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Research article

Reproductive assessment and preliminary evaluation of assisted reproductive technologies in drills (*Mandrillus leucophaeus*)

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Abstract
The drill (*Mandrillus leucophaeus*) is listed as endangered by the IUCN and its population is decreasing due to habitat loss and human activity. The European Endangered Species Programme (EEP) aims to consolidate a self-sustaining ex-situ population, and there is a need to develop appropriate assisted reproductive technologies (ART) that could help to increase breeding rates and/or genetic variability, or at least preserve sexual cells for the future. In 2006, a new breeding male drill arrived at Barcelona Zoo but was unable to mate appropriately with females during oestrus. Several attempts at sperm cell recovery by electro-ejaculation were performed on the breeding male in order to ascertain his fertility, while female oestrous cycles were visually monitored every month. In all, five electro-ejaculation and artificial insemination (AI) attempts were undertaken. Good samples of sperm were gathered and preserved, but the optimal moment for insemination needs more investigation, as no female got pregnant. To our knowledge, this is the first report of attempted AI in drills under anaesthesia and provides some valuable information for the future development of ART in endangered cercopithecids.

Introduction

The drill (*Mandrillus leucophaeus*) is a large terrestrial forest-dwelling primate, endemic to a restricted range of rainforests in southeast Nigeria and western Cameroon (Macdonald 2006; Wood 2007). The drill is one of Africa's most endangered primates as a result of habitat fragmentation and hunting pressure (Linder and Oates 2011). It is currently considered as Endangered on the IUCN's Red List (Oates and Butynski 2008) and is on Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES 2008).

Drills have suffered a population decline of over 50% in the last 30 years (Astaras et al. 2008; Wood 2007); by 1986 the wild population was estimated at fewer than 10,000 individuals, with recent estimates at fewer than 3,000 individuals, and the species has been prioritised by the IUCN/SSC Action Plan for African Primates (Oates 2005). This plan recommended several actions including ex-situ captive breeding programmes. However, there is only a small captive population worldwide, with 78 individuals in Europe, in 14 EAZA institutions at the time of writing.

Recent studies in the wild have reported variable sizes for drill groups, ranging from five to 400 members (Wild et al. 2005; Astaras et al. 2008), although multimale hordes can dissolve into subunits (Gartlan 1970). However, in captivity the current EEP strategy establishes breeding groups based on one male with two or three unrelated females or matrilineal female groups (Martin 2003).

Drills are characterised by high sexual dimorphism (Elton and Morgan 2006; Marty et al. 2009). From data compiled in captive animals, the average length of the menstrual cycle in drills is 29–31 days, including 2–4 days of menses, which can vary between females in terms of duration and blood amount (Zinner and Deschne 2000). During the follicular phase of the reproductive cycle, females show a gradual increase in perineal tumescence with prominent and conspicuous vulval swelling (Zinner and Deschne 2000). These variations in tumescence and coloration reflect changes in oestrogen and progesterone secretion during the menstrual cycle, with peak swelling (two weeks after menses) coinciding with ovulation (Dixson 1983).

On average, female drills experience their first swelling at 2.7±0.63 years old (Wood 2007). The male, by olfactory and

Figure 1. Male inspecting female's anogenital area (Photo M. Martin).

tactile inspection of the genital area, receives complementary information about female receptivity (Figure 1). This species is a relatively slow reproducer since females have a single offspring at a time, with an interbirth interval of approximately 17–19 months (Knieriem 2007; Wood 2007).

