FULL PAPER *Theriogenology*

Electroejaculation and Semen Cryopreservation of Free-Ranging Japanese Black Bears (*Ursus thibetanus japonicus*)

Tsukasa OKANO[1)], Tetsuma MURASE[1,2)] and Toshio TSUBOTA[1,3)]

[1)]The United Graduate School of Veterinary Science, Gifu University, Gifu 501–1193, [2)]Laboratory of Theriogenology, Faculty of Applied Biological Sciences, Gifu University, Gifu 501–1193, [3)]Laboratory of Zoo and Wildlife Medicine, Faculty of Applied Biological Sciences, Gifu University, Gifu 501–1193, Japan

(Received 6 April 2004/Accepted 29 June 2004)

ABSTRACT. The Japanese black bear (*Ursus thibetanus japonicus*) is endangered for extinction in some areas of Japan, and semen collection and cryopreservation are an important means to preserve genetic resources. The aim of this study was to characterize and cryopreserve semen of free-ranging Japanese black bears. Semen was collected by electroejaculation procedure from 4 free-ranging Japanese black bears at the capture point in the field. Ejaculates containing motile sperm were recovered from all of the animals and ejaculate volume, total sperm count, % motility (percentage of motile spermatozoa), % viability (percentage of spermatozoa that excluded eosin) and % abnormal morphology (range (mean)) were 0.65–2.20 (1.51) m*l*, 99–1082 (490) $\times$ 10^6, 5–100 (31), 42–97 (66) and 20–87 (53), respectively. Three of the 4 ejaculates were diluted with an egg yolk-TRIS-citrate-glucose extender and cryopreserved in liquid nitrogen. Motile spermatozoa were observed after freezing and thawing in all cases. This study showed that electroejaculation would be a useful method for collecting semen from free-ranging Japanese black bears and that at least motile spermatozoa would be obtained by fre ezing the thus collected electroejaculates.

KEY WORDS: cryopreservation, electroejaculation, free-ranging, Japanese black bear, semen.

J. Vet. Med. Sci. 66(11): 1371–1376, 2004

The Japanese black bear (*Ursus thibetanus japonicus*) is a large mammal that inhabits the islands of Honshu and Shikoku in Japan. In recent years, their habitats have become fragmented and shrunken by human activity, and this species even faces extinction in some areas [7]. What is worse, bears have been killed to protect forestry, agriculture, livestock and humans from damage caused by them.

Endangered and threatened wild animals are now attracting attention as a genetic resource and for species conservation, and semen has been recovered from various captive non-domestic animals, including fat-tailed dunnarts, koalas, brushtail possums, long-footed potoroos, northern brown bandicoots and ring-tailed possums [32], blesboks, dorcas gazelles and onagers [11], chacma baboons [3], jaguars [21], Indian tigers and Asiatic lions [29] and cheetahs [34]. As to Ursidae, there are reports on electroejaculation and/or semen cryopreservation in giant pandas [4, 20, 22, 24], Hokkaido brown bears [13, 14] and Japanese black bears [16]. However, these studies were done with captive animals, and there are no reports on semen collection and cryopreservation in free-ranging bears. Cryopreservation of semen collected from free-ranging animals is one of the most important methods for preservation of genetic resources and useful for artificial breeding. Moreover, characteristics of semen from wild animals may be essential for understanding reproductive status under field conditions, although studies using captive animals, kept under controlled conditions, may provide valuable data to analogize state under field conditions. It is easy to expect that the state of captive animals may differ from that of free-ranging ones to some extent, because of differences in their living environment and diet. It is difficult to obtain semen from free-ranging Japanese black bears due to limited access to the animals and seasonality of their sperm production [17]. Fundamental data on reproductive characteristics are of primary importance to understand the effects of genetics and diseases on fertility, establish captive breeding colonies or chose an appropriate conservation strategies for wild populations [35].

The objective of this study was to characterize and preserve semen collected by electroejaculation in free-ranging Japanese black bears.

MATERIALS AND METHODS

Capture and handling: A total of 4 free-ranging, mature male Japanese black bears that inhabited Neo Village, Gifu Prefecture, Japan, were captured by barrel-type traps in July each year of 2000–2002. Bears were handled under field conditions at the site of capture and released at the same site after the experiment. Trapped bears were immobilized by an intramuscular administration of Zolazepam HCl and Tiletamine HCl (Zoletil, Virbac, Carrors, France: 9 mg/kg) via a dart blowgun. After immobilization, bears were weighed and their physical condition was monitored for body temperature, pulse and respiration. The second *dentes premolare* from either the left or right upper side was extracted for age determination.

