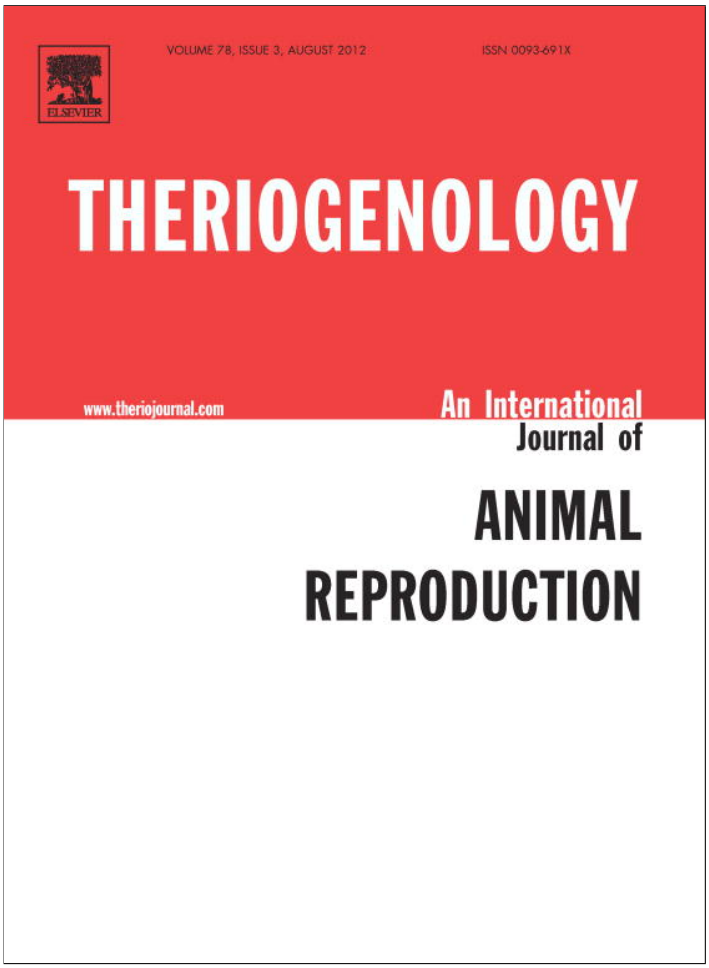

Available online at www.sciencedirect.com

SciVerse ScienceDirect

Theriogenology

Theriogenology 78 (2012) 696–701

www.theriojournal.com

Technical note

Improved semen collection method for wild felids: Urethral catheterization yields high sperm quality in African lions (*Panthera leo*)

I. Lueders[a,*], I. Luther[b,c], G. Scheepers[d], G. van der Horst[b]

[a] *Geolifes-Animal Fertility and Reproductive Research, Frohmestr. 7, 22 457 Hamburg, Germany*
[b] *Department of Medical Biosciences, University of the Western Cape, Private Bag X17, Bellville 7535, South Africa*
[c] *National Zoological Gardens of South Africa, 323 Boom Street, Pretoria 0110, South Africa*
[d] *Zodiac Dierekliniek, P O Box 460, brits 0250, South Africa*

Received 13 December 2011; received in revised form 17 February 2012; accepted 19 February 2012

Abstract

For wild and domestic felids, electroejaculation (EE) is the most common semen collection method. However, the equipment is expensive, there is a risk of urine contamination and animals usually show strong muscular contraction despite general anesthesia. Accordingly, we tested the feasibility of a different approach using urethral catheterization (UC) in seven African lions, previously described for domestic cats only. After general anesthesia with the α2-agonist medetomidine (which also stimulates semen release into the urethra) and ketamine, a transrectal ultrasound was performed to locate the prostate. A commercial dog urinary catheter (2.6 or 3.3 mm in diameter) was advanced approximately 30 cm into the urethra to allow semen collection into the lumen of the catheter by capillary forces. After retraction, sperm volumes between of 422.86 $\pm$ 296.07 μl yielded motility of 88.83 $\pm$ 13.27% (mean $\pm$ SD) with a mean sperm concentration of 1.94×10^9/ml. Here we describe a simple, field friendly and effective method to attain highly concentrated semen samples with excellent motility in lions and potentially other wild felid species as an alternative to electroejaculation.