Several studies have been carried out in an attempt to improve drill reproduction due to the species' apparently sub-optimal reproductive success in captivity. In the mid 1990s, it was suggested that the low reproductive success of captive animals was due to a high incidence of abnormal behaviour such as inactivity, lethargy, passivity and depression, probably due to environmental conditions (Terdal 1996). To fully understand and address the relatively low reproductive output in zoo populations, Wood (2007) recommended a full comparative study using studbook information, behavioural interactions and facility conditions. Moreover, Durrant et al. (1999) demonstrated that drill semen can successfully be cryopreserved using a simple cooling and thawing technique in order to ensure their genetic contribution through assisted reproductive technologies. However, we could find only one trial of artificial insemination in drills in the literature, at Los Angeles Zoo, where two attempts were carried out without anesthaesia, and no pregnancy resulted (Hogan 1991; Cox 1995).

Despite the drill's low reproductive success in captivity, Barcelona Zoo successfully bred drills within the (European Endangered Species Programme) from 1999 to 2001, with seven surviving offspring. In June 2006 the current breeding male died, and a new male was introduced to the Barcelona group. As the new individual showed serious deficits in social and breeding behaviour, and after consulting the EEP coordinator, the drill group was subjected to fertility assessment and ART procedures. The aims were the successfully recovery of semen by electro-ejaculation, the estimation of ovulation time in females, and to undertake some artificial insemination cycles.

Methods

Animals

In 2006, Biko (ESB number 451; born 22 May 1996) was recommended as the new breeding male for Barcelona Zoo's group. He was introduced to the group, which consisted of four females: Cabinda (ESB 302; born 1985), Inga (ESB 443; born 31 August 1995), Ilachy (ESB 560; born 7 September 2001) and Kuna (ESB 521; born 1 September 2000). Cabinda and Inga were proven breeders, whereas Ilachy and Kuna were young females of breeding age but still nulliparous.

Reproductive assessment in females

Female drills exhibit prominent and conspicuous sexual swellings and reddening in the vulval and perineal areas around ovulation. These swellings generally reach maximum size around the time of ovulation and reflect changes in oestrogen and progesterone secretion during the menstrual cycle (Dixson, 1983). When maximally swollen (phase 4), the skin is red or pink coloured and shiny, and in detumescence (phase 0) the skin is wrinkled and the colour faded (Figure 2). When the next ovarian follicle matures, inflation of the perineal area starts again (phase 1) and in the middle of the cycle the female menstruates (phase 2). The increase in tumescence and skin coloration continues (phase 3) until they reach maximum again (phase +4). Keepers evaluated the sexual cycle of the females daily, carrying out visual monitoring after a period of training to learn to recognise and identify the different grades of swelling. Records were made of blood in the vulval area, mating and birth.

Vaginal smears, ultrasonography and plasma hormone profiles (oestradiol and progesterone) were also performed. All procedures were done under anaesthesia before AI. Females were pre-medicated with benzodiazepines (diazepam at 1–2 mg/kg PO or Midazolam at 0.4–0.5 mg/kg IM (dart)), and darted 15–60 minutes later with a combination of Zoletil® (2–3 mg/kg) and medetomidine (0.04–0.06 mg/kg) for anaesthetic induction. This protocol resulted in consistent surgical anaesthetic planes for 30–40 minutes. Isofluorane via face mask or endotracheal tube was used to maintain anaesthesia.

Vaginal smears were obtained by introducing a cotton-tipped swab into the vagina (Fig. 3). After rubbing the swab against the vaginal wall, the cells were transferred to a glass slide by gently