Electroejaculation: Electroejaculation was performed according to Kojima *et al.* [16] with some modifications. Briefly, we used RV GEAR portable power (AC 100 V, DC 12 V; National, Osaka, Japan) as a power supply in the field. The animals were restrained in dorsal the position and the preputial area was shaved. The penis was washed succes-

sively with diluted benzalkonium chloride, warm tap water and sterile physiological saline. The bladder was emptied by catheterization (5-fr. polypropylene urinary catheter; Sovereign, Sherwood Medical, MO, U.S.A.). A collection catheter whose tip had been cut off to widen the hole (8-fr. Sovereign) was inserted into the urethra about 10 cm deep and held during collection, and a sterile glass test tube (15 × 100 mm) covered the tip of the collection catheter to collect the semen. The rectal probe used was a tube made of vinyl chloride (20 × 500 mm) with two copper rings (8 mm wide) spaced 20 mm apart. The copper electrodes were connected to electrical stimulator (Electric Stimulator, sine wave; Fujihira Industry, Co., Ltd., Tokyo, Japan). The probe was inserted 12 to 21 cm into the rectum and positioned so that it was in contact with dorsal surface of the rectum. Stimulation began at 1 V, and then raised in increments of 1 V up to 10 V (This same voltage was used for up to 11 stimuli). Each stimulus lasted 5 seconds with a subsequent pause for 10 seconds. This cycle was repeated either until semen was ejaculated or until 20 stimuli (1 to 10 V once each and 10 V × 10 times) were given. When we judged ejaculation might be occurring soon at the next stimuli, we gave an additional 5 stimuli. When semen was not ejaculated during the first set of 20 stimuli, another set of stimuli was given after 1 to 2 min. If the amount of collected semen was small, another set of stimuli was carried out. A maximum repetition of 4 sets was performed. Semen collected was forced into the test tube by gently supplying air.

Semen examination and cryopreservation: Examination and cryopreservation of semen was performed according to the method that we used in dogs [16] with some modifications. Briefly, when more than one tube of semen sample was collected during one anesthesia period, each was analyzed. The appearance was examined. The presence of urine was determined by its yellow color. Ejaculate volume was measured with the aid of a micropipette. Sperm concentration was estimated by haemocytometer. Motility was evaluated by subjective observation under microscope (× 400) at 38°C and categorized into 5 groups: +++ (motile with progression at a high speed), ++ (motile with progression at a moderate speed), + (motile with progression at a low speed), ± (motile without progression) and – (immotile). Each category was presented in percentage (%), and the results are shown as percentage of spermatozoa exhibiting a motility grade of +++ (% +++ motility) or that of spermatozoa with ± or higher motility grades (% motility). Viability was assessed by a supravital stain with eosin (Eosin Y; Merck, Darmstadt, Germany) and nigrosin (Tokyo Chemical Industries Co., Ltd., Tokyo, Japan). Spermatozoa were fixed by mixing with an equal volume of 2% v/v glutaraldeyde in 0.165 M sodium cacodylate/HCl, pH 7.3. Samples were fixed for over 30 min at ambient temperature then stained for 2 hr at room temperature with Giemsa staining solution (7.5% v/v commercial Giemsa solution in 5 mM phosphate). A total of 100 spermatozoa were counted under a microscope (× 1,000), and the proportion (%) of morphologically abnormal spermatozoa was obtained (% abnormal morphology). Slides were prepared with the glutaraldehyde-fixed sample and a total of 100 sperm were counted. Acrosomes with a dense apical ridge were considered acrosome-intact and the proportion (%) of spermatozoa with intact acrosomes was expressed (% intact acrosomes)[28].

The semen considered to be highly contaminated with urine was centrifuged at 500 × g for 5 min, and the supernatant was discarded to remove urine. Then, pelleted spermatozoa were resuspended in sterile physiological saline.