Keywords: Urethral catheter; Electroejaculation; Wild cats; Semen sampling

1. Introduction

Semen collection in zoo and wild animals is warranted for fertility assessment, genome banking and preservation as well as for assisted reproduction or research purposes. Various collection methods are described in domestic as well as different wild mammalian species. Generally, there are three approaches: i. non-repeatable methods, such as collection of sperm from the epididymis or vas deferens after 1) castration/sterilization or death (e.g., cat [1], vervet monkey [2]); 2) repeatable methods in trained or conditioned animals, such as use of artificial vaginas (AV) and phantoms (e.g., horse [3]), triggered spontaneous ejaculation (e.g., dolphin [4]); manual stimulation of the penis (e.g., dog [5]); or the prostate (e.g., elephant [6]); and 3) repeatable methods in anesthetized or sedated animals, comprised of electroejaculation (EE) (e.g., rhino [7]), or chemical induction of ejaculation (e.g., horse [8]).

In domestic cats, different semen extraction methods are described [9]. Repeatable methods reported are electroejaculation [10], training of males using an

* Corresponding author. Tel: 0049 163 2641197.
E-mail address: imke.lueders@gmail.com (I. Lueders).

0093-691X/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2012.02.026

Table 1
Animals examined during this study and sperm analysis results.

Animal no	Age (yrs)	Catheter diameter (mm)	Volume (μl)	Concentr ($\times 10^9$ /ml).	Total conc. per ejaculate ($\times 10^9$)	% motile HF10	% prog HF10	% motile BO	% progBO
#1	6	3.3	500	2.29	1.15	89.2	71.3	95.6	87.6
#2	6	3.3	260	4.86	3.65	97.7	78.2	97.2	84.2
#3	6	3.3	1000	1.33	1.33	94.4	84.1	97	93.5
#4*	4	3.3	100	0.125	0.013	39	18.9	62.5	35
#5*	10	2.6	500	2.53	1.27	94.8	88.3	98	95.5
#6	4	3.3	400	0.228	0.092	85.9	67	79.5	68.5
#7	4	2.6	200	2.22	0.444	88	74.8	92	82
Mean	**5.71**		**422.86**	**1.94**	**1.14**	**84.14**	**68.94**	**88.83**	**78.04**
SD	**2.14**		**±296.07**	**±1.61**	**±1.24**	**±20.35**	**±23.23**	**±13.27**	**±20.95**

HAM F-10, Ham's F-10 culture medium; BO, Bovine culture medium.
* , proven breeder.

artificial vagina and a queen in heat [11], and recently urethral catheterization (UC) [12]. The latter method ("Zambelli" method) has been described only in domestic cats [12]. This innovative method utilizes the α-adrenergic effect of the drugs used for anesthesia. Alpha-adrenoreceptors are believed to mediate the response to adrenergic agonists for contraction of the ductus deferentes [13]. Therefore, narcotics, such as medetomidine or xylazine (α2-agonists) appear to enhance the contraction of the deferent ducts and the ampullae ductus deferens [8]. Subsequently, semen is released into the urethra. Higher sperm concentrations were retrieved when administering medetomidine compared to ketamine anesthesia in cats [14]. The highly concentrated semen may be collected through simple insertion of a catheter [12,15]. The collected sperm had freezing and *in vitro* fertilization abilities comparable to semen retrieved via electroejaculation [12].

Because of practical, technical and safety reasons, larger wild felids in captivity need general anesthesia to be handled. Even though the training for AV was successful in a cheetah [16], currently, electroejaculation is the method of choice in untamed or free-ranging felid species [17–20]. However, expensive equipment and an experienced operator are required. Furthermore, because of the electric stimulus, contraction of the bladder and subsequent urine contamination of the semen sample do occur in many instances. Therefore, we investigated the feasibility of the "Zambelli" method in the African lion as a model for large wild cat species. Here we demonstrate that UC is a less invasive alternative to EE and therefore may also be applicable to other wild felids.

2. Materials and methods

2.1. Lions

Seven captive bred, adult male African lions (*Panthera leo*) kept at a breeding ranch in South Africa were used for this trial. The animals were from 4 to 10 years of age. They were either breeders housed with four to six females or non-breeders housed in bachelor groups consisting of three to six brothers and half-brothers (Table 1). All animals were maintained in outdoor enclosures structured with trees and shelters. The bachelor groups had visual and olfactory contact with females. All animals were healthy and in good body condition. The research work on the animals was carried out in accordance with the Directive 2010/63/EU EEC for animal experiments.

2.2. Anesthesia

The animals were immobilized for routine health checks or translocation using a combination of 12 mg medetomidine and 150 mg ketamine. The drugs were delivered intramuscularly via remote dart gun in 3.0 ml darts.