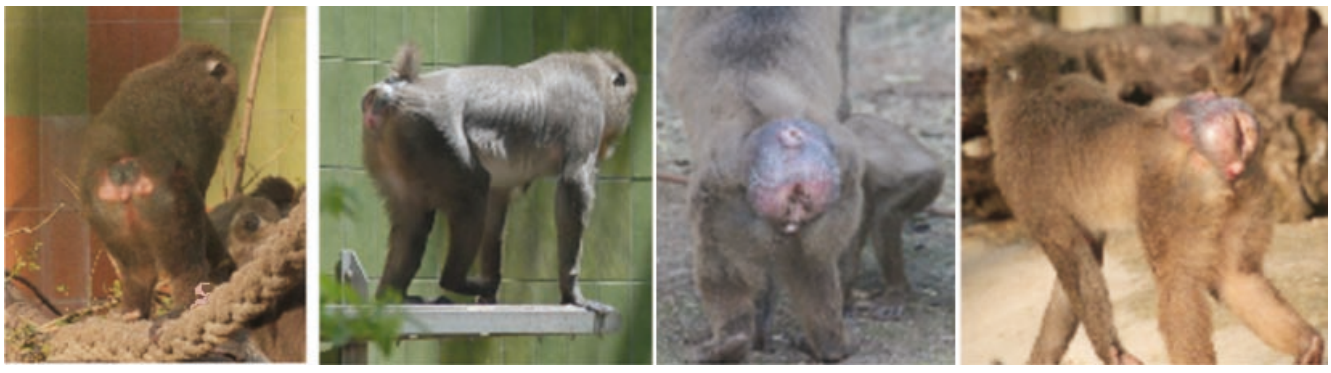

Figure 2. Grade of sexual swelling in female. From left to right: detumescence (0); menstruation (2); maximal swelling (skin not shiny) (4); maximal swelling with shiny skin (+4), the optimal state for mating (Photos: E. Roque).

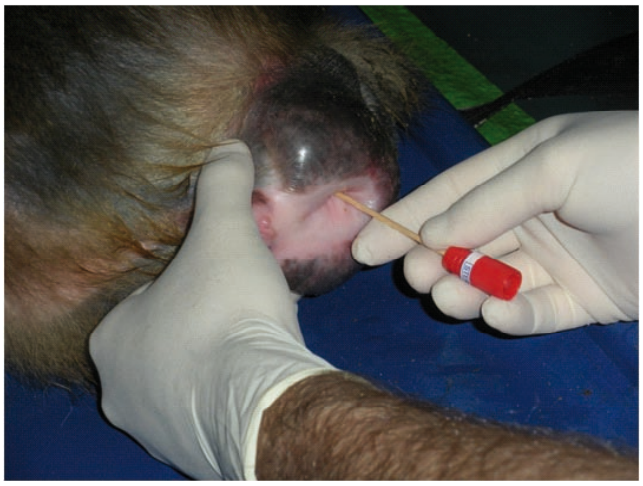

Figure 3. Introducing a cotton-tipped swab into the vagina for vaginal cytology.

rolling the swab. Finally, the smear was allowed to air dry and stained with a Diff-Quick® stain (Baxter S.A., Valencia, Spain). The results of the smear were recorded as the proportions of leucocytes, basal, parabasal, intermediate and superficial cells counted (Thrall and Olson, 1999). Basal cells are small with a discrete amount of cytoplasm and a hyperchromatic nucleus. Parabasal cells are small and rounded, with round nuclei and a small amount of cytoplasm. Large and small intermediate cells have a large cytoplasm and are denoted polygonal, rounded or oval. Superficial cells, the largest and oldest epithelial cells of the vagina, bear a pyknotic nucleus and are occasionally enucleate. The appearance and the relative proportions of these cell types were used to determine the stage of the sexual cycle (Mayor et al. 2007). During proestrus, leucocytes begin to decrease in number, and eventually disappear from smears. Epithelial cells increase in size, and become rounded. Oestrous smears consist predominantly of enucleate, cornified epithelial cells, other cell types being absent. During metaoestrus the cornified epithelial cells begin to clump, and there is a gradual reinvasion of leucocytes until dioestrus is reached, in which leucocytes are the predominant cell type (Izard and Simons 1986).

Blood samples (1.0 ml) were collected into siliconised tubes by femoral venipuncture for plasma oestradiol and progesterone determination. Plasma was removed after centrifugation at 2000×g for 15 min within 2 h of collection and stored at −20◦ C until assayed. Plasma concentrations were measured by a direct, non-extraction radioimmunoassay (ImmuChemTM 17B-Estradi, Biomedicals, Inc.).