The semen sample was diluted about 2- to 4-fold by visual estimate at ambient temperature in the field with an egg yolk-TRIS-citrate-glucose extender supplemented with 0.52 mg potassium penicillin G/m*l* and 0.8 mg streptomycin sulphate/m*l* (first extender [26, 27]). Diluted semen was encaged in a styrofoam container, and cooled moderately in a container with a cooling material for 30–60 min during transfer from the site of capture to a refrigerator placed in the car. By the time we arrived at the laboratory, the diluted semen had been cooled down to 4°C in the refrigerator. The semen was further incubated at 4°C for a total of 3 hr from the addition of the first extender. An equal volume of the second extender, consisting of the first extender supplemented with 16 v/v% glycerol, was then added at 4°C. Thus, the final concentration of glycerol was always 8 v/v%. The semen was immediately loaded into 0.5 m*l* straws at about 5°C, and placed horizontally 6 cm above the surface of liquid nitrogen with a height of 8 cm contained in a styrofoam container (inner size: 17.5 × 24.5 × 17.5 cm). The straws were kept thus for 15 min, then immediately plunged into the liquid nitrogen and stored until examination. The semen collected from Bear No. 1 was not processed for cryopreservation. Straws were allowed to thaw at 38°C for a few minutes and used immediately for examination. Frozen-thawed semen was examined in a similar way to the method of fresh semen.

Statistics: Values were expressed as range and mean.

RESULTS

Electroejaculation: Capture and anesthesia of bears for electroejaculation was performed in safety with no abnormal changes in body temperature, heart rate or respiration. Body weight and estimated age were 52–70 kg and 4–10 years old, respectively (Table 1). All bears began erection at the first cycle (7–15 stimuli) of stimulation. After erection, ejaculation of semen and/or urea occurred at 1–4 cycles.

Fresh semen: Ejaculates containing motile spermatozoa were collected in all of the 4 trials. One tube of ejaculate sample was collected from Bears No. 1, 2 and 3 and analyzed, but 3 tubes of ejaculate were collected from Bear No. 4, one of which sample was examined (Table 1). Because the other 2 samples contained too low sperm concentration and/or too much urine to be enough for one straw of frozen-thawed sample, they were not processed for cryopreservation, and thus their results are omitted. It was considered

Table 1. Body weight, age, and characteristics of electroejaculates from 4 free-ranging Japanese black bears

Date	Bear identification No.	Body weight (kg)	Age (yr)[a)]	Appearance of ejaculate	Total ejaculate volume (m*l*)	Sperm concentration ($\times 10^6$/m*l*)	Total sperm count ($\times 10^6$)	% +++ Motility[b)]	% Motility[c)]	% Viability[d)]	% Abnormal morphology	% Intact acrosome
2000/7/26	1	64	4	Yellow, transparent	1.2	195	234	0	10	49	33	90
2001/7/10	2	70	7	White-yellow, milky	0.65	1665	1082	95	100	97	20	97
2001/7/18	3	52	5	Yellow, transparent	2	273	546	0	5	76	87	96
2002/7/27	4	68	10	Yellow, transparent	2.2	25	99	0	10	42	72	65
	Mean	63.5	6.5	–	1.51	540	490	24	31	66	53	87

a) Determined by *dentes premolare*.
b) The percentage of motile spermatozoa with progression at a high speed.
c) The percentage of motile spermatozoa.
d) The percentage of spermatozoa that excluded eosin.

Table 2. Characteristics of frozen then thawed spermatozoa from 3 free-ranging Japanese black bears[a)]

Bear identifi–cation No.	% +++ Motility[b)]	% Motility[c)]	% Viability[d)]	% Abnormal morphology	% Intact acrosome	Sperm Concentration ($\times 10^6$/m*l*)	Wash[e)]
2	0	15	26	77	22	185	–
3	0	4	42	85	28	105	+
4	0	20	22	51	55	65	+
Mean	0	13	30	71	35	118	–

a) Characteristics after washing of spermatozoa, if done.
b) The percentage of motile spermatozoa with progression at a high speed.
c) The percentage of motil espermatozoa.
d) The percentage of spermatozoa that excluded eosin.
e) The semen considered to be highly contamined with urine was washed by centrifugation.

that contaminations with a little urine occurred in all the ejaculates, because they appeared yellow, but the ejaculate of Bear No. 2 contained very little urine (the color was much less yellow than the others). The characteristics of the 4 ejaculates ranged widely; ejaculate volume, sperm concentration and total sperm count were 0.65–2.20 m*l*, 25–1665 $\times$ 10^6/m*l* and 99–1082 $\times$ 10^6, respectively. Spermatozoa from Bear No. 2 was very vital with % +++ motility, % motility and % viability being 95, 100 and 97, respectively. The others showed less vital motion with % +++ motility, % motility and % viability being 0, 5–10 and 42–76, respectively. The % abnormal morphology was high in Bears No. 3 and 4 (87 and 72, respectively), and hair-pined tail was most frequently observed. Percent intact acrosome ranged from 65 (Bear No. 4) to 97 (Bear No. 2).

