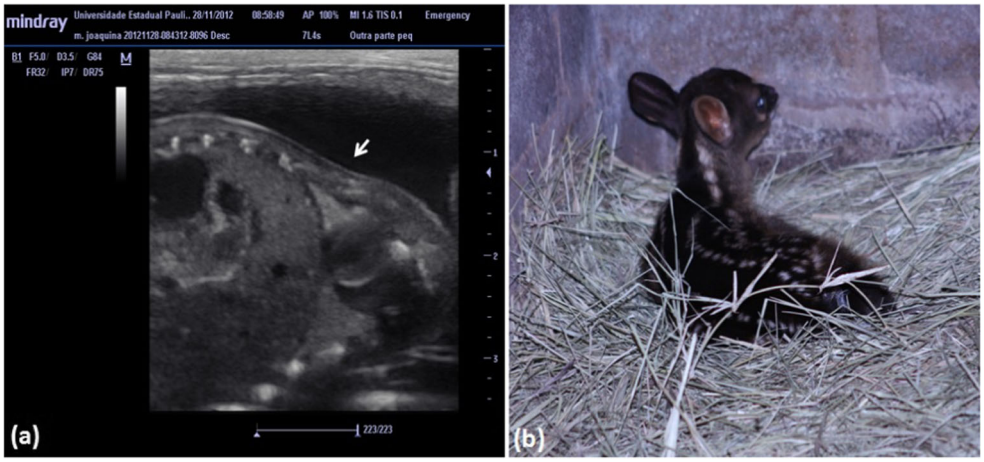

Fig. 2 **a** Ultrasound image (B-mode) showing the fetus, 90 days after AI of a female *Mazama nemorivaga*. **b** Male fawn of the species *M. nemorivaga*, born after transcervical AI by cervix retraction into the vaginal canal

Conclusion

The technique of transcervical artificial insemination, with cervical traction and the use of an inseminating applicator for sheep (with a joint expander function), proved to be effective for the species *M. nemorivaga*, resulting in an uncomplicated pregnancy and the birth of a healthy fawn. Based on the findings discussed herein, this technique could be considered a promising approach to artificial insemination in Neotropical deer, not only because of the short time it took to complete the procedure but because it is also relatively simple, low cost, and minimally invasive.

Compliance with ethical standards All experimental procedures were compliant with the guidelines on the Ethics and Animal Welfare and had been approved by the College of Agricultural and Veterinary Sciences (FCAV) Animal Care Committee, São Paulo State University, Jaboticabal, SP, Brazil (protocol no. 000180/11).

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