Ultrasonographic examinations were conducted by serial transabdominal ultrasonography using a portable B-mode ultrasound scanner equipped with a 5.0 MHz sector-array transducer (Pie Medical, Maastricht, Holland). Females were scanned in lateral recumbence on either the left or right abdominal walls. The duration of the ultrasonography exams ranged from 5 to 10 min per female, scanning the ovaries for the existence of antral follicular structures and uterus (Mayor et al. 2005). The endometrial pattern was classified into two types (Oliveira et al. 1997): pattern I corresponded to multilayers presenting “three marked lines” consisting of hyperechogenic external lines in the limit between endometrium and myometrium, and a central line of identical characteristics separated by a central hypoechogenic region corresponding to endometrial tissue. Pattern II was fully homogeneous and hyperchogenic in relation to the adjacent myometrial tissue. The presence of pattern I appears more likely to favour pregnancy in women after embryo transfer (Oliveira et al. 1997). All examinations were performed by the same operator.

Reproductive assessment in the male

For the reproductive assessment the male was pre-medicated with benzodiazepines (diazepam at 1–2 mg/kg PO or midazolam at 0.4–0.5 mg/kg IM (dart)), and darted 15–60 minutes later with a combination of Zoletil® (2–3 mg/kg) and medetomidine (0.04–0.06 mg/kg) for anesthetic induction. As Zoletil® produced a marked muscular stiffness for a successful electro-ejaculation, an alternative combination was used for the induction based on ketamine (2.9–3.3 mg/kg) plus medetomidine (140–160mg/kg). This protocol resulted in consistent surgical anaesthetic planes for 30–40 minutes. Isofluorane via face mask or endotracheal tube was used to maintain anaesthesia.

After the examination of external genital organs and testis, electro-ejaculation cycles were performed by trans-rectal stimulation using a LifeStim electroejaculator (Minitüb; Tiefenbach, Germany). Ten stimulation cycles of up to 40V and 100–250 mA were applied. The ejaculates were diluted in Kenney’s medium (Kenney et al. 1975) and shipped at room temperature to the