Frozen-thawed semen: The characteristics of spermatozoa after freezing and thawing are shown in Table 2. After thawing, motile spermatozoa were observed in all samples; % +++ motility, % motility, % viability, % abnormal morphology, % intact acrosome and sperm concentration were 0, 4–20, 22–42, 51–85, 22–55 and 65–185$\times$ 10^6/m*l*, respectively. However the motion of spermatozoa was slow in all samples after thawing.

DISCUSSION

Electroejaculation: This is the first report of erectroejaculation and semen cryopreservation in free-ranging bears (Ursidae). There are several reports on semen collection by electroejaculation in other free-ranging mammalian species, such as African elephant [10], jaguar [21], seal [18], Eastern grey kangaroo [15] and koala [35], while fewer reports about cryopreservation of erectroejaculates from free-ranging mammals such as African elephant [12], impala, wart hog, elephant and lion [5]. The inherent difficulties in collecting physiological data from free-ranging bears are reflected in the small number of samples, representing the sparse number of we had capture opportunities over the 3-year period. The results showed, at least, that semen could be collected successfully from free-ranging Japanese black bears by electroejaculation. The data on semen characteristics may be useful to establish normative reproductive values in this species. Electroejaculation would be a safe and reliable method for collecting semen from free-ranging Japanese black bears, as has been recognized in other species [5, 10, 12, 15, 18, 21, 35]. Semen samples obtained under such conditions could subsequently be used in genetic analyses and tests of sexual maturity and competence.

Fresh semen: The reported sperm concentration of the captive Japanese black bear (mean 361×10^6/m*l* [16]), Hokkaido brown bear (mean 590×10^6/m*l* [13]) and Giant panda (mean 645×10^6/m*l* [22]) was similar to that observed in this study (mean 540×10^6/m*l*). On the other hand, the reported total ejaculate volume of the captive Japanese black bear (mean 0.2 m*l* [16]), Hokkaido brown bear (mean 3.7 m*l* [13]), Giant panda (mean 6.5 m*l* [22]) and free-ranging Japanese black bear (mean 1.5 m*l*; this study) differed from each other to some extent. This might be due not only to species difference but also to the variation in electric stimulation methods and the degree of urine contamination.

The % +++ motility, % motility and % viability in captive Japanese black bears shown earlier [16] were superior to those observed in this study. This might be due to urine contamination. In this study, although urine contaminations occurred in all the ejaculates, spermatozoa in the ejaculate containing very little urine was very vital (Bear No. 2). African elephant sperm motility was rapidly depressed if the semen sample was contaminated with urine [12]. Thus, avoidance of urine contamination may be important for high sperm motility rate in electroejaculates [14].

Sperm abnormal morphology rate was higher in this study (mean 53%) than the captive Japanese black bear (mean 11%, [16]) and Giant panda (mean 11.5%, [22]). The main abnormal form of sperm in this study was a hair-pined tail.

Was this many abnormal spermatozoa led by hyperosmotic condition from urine contamination? Osmotic pressure of urine and semen in Japanese black bear is estimated to be about 1,000 and 290 mOsm, respectively by values reported in other mammalian species [19, 33]. Urine contamination into semen causes exposure of spermatozoa to hyperosmotic condition. In general, spermatozoa are relatively tolerant in high osmotic pressure condition [19]. Although even at hyperosmotic condition (1,000 mOsm), sperm membrane in some mammalian species is caused nearly no damage [1, 25, 30], abrupt return to isotonicity from a hyperosmotic stress causes extensive sperm membrane damage [1, 25, 30, 31].

In this study, the semen contaminated with urine was centrifuged and the supernatant was discarded before pelleted spermatozoa were resuspended in sterile physiological saline. Because in this process, osmotic pressure changed from hyperosmotic to isotonic, it was possible that spermatozoa might have been damaged, leading to abnormal sperm morphology. However, intact acrosome rate in this study is relatively high (mean 87 %), showing that the damage of sperm membrane was a little.