The lions were accessible for semen analysis 10 to 15 min after darting. The procedures took 1.0 to 1.5 h after which anesthesia was reversed with 43.75 mg of yohimbine (7 ml/ lion) hand injected IV. The animals subsequently recovered within a few minutes from anesthesia.

2.3. Semen collection

Each lion received an enema to facilitate a transrectal ultrasound of the prostate to determine the depth of urethral catheterization (Fig. 1). The ultrasound was

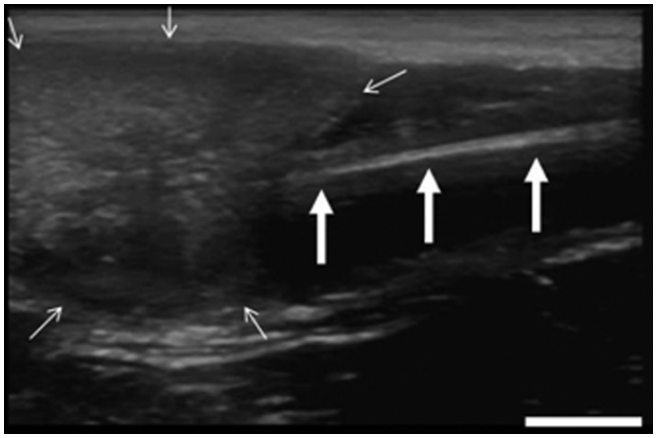

Fig. 1. Ultrasound image of the urinary catheter (large arrows) within the urethra advanced up to the prostate (small arrows). Transrectal scanning, in longitudinal section (bar indicates 1 cm).

performed using a portable ultrasound device (Logic e, General Electrics Healthcare, GmbH, Solingen, Germany) equipped with a 7 to 10 MHz rectal, linear probe mounted to a PVC extension. The straight, hollow extension had a diameter of 2 cm and total length of 30 cm with a slightly bend handle of 15 cm. After ultrasonography, the region around the penis was cleaned with water and dried. The penis was subsequently extended beyond the prepuce and the shaft was fixed with a surgical forceps (Fig. 2). The penis was then cleaned with ProntoSan spray (polyhexanid-betain-complex, Prontomed, GmbH, Hiddenhausen, Germany) and wiped with sterile gauze. The catheterization of the urethra started 20 to 40 min into anesthesia to allow the effect of medetomidine to induce spermatozoa release. A commercial dog urinary catheter (Buster, sterile dog catheter, WDT, Garbsen, Germany), either 2.6 mm × 500 mm (Lion Number 5 and 7) or 3.3 × 500 mm (Lion Number 1–4, 6), was lubricated on the tip with non-spermicidal sterile lubricant (Priority Care, First Priority, Inc, IL, USA) before insertion into the external urethral opening. Differing from the original trials in tomcats [12], the catheters were not cut at the tip, but were used as they came, with a rounded closed tip and two lateral openings. A second examiner palpated the urethra transrectally to check for catheter passage at the point of the pelvic flexure. The catheter was advanced until reaching the prostate (Fig. 1). Immediately, the catheter was slowly retracted and checked for semen content (Fig. 2). Semen was ejected from the catheter with a syringe directly into a prewarmed plastic 1.5 mL Eppendorf tube. In three lions, the catheter was inserted a second time.

2.4. Sperm analysis

A Nikon E50i microscope with a warmed stage (37 °C) was used for all sperm motility studies. A Basler 602f 100fps digital camera was fitted to the microscope and was connected to a computer. In conjunction with the Basler camera the Sperm Class Analyzer (SCA) (version 5.1.0.1, Microptic SL, Barcelona, Spain) is a computer aided semen analysis system (CASA) that accurately measures sperm concentration, the percentage of motile sperm, the percentage progressively motile sperm and eight kinematic parameters of sperm. In this investigation the kinematic parameters were excluded. For each semen sample, 2 to 5 μl was loaded using a micro pipette into a prewarmed "chambered" CE Leja slide (20 μm deep with either a 2 or 5 μl volume, Leja Products B.V., Nieuw Vennep, the Netherlands). In most instances sperm concentration was too high to evaluate sperm motility accurately in neat semen. Semen was accordingly diluted in either Ham's F-10 culture medium (Ham F-10) or Bovine culture medium (BO) based on the previously published modified version [21]. At least 500 motile sperm were analyzed with SCA for each lion in each medium. Sperm motility was captured with the SCA system at 100 frames per sec. Three samples of neat semen from lion Number 1, 3 and 5 were reanalyzed 6 h after collection and storage at 4 °C. For motility analysis, samples were also diluted in BO medium as described above. In three lions, ejaculate smears were stained with SpermBlue (Microptic SL, Barcelona, Spain), to assess sperm vitality and morphology. Statistics were performed using GraphPad InStat (GraphPad Soft-