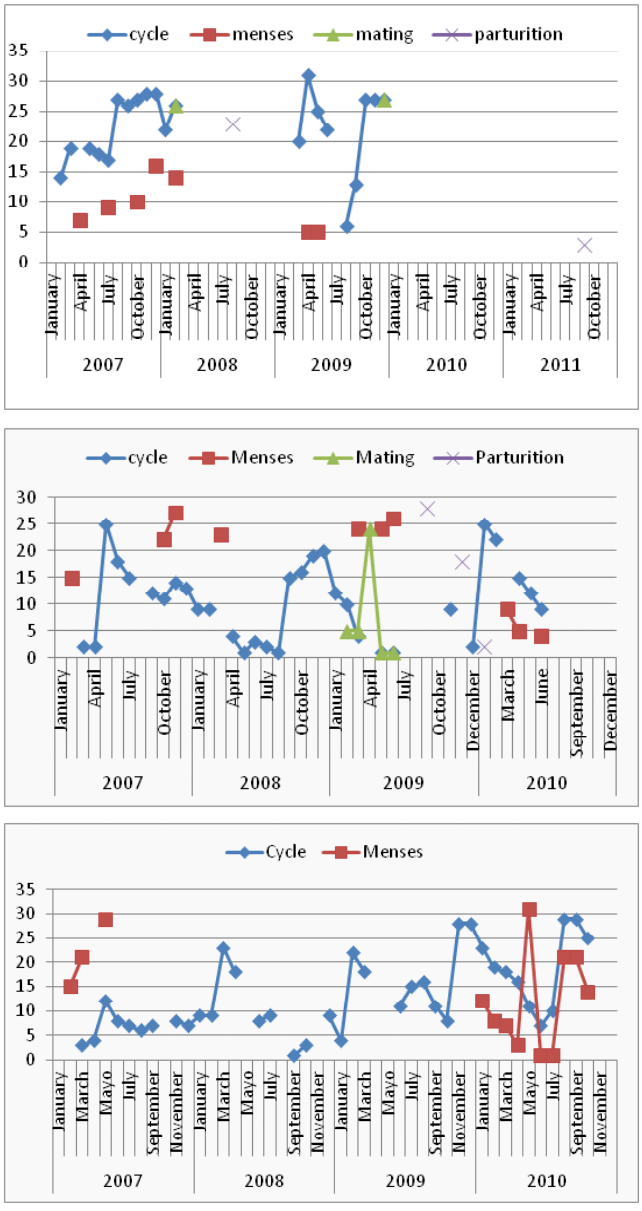

Figure 4. Details of female reproductive cycles. Top: Inga; middle: Ilachy; bottom: Kuna.

Table 1. Semen characteristics obtained after electro-ejaculation cycles.

Date	**Volume (ml)**	Viability (%)	Concentration (spermatozoa/ml)	Total abnormalities (%)	Motility score (from 1 to 4)	Progressivity score (from 1 to 4)	**AI**
15 March 2007	20	78.00%	23.95 x 10^6	46.75% (tails 35.50%)	3	1	No
08 June2007	16	67%	6.42 x 10^6	32.40% (tails 15.50%)			No
22 November 2007	a drop						Yes
07 March 2008	0.5	72.50%	2 x10^6	18.50% (tails 8%)	4	3	No
22 November 2008	2						Yes
17 February 2010	0.5	91.89%	10.92 x 10^6	65.54% (tails 50.13%)	3	3	No
19 March 2010	0.2	76.78%	91.226 · 10^6	16.11% (tails 11.38%)	3	3	Yes (23 March 2010)
11 March 2010	2	71.43%	0.57 · 10^6	50.00% (tails 48.57%)	4	4	No

laboratory. Sperm motility and progressivity were measured in 200 sperm cells under an optical microscope, while sperm viability and total morphological abnormalities were determined after eosin–nigrosin staining (Bamba 1988). The sperm motility of each sample was recorded and analysed on a scale that ranged from 0 to 4, which corresponded to the percentage of sperm showing movement: 0 to 25, 25 to 59, 60 to 84, or 85 to 100%, respectively (modified from Roca et al. 2000). Sperm progressivity was recorded following a subjective scale that ranged from 0 to 4 as follows (WHO, 1992): Grade 1 (progressive sperm): sperm with fast and straight movement; Grade 2 (non-linear motile sperm): motile sperm with curved or crooked motion; Grade 3 (non-progressive sperm): sperm that do not move forward; Grade 4 (no motile sperm): immotile sperm that fail to move at all.

After the initial determination of sperm parameters, samples were prepared for the artificial insemination procedure. When necessary, sperm were stored at refrigeration temperature (4° C) and/or via cryopreservation using a conventional protocol with glycerol as a cryoprotectant.

Artificial insemination

Once the semen samples were obtained by electro-ejaculation, they were pooled and prepared with Kenney medium to obtain a final insemination volume of 0.5 ml.

The female was placed in a prone position after anaesthesia and treated with 500 IU hCG. A vaginal or intracervical insemination was then performed. For the intracervical insemination, a Cook insemination probe (13 cm long) was introduced to the cervix through a vaginoscope. A small amount of Kenney medium was introduced first, followed by the seminal dosage (round 0.5 ml) and finally 1 ml of Kenney medium to facilitate the sperm passing through the probe. After the AI, the female's abdominal area was positioned in an elevated position to help the seminal dosage reach the uterus.

Results

All females showed regular cycles with a mean length of 31.2 days (Fig. 4). However, observations of menses were irregular and it was not detected in all cycles. The peri-ovulatory periods (final 5 days of maximal swelling) of the three female drills rarely overlapped.