Furthermore, in the study of Japanese black bears reported by Kojima *et al.* [16], abnormal morphology rate of spermatozoa contaminated with urine was mean 25 % (n = 8) and was much lower than our result. This would also show that main causes of high abnormal morphology rate in this study are not only urine contamination.

A close relation seems to exist between genetic diversity and sperm pleiomorphism [29]. In particular, a high percentage of pleiomorphic spermatozoa in ejaculates were associated with low genetic diversity [34]. However, the main abnormality in this study was a hair-pined tail and it was not clear whether or not this type of abnormality is genetically based.

The frequency of several abnormalities differed between the nonbreeding and breeding seasons in roe deer [6]. In this study and the study of captive bear reported by Kojima *et al.* [16], collection of semen was performed in estimated mating season [36]. If there were a difference of spermatogenetic season between free-ranging and captive bears, collections at similar season would have resulted in different abnormal morphology rates.

In order to clarify the reasons for this high abnormal morphology rate in the future, further research is expected.

Age of sexual maturation in wild male Japanese black bears was estimated to be 3–4 years [23]. In this study, all the captured bears were considered to be over 4 years old. The mating season of the Japanese black bear is from June to August. Captive bears tend to mate in mid-July more frequently [36]. Because all collections were obtained in July, these data confirm that free-ranging Japanese black bears normally produce spermatozoa in July. Additional studies on the relation between seminal traits and endocrinological status would elucidate the variation in seminal traits.

Frozen-thawed semen: When semen was collected in the field from free-ranging bears and cryopreserved, severe conditions were obligated. Therefore, we used the modified method of cryopreservation (dilution rates, final sperm concentration, cooling and equilibration time) for free-ranging animals to carry out effective and smooth collection. The method allowed us to expand the time up to glycerolization and freezing process (1 to 3 hr), which time was spent during transportation by car from the handling site to the laboratory.

This study showed that frozen-thawed spermatozoa were motile, but the result was not as good as that of other captive ursid [13, 22]. Experiments will be required to further improve the post-thaw motility including % +++ motility and % motility, probably by using captive Japanese black bears as a model.

% Motility of spermatozoa from Bear No. 4 increased after freezing-thawing procedure (10 to 20%). The motility of fresh spermatozoa can be improved markedly by diluting raw semen with a diluent [9]. This phenomenon sometimes observed in urine contaminated and long time left semen. This improvement of motility would be from replenishment of an energy substrate for movement or dilution of a harmful matter of urine or seminal plasma. Thus it appeared that motility of spermatozoa from Bear No. 4 may have improved by addition of the diluent, and even after a decrease by freezing-thawing procedure, the motility may have remained at a higher level than before dilution.

Abnormal morphology rate of spermatozoa from Bear No. 3 changed little by freezing-thawing procedure (87 to 85%), while that from Bear No. 2 increased after freezing-thawing procedure extensively (20 to 77%). First reason for

this may be from difference in cryoprotective ability of spermatozoa among individuals [8]. Spermatozoa form Bear No. 2 might be weak for cryopreservation. Second reason for this may be form sudden changes in temperature. Spermatozoa with looped tails are sometimes caused by abrupt temperature changes [2]. The process of freezing operation in Bear No. 2 spermatozoa might have forced abrupt temperature changes upon spermatozoa.

In summary, we recovered semen from free-ranging Japanese black bears in the wild by electroejaculation and found that at least motile spermatozoa were confirmed to be in the frozen-thawed semen samples.

ACKNOWLEDGMENTS. The authors wish to thank the members of the Gifu University Japanese Black Bear Research Group for their assistance in the capture and handling of bears. This study was supported in part by the project Habitat Condition Survey of the Japanese Black Bear in Neo Village, Gifu Prefecture, Japan, and by a Grant-in-Aid for Scientific Research (The 21st Century Center-of-Excellence Program) from the Ministry of Education, Sports, Science and Technology of Japan (E-1).