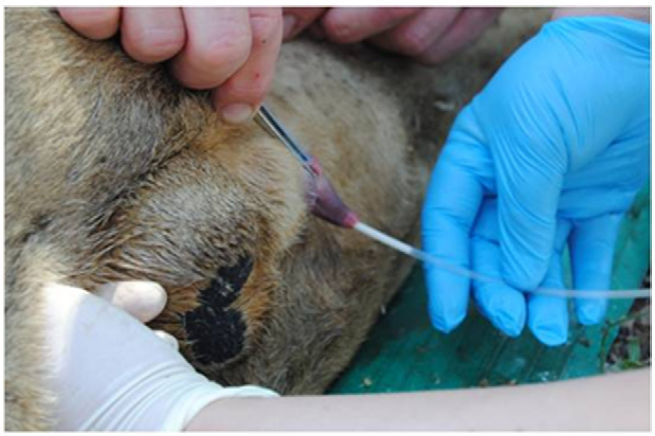

Fig. 2. Retraction of the urethral catheter yielding a thick white semen sample. Note the transrectal palpation (white gloved hand) to determine the passage of the pelvic flexure by the catheter within the urethra. Photo credit: Steve and Bieke De Raeymaeker.

Table 2
Results of semen samples assessment for pH, vitality and morphology.

Animal no	pH	% normal morphology	% abnormal morphology	Vitality: % live
#1	7.5	56.0	44.0	*Nm*
#2	7.0	48.0	52.0	*Nm*
#3	*nm*	75.0	25.0	*Nm*
#4*	*nm*	17.0	83.0	65.0
#5*	7.0	61.0	39.0	77.0
#6	*nm*	60.0	40.0	*Nm*
#7	*nm*	5.0	95.0	57.0
Mean	**7.2**	**46.0**	**54.0**	**66.3**
SD	**±0.3**	**±25.5**	**±25.5**	**±10.1**

ware, Inc., La Jolla, CA, USA). Values are presented as mean ± standard deviation (SD).

3. Results

Semen was obtained from all seven animals whether in breeding groups or housed with other males only. In one case (Lion no. 1), digital rectal massage resulted in partial ejaculation, and about 30 μl of sperm were collected from the tip of the penis before catheterization. Catheter diameter of 2.6 and 3.3 mm in diameter worked equally well. The catheter was advanced about 25 to 35 cm into the urethra until the prostate was reached. The semen samples were collected within the first 4 to 12 cm from the tip of the catheter. Volumes attained varied between 100 μl and 1000 μl (mean: 422.86 ± 296.07 μL Table 1). Sperm concentration ranged from 125 × 10^6 /ml to as high as 4.86 × 10^9/ml (mean: 1.94 × 10^9/mL). The mean percentage of motile sperm was 84.14 ± 20.35% in Ham F-10 and 88.83 ± 13.27% in BO, respectively (Fig. 2). Both media sustained very high percentage sperm motility. Although no significant differences was seen in the absolute motility in BO and Ham's F-10 medium (paired *t* test: P = 0.23, *t* = 1.340, df = 6), the percentage of progressive motility was significantly higher in BO medium (paired *t* test: P = 0.004. *t* = 4.451, df = 6). All but one lion (#4) showed motility >79.5% with progressively motile spermatozoa of 67 to 95%. A high percentage of 54.0 ± 25.5% abnormal sperm of was observed (Table 2). Vitality staining was performed in three lions and revealed 66.3 ± 10.1% live sperm (Table 2). The pH of the ejaculates was at 7.2 ± 0.3 in three lions (Fig. 1). A second catheterization in three animals yielded no additional sperm retrieval. Three samples of neat semen (lion Number 1, 3 and 5) were reanalyzed after 6 h at 4 °C and showed a 5% decrease in motility.

4. Discussion

The UC after medetomidine induction of seven lions showed consistent results. Capillary forces worked as good as in tomcat catheters described in previous studies, despite the larger diameter of dog catheters. Ejaculate volumes obtained were small, but had higher sperm concentrations. This is comparable to the findings in domestic cats, where UC semen volumes were smaller but higher concentrated compared to EE semen samples [12].