Biko displayed a marked inability to cope when the females approached him during oestrus. He showed no interest in the females, and occasionally, he attacked the approaching female, biting their hind quarters. This resulted in significant wounds which required medical treatment, and also caused Cabinda's death in 2006. After that, the male (and occasionally females) was treated with narcoleptics to reduce tension during sexual approaches. The use of narcoleptics worked quite well and mating was observed with Inga and Ilachy in January 2008 and March 2009, respectively. However, despite the fact that medical treatment reduced Biko's aggressiveness, Kuna avoided any kind of approach. Although no

Table 2. Artificial insemination attempts. No pregnancies resulted.

Date	Female	Semen source	Volume (ml)	AI method
22 November 2007	Inga	Electro-ejaculation	a drop	Vaginal
22 November 2008	Inga	Electro-ejaculation	2	Vaginal
17 February 2010	Kuna	Electro-ejaculation	0.5	Intracervical
23 March 2010	Kuna	Frozen sample from electro-ejaculation (19 March 2010)	0.2	Vaginal
11 June 2010	Kuna	Electro-ejaculation	2	Intracervical

Figure 5. Urethral plug obtained after electro-ejaculation. The presence of urethral plugs, probably of bulbourethral gland origin, after electro-ejaculation in other species has been related to the place where the probe is located (sacral vs lumbar region; Concannon et al., 1996) or due to high voltage and/or amperage (Freund, 1969).

Table 3. Reproductive assessment in Kuna.

Date	Oestradiol (pg/ml)	Progesterone (ng/ml)	Swelling record	Ultrasonography	Vaginal smear
21 January 2010	795	0.23	At the beginning of Phase 4	No antral ovarian follicles detected. Triple endometrial line: Pattern 1.	Proestrus
17 February 2010			At the beginning of Phase 4		
19 March 2010	63.2	0.71	End of Phase 4	No antral ovarian follicles detected. Triple endometrial line: Pattern 1.	Proestrus
23 March 2010	177	1.51	At the beginning of Phase 2		Proestrus
11 March 2010	103		Phase 3	Ovarian follicles of different diameters. Endometrial Pattern 2.	Dioestrus

copulations were seen by zoo keepers, some signs (alopecia on both sides of her body) were detected suggesting that the male tried to copulate with Kuna.

From 2007 to 2010, eight electro-ejaculation cycles were performed on the male. Table 1 shows the general characteristics of the ejaculates and sperm cell features. Five AI attempts were performed (Table 2), two on Inga (a proven breeding female) and three on Kuna, all of them unsuccessfully. For the first attempt with Inga in 2007, only a very small amount of semen was obtained from the male, perhaps due to copulation attempts in the days before the procedure. For the following attempt (2008), the male was treated with perphenazine (8 mg PO daily) for 8–10 days before the AI in order to avoid an undesirable ejaculation before the procedure, and 2 ml of semen were obtained. Neither vaginal insemination resulted in pregnancy.

As Inga and Ilachy got pregnant in 2008 and 2009, respectively, all efforts were then focused on Kuna. Hormonal levels, reproductive ultrasonography and vaginal smears were done on Kuna at the same time as the procedure in order to correlate the results with those obtained from the swelling score, and determine the phase of the menstrual cycle (Table 3).

In the first AI attempt with Kuna (February 2010), six seminal fractions were obtained by electro-ejaculation. The first fraction a had very watery consistency, the second, fourth and sixth fractions had a coagulated consistency (Fig. 5), while the third and fifth samples were urine only. The first fraction (0.5 ml), with a high percentage of viable sperm (Fig. 6) and straight movement, was diluted 1:1 (v/v) with Kenney medium and prepared as the insemination dose. Kuna had a sexual swelling score of four, considered to be the optimal time for insemination; however, ultrasonography and a vaginal smear did not reveal the same phase of the menstrual cycle (proestrus, Fig. 7). This suggested that the female would enter oestrus 3–4 days after the AI. Despite this, an intracervical insemination was performed after a dosage of hCG in the hope that sperm would remain inside the uterus, but no pregnancies resulted from this procedure.