REFERENCES

1. Agca, Y. and Critser, J. K. 2002. Cryopreservation of spermatozoa in assisted reproduction. *Semin. Reprod. Med.* **20**: 15–23.
2. Arthuer, G. H., Noakes D. E. and Person, H. 1982. Reproductive abnormalities of male animals. pp. 431–468. *In*: Veterinary Reproduction and Obstetrics, 5th ed., Bqillière Tindqll, London.
3. Bornman, M. S., van Vuuren, M., Meltzer, D. G., van der Merwe, C. A. and v Rensburg, S. J. 1988. Quality of semen obtained by electroejaculation from Chacma baboons (*Papio ursinus*). *J. Med. Primatol.* **17**: 57–61.
4. Feng, W. H., Zhao, J. and Fujihara, N. 1997. Physiological characteristics of electro-ejaculated giant panda (*Ailuropoda melanoleuca*) semen. *Jpn. J. Zoo Wildl. Med.* **2**: 107–112.
5. Gilmore, J. A., McGann, L. E., Ashworth, E., Acker, J. P., Raath, J. P., Bush, M. and Critser, J. K. 1998. Fundamental cryobiology of selected African mammalian spermatozoa and its role in biodiversity preservation through the development of genome resource banking. *Anim. Reprod. Sci.* **53**: 277–297.
6. Goeritz, F., Quest, M., Wagener, A., Fassbender, M., Broich, A., Hildebrandt, T. B., Hofmann, R. R. and Blottner, S. 2003. Seasonal timing of sperm production in roe deer: interrelationship among changes in ejaculate parameters, morphology and function of testis and accessory glands. *Theriogenology* **59**: 1487–1502.
7. Hazumi, T. 1999. Status and management of the Asiatic black bear in Japan. pp. 207–211. *In*: Bears Status Survey and Conservation Action Plan (Servheen, C., Herrero, S. and Peyton, B. eds.), IUCN, Gland, Switzerland and Cambrige, U. K.
8. Holt, W. V. 2000. Fundamental aspects of sperm cryobiology: the importance of species and individual differences. *Theriogenology* **53**: 47–58.
9. Howard, J. G. 1993. Semen collection and analysis in carnivores. pp. 390–399. *In*: Zoo & Wild Animal Medicine - Current Therapy 3 (Flowler, M. E. ed.), B. W. Saunders, Philadelphia.
10. Howard, J. G., Bush, M., de Vos, V. and Wildt, D. E. 1984. Electroejaculation, semen characteristics and serum testosterone concentrations of free-ranging African elephants (*Loxodonta africana*). *J. Reprod. Fertil.* **72**: 187–195.
11. Howard, J. G., Pursel, V. G., Wildt, D. E. and Bush, M. 1981. Comparison of various extenders for freeze-preservation of semen from selective captive wild ungulates. *J. Am. Vet. Med. Assoc.* **179**: 1157–1161.
12. Howard, J. G., Bush, M., Vos. V. d., Schiewe, M. C., Pursel, V. G. and Wildt, D. E. 1986. Influence of cryoprotective diluent on post-thaw viability and acrosomal integrity of spermatozoa of the African elephant (*Loxodonta africana*). *J. Reprod. Fertil.* **78**: 295–306.
13. Ishikawa, A., Matsui, M., Sakamoto, H., Katagiri, S. and Takahashi, Y. 2002. Cryopreservation of the semen collected by electroejaculation from the Hokkaido brown bear (*Ursus arctos yesoensis*). *J. Vet. Med. Sci.* **64**: 373–376.
14. Ishikawa, A., Matsui, M., Tsuruga, H., Sakamoto, H., Takahashi, Y. and Kanagawa, H. 1998. Electroejaculation and semen characteristics of the captive Hokkaido brown bear (*Ursus arctos yesoensis*). *J. Vet. Med. Sci.* **60**: 965–968.
15. Johnston, S. D., Blyde, D., Gamble, J., Higgins, D., Field, H. and Cooper, J. 1997. Collection and short-term preservation of semen from free-ranging eastern grey kangaroos (*Macropus giganteus: Macropodidae*). *Aust. Vet. J.* **75**: 648–651.
16. Kojima, E., Tsuruga, H., Komatsu, T., Murase, T., Tsubota, T. and Kita, I. 2001. Characterization of semen collected from beagles and captive Japanese black bears (*Ursus thibetanus japonicus*). *Theriogenology* **55**: 717–731.
17. Komastu, T., Tsubota, T., Kishimoto, M., Hamasaki, S. and Tiba, T. 1994. Stem cell in connection with sexual maturity and spermatogenesis in male Japanese black bear (*Selenarctos thibetanus japonicus*). *J. Reprod. Dev.* **40**: 65–71 (in Japanese).
18. Lawson, J. W., Parsons, J. L., Craig, S. J., Eddington, J. D. and Kimmins, W. C. 1996. Use of electroejaculation to collect semen samples from wild seals. *J. Am. Vet. Med. Assoc.* **209**: 1615–1617.
19. Masuda, H. 2001. Spermatozoa. pp. 103–110. *In*: Textbook of Theriogenology, 2nd ed. (Mori, J., Kanagawa, H. and Hamana, K. eds.), Buneido, Tokyo (in Japanese).
20. Masui, M., Hiramatsu, H., Nose, N., Nakazato, R., Sagawa, Y., Tajima, H. and Saito, K. 1989. Successful artifical insemination in the giant panda (*Ailuropoda melanoleuca*) at Ueno zoo. *Zoo Biol.* **8**: 17–26.
21. Morato, R. G., Conforti, V. A., Azevedo, F. C., Jacomo, A. T. A., Silveira, L., Sana, D., Nunes, A. L. V., Guimaraes, M. A. B. V. and Barnabe, R. C. 2001. Comparative analyses of semen and endocrine characteristics of free-living versus captive jaguars (*Panthera onca*). *Reproduction* **122**: 745–751.
22. Olson, M. A., Yan, H., DeSheng, L., Hemin, Z. and Durrant, B. 2003. Comparison of storage techniques for giant panda sperm. *Zoo Biol.* **22**: 335–345.
23. Okano, T., Murase, T. and Tsubota, T. 2003. Spermatogenesis, serum testosterone levels and immunolocalization of steroidgenic enzymes in the wild male Japanese black bear (*Ursus thibetanus japonicus*). *J. Vet. Med. Sci.* **65**: 1093–1099.
24. Platz, C. C. Jr., Wildt, D. E., Howard, J. G. and Bush, M. 1983. Electroejaculation and semen analysis and freezing in the giant panda (*Ailuropoda melanoleuca*). *J. Reprod. Fertil.* **67**: 9–12.
25. Pukazhenthi, B., Noiles, E., Pelican, K., Donoghue, A., Wildt, D. and Howard, J. 2000. Osmotic effects on feline spermatozoa from Normospermic versus teratospermic donors. *Cryobiology* **40**: 139–150.