Ejaculates retrieved from captive Asian lions (*Panthera leo persica*) via EE were reported to have a volume of 3.94 ± 2.4 ml and a concentration of 52.1 ± 25.1 × 10^6/ml [22]. In a study on wild African lions, ejaculate volumes were as high as 9.4 ± 1.4 ml with a concentration of 236.0 ± 93.0 × 10^6/ml [23]. However, our UC data show an average sperm concentration that is almost ten times higher than in the investigations of Wildt, et al. 1987 [23]. It would be necessary to directly compare the same individuals with the two collection methods, EE and UC, to draw final conclusions. Yet, similar results were retrieved comparing EE and UC samples in tomcats [12]. Zambelli, et al. 2008 also showed that the sperm quality parameters did not differ between EE and UC sperm samples in domestic cats [12]. However, besides a higher concentration, with a lower total volume, UC semen showed lower pH values [12]. Although we could not find any published data on African lion semen pH, the values determined in our study were considerably lower compared to reports on Asian lion semen pH obtained via electroejaculation [22]. In the Asian lion the pH was 7.9 on average compared to our observations of 7.2. The stimulation during EE may increase accessory sex gland fluid content in the collected ejaculates compared to the UC method. In tomcats, small differences in seminal plasma proteins in EE ejaculates were attributed to prostatic and bulbourethral contribution, which may be less during UC [15].

The BO medium showed better results in maintaining high numbers of progressively motile sperm. Due to the use of sophisticated equipment, we measured the motility at 100 frames per sec, therefore picking up even slight differences. We also found a high number of abnormal sperm. The occurrence of teratospermie is not unusual in felids [18,22,23]. It appears that lions compensate through higher frequency of copulation and high concentration of sperm. However, Lion 4 had an unusual low motility and a high percentage of abnormal sperm (Tables 1 and 2). His offspring was reported to show additional toes and fur discoloration, which could possibly be related to his diminished

sperm quality. In *in vitro* fertilization trials, there were no differences reported in the ability to fertilize between spermatozoa obtained via UC and EE in tomcats [14]. This also needs to be determined in lions and other felids in the future.

Urine contamination, a common problem during electroejaculation, may be completely avoided, if the UC is applied correctly. Therefore, in this study, the depth of the prostate was previously ultrasonographically determined to avoid catheterization of the urinary bladder (Fig. 1). The catheter therefore should not be pushed further than about 30 cm into the urethra of an adult lion.

This inexpensive and field-friendly method does not involve any costly equipment. The only prerequisite is anesthesia performed with an α2-agonist to achieve the α-adrenergic effect on the vas deferens. Additional rectal massage and prostate stimulation (e.g., during transrectal ultrasonography of this gland) may help with sperm release into the urethra.

Because of the excellent quality (high concentration, motility and longevity) of the sperm samples obtained through UC, semen could be used for artificial insemination or cryopreservation. The catheter collected semen may be preferred for freezing because it appears to contain less seminal plasma that may be damaging to sperm during freezing in some species [24]. In rare and endangered felids, this method may be applicable to advance the research on freezing techniques and assisted reproduction measures. Semen collection in a Bengal tiger (*Pathera tigris tigris*) (our unpublished data) and a cheetah (*Acinonyx jubatus*) (personal Communication Dr Peter Rich, African Lion Safari, Ontario, Canada) applying the described method, showed similar good results. These findings underline the successful use in different felid species. In summary, we showed that UC is a simple and repeatable semen collection method not only in tomcats [12], but also in the African lion and it appears to have an excellent potential in other wild felids.

Acknowledgments

Prof. van der Horst received 10.000 RSA, Rand from Microptic SL, Barcelona, Spain to finance travels, accommodation and disposables during this study for all authors.

The work described has been carried out in accordance with the Directive 2010/63/EU EEC for animal experiments.

The authors thank Willi sen, Willi jr., and Gilian Jacobs from Ukutula Lion Park, RSA for making their animals available to us, Prof. Antoinette Kotze (National Zoological Gardens, Pretoria, RSA) for technical support as well as Microptic SL, Barcelona, Spain for funding this trial.