For the second AI attempt with Kuna (March 2010), two aqueous fractions were recovered from the electro-ejaculation (0.2 ml). Vaginal smears revealed that Kuna was in proestrus and the procedure was postponed for four days. The sperm sample was then frozen with a commercial extender (Gent B®; Minitüb). Four days later, straws were thawed and re-evaluated for quality. Semen had lower quality but were still motile. Kuna showed less peri-anal swelling than we expected, and ultrasonography and vaginal smears revealed that it was not the best time for AI. However, given Kuna's hormone levels a vaginal insemination was performed, again with no success, and Kuna's menses was detected sixteen days later.

In the last AI attempt performed on Kuna (June 2010) a total of six samples were achieved from the electro-ejaculation procedure, and semen of good quality was obtained from the last ones. Kuna

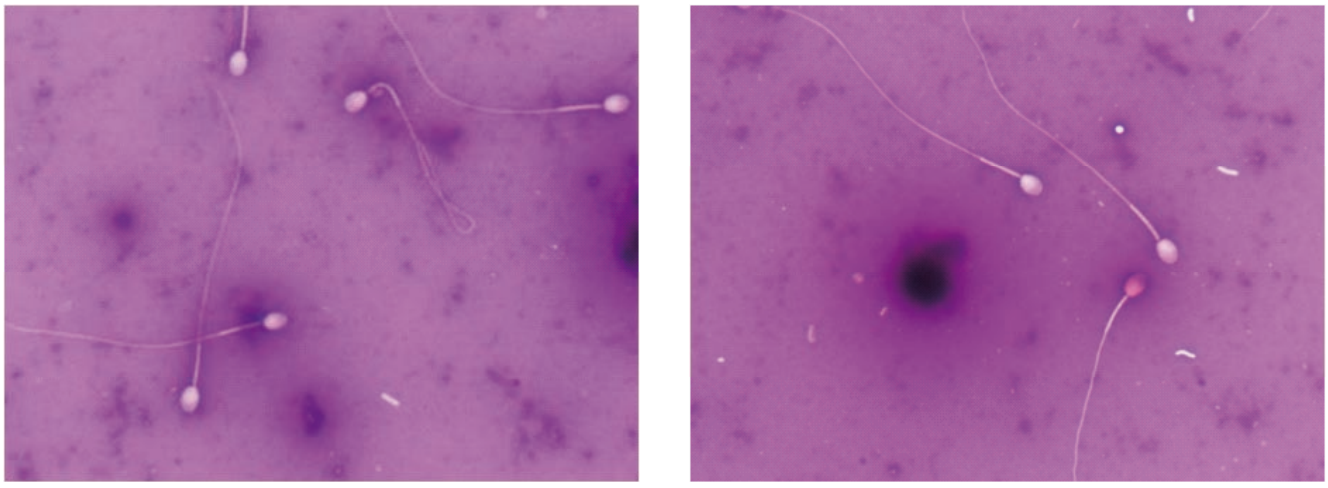

Figure 6. Sperm cells after eosine-nigrosin staining showing (left) five viable sperm cells and (right) two viable (above) and one non-viable sperm cell (below).

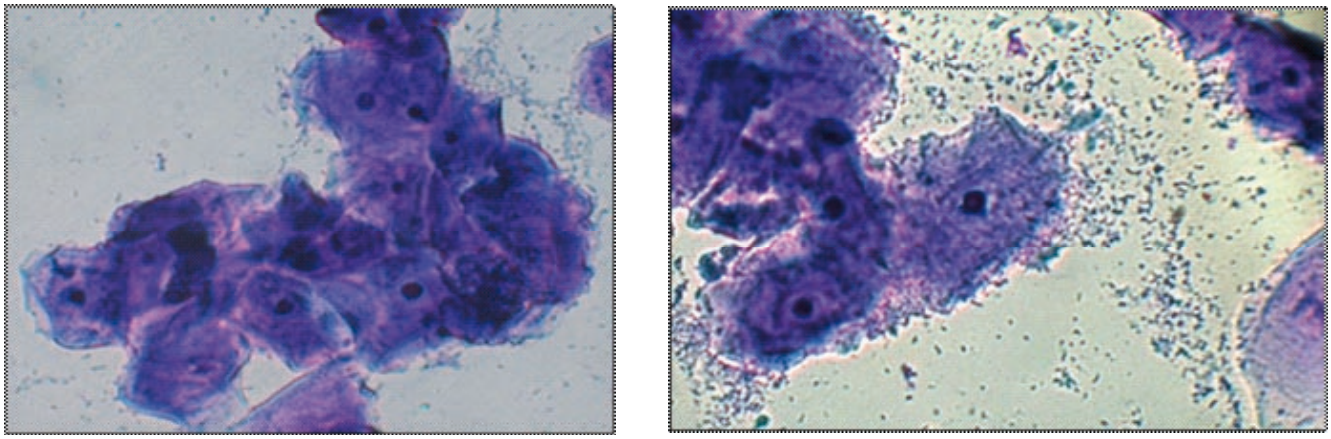

Figure 7. Vaginal smears from Kuna. Proestrus (left) and dioestrus (right) phases of the menstrual cycle.

had a swelling score of three with ultrasonography showing some ovarian follicles of various diameters. The vaginal smear showed some total keratinised cells, indicative of dioestrus (Fig. 7). An intracervical insemination was performed, again with no results.

Discussion

The EEP population of drills has been growing in recent years as a result of the regrouping of some individuals and better knowledge of their management. Nevertheless, artificial breeding techniques could be needed in the near future for this and other cercopithecid species, not only to provide breeding opportunities but also as a possible way to increase genetic diversity in captive populations by insemination with samples from genetically important animals.

At the time this research was initiated the behavioural difficulties with mating shown by the new male in Barcelona, and the impossibility of finding another suitable male in the EEP for the female group, led us to try to find an appropriate ART. Little experience exists with artificial breeding techniques in drills, so the first steps were to find simple and effective methods of evaluating female sexual cycles and recovering semen samples.

Our methods for sperm collection and preparation had good results in terms of viability, motility, and morphological normalcy after electro-ejaculation and cryopreservation. Our results (Maya-Soriano et al. 2010) were consistent with those presented by Durrant et al. (1999), who achieved good viability after thawing sperm samples from two non-breeding males.

The problem we have not been able to solve is how to determine the optimal day for AI in the female, which may at least in part explain why our attempts at AI had no positive results. We obtained inconsistent results using swelling rate of the external genitalia, vaginal smears, ultrasonography or hormone levels. It would probably be possible to determine the best time for insemination exactly by performing serial ultrasonography of the endometrium at different stages of the menstrual cycle, but intensive animal manipulation is not easy, and also not recommended for welfare reasons. More investigations need to be undertaken in order to develop non-invasive procedures for monitoring the menstrual cycle, such as urinary or faecal hormone analysis.

As natural breeding was achieved some time after the AI attempts (see Table 1), we stopped research on the development of ART in drills at Barcelona Zoo. Nevertheless, we hope the information we gathered can help to further the artificial breeding programmes for cercopithecids that will certainly be necessary in the near future.

Conclusion

In this paper, we provide information that could help in the development of artificial reproductive techniques for cercopithecids. ART could be a valuable tool in populations with a lack of natural breeding or in which there is a need to increase genetic variability. We have demonstrated that successful sperm recovery by electro-ejaculation can be achieved with good results. However, more research is needed to identify the best time for AI in female drills.

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