References

[1] Hay MA, Goodrowe KL. Comparative cryopreservation and capacitation of spermatozoa from epididymis and vasa deferentia of the domestic cat. J Reprod Fertil Suppl 1993;47:297–305.
[2] Van der Horst G, Seier JV, Spinks AC, Hendricks S. The maturation of sperm motility in the epididymis and vas deferens of the vervet monkey, *Cercopithecus aethiops*. Int J Androl 1999;22:197–207.
[3] Richardson GF, Wenkoff MS. Semen collection from a stallion using a dummy mount. Can Vet J 1976;17:177–80.
[4] Keller KV. Training of the Atlantic bottlenose dolphins (*Tursiops truncates*) for artificial insemination. Int Assoc Aquatic Anim Med 1986;14:22–4.
[5] Kutzler MA. Semen collection in the dog. Theriogenology 2005;64:747–54.
[6] Schmitt DL, Hildebrandt TB. Manual collection and characterization of semen from Asian elephants (*Elephas maximus*). Anim Rprod Sci 1998;53:309–14.
[7] Roth TL, Stoops MA, Atkinson MW, Blumer ES, Campbell MK, Cameron KN, et al. Semen collection in rhinoceroses (Rhinoceros unicornis, Diceros bicornis, Ceratotherium simum) by electroejaculation with a uniquely designed probe. J Zoo Wildl Med 2005;364:617–27.
[8] Johnston PF, DeLuca JL. Chemical ejaculation of stallions after the administration of oral imipramine followed by intravenous xylazine. Proc Ann Conv AAEP 1998;44:12–5.
[9] Zambelli D, Cunto M. Semen collection in cats: techniques and analysis. Theriogenology 2006;66:159–65.
[10] Platz CC, Seager SW. Semen collection by electroejaculation in the domestic cat. J Am Vet Med Assoc 1978;173:1353–5.
[11] Dooley MP, Pineda MH, Hopper JG, Hsu WH. Retrograde flow of spermatozoa into the urinary bladder of cats during electroejaculation, collection of semen with an artificial vagina, and mating. Am J Vet Res 1991;52:687–91.
[12] Zambelli D, Prati F, Cunto M, Iacono E, Merlo B. Quality and in vitro fertilizing ability of cryopreserved cat spermatozoa obtained by urethral catheterization after medetomidine administration. Theriogenology 2008;69:485–90.
[13] MacDonald A, McGrath JC. The distribution of adrenoceptors and other drug receptors between the two ends of the rat vas deferens as revealed by selective agonists and antagonists. Br J Pharmacol 1980;71:445–58.
[14] Zambelli D, Cunto M, Prati F, Merlo B. Effects of ketamine or medetomidine administration on quality of electroejaculated sperm and on sperm flow in the domestic cat. Theriogenology 2007;68:796–803.
[15] Zambelli D, Raccagni R, Cunto M, Andreani G, Isani G. Sperm evaluation and biochemical characterization of cat seminal plasma collected by electroejaculation and urethral catheterization. Theriogenology 2010;74:1396–402.
[16] Durrant BS, Schuerman T, Millard D. Noninvasive semen collection in the cheetah. Proc Ann Mtg Am Assoc Zoo Pks Aquar, 1985; pp. 564–56.
[17] Wildt DE, Bush M, Howard JG, O'Brien SJ, Meltzer D, van Dyk A, et al. Unique seminal quality in the South African

cheetah and a comparative evaluation in the domestic cat. Biol Reprod 1983;29:1019–25.
[18] Wildt DE, Phillips LG, Simmons LG, Chakraborty PK, Brown JL, Howard JG, et al. A comparative analysis of ejaculate and hormonal characteristics of the captive male cheetah, tiger, leopard, and puma. Biol Reprod 1988;38:245–55.
[19] Jayaprakash D, Patil SB, Kumar MN, Majumdar KC, Shivaji S. Semen characteristics of the captive Indian leopard, *Panthera pardus*. J Androl 2001;22:25–33.
[20] Goritz F, Neubauer K, Naidenko SV, Fickel J, Jewgenow K. Investigations on reproductive physiology in the male Eurasian lynx (Lynx lynx). Theriogenology 2006;66:1751–4.
[21] Brackett BG, Zuelke KA. Analysis of factors involved in the in vitro production of bovine embryos. Theriogenology 1993;39: 43–64.
[22] Shivaji S, Jayaprakash D, Patil SB. Assessment of inbreeding depression in big cats: testosterone levels and semen analysis. Curr Sci 1998;75:923–30.
[23] Wildt DE, Bush M, Goodrowe KL, Packer C, Pusey AE, Brown JL, et al. Reproductive and genetic consequences of founding isolated lion populations. Nature 1987;329:328–31.
[24] Moore AI, Squires EL, Graham JK. Effect of seminal plasma on the cryopreservation of equine spermatozoa. Theriogenology 2005;63:2372–81.