26. Rota, A., Iguer-Ouada, M., Verstegen, J. and Linde-Forsberg, C. 1999. Fertility after vaginal or uterine deposition of dog semen frozen in a tris extender with or without Equex STM paste. *Theriogenology* **51**: 1045–1058.
27. Rota, A., Strom, B., Linde-Forsberg, C. and Rodriguez-Martinez, H. 1997. Effects of equex STM paste on viability of frozen-thawed dog spermatozoa during *in vitro* incubation at 38°C. *Theriogenology* **47**: 1093–1101.
28. Shams-Borhan, G. and Harrison, R. A. 1981. Production, characterization, and use of ionophore-induced, calcium-dependent acrosome reaction in ram spermatozoa. *Gamete. Res.* **4**: 407–432.
29. Shivaji, S., Jayaprakash, D. and Patil, S. B. 1998. Assessment of inbreeding depression in big cats: testosterone levels and semen analysis. *Curr. Sci.* **75**: 923–930.
30. Songsasen, N. and Leibo, S. P. 1997. Cryopreservation of mouse spermatozoa. II. Relationship between survival after cryopreservation and osmotic tolerance of spermatozoa from three strains of mice. *Cryobiology* **35**: 255–269.
31. Songsasen, N., Yu, I., Murton, S., Paccamonti, D. L., Eilts, B. E., Godke, R. A. and Leibo, S. P. 2002. Osmotic sensitivity of canine spermatozoa. *Cryobiology* **44**: 79–90.
32. Taggart, D. A., Leigh, C. M., Steele, V. R., Breed, W. G., Temple-Smith, P. D. and Phelan, J. 1996. Effect of cooling and cryopreservation on sperm motility and morphology of several species of marsupial. *Reprod. Fertil. Dev.* **8**: 673–679.
33. Tsuda, T. 1997. Urine. p. 198. *In*: Physiology of Domestic Animal, 2nd ed., Yokendo, Tokyo (in Japanese).
34. Wildt, D. E., Bush, M., Howard, J. G., O'Brien, S. J., Meltzer, D., Van Dyk, A., Ebedes, H. and Brand, D. J. 1983. Unique seminal quality in the South African cheetah and a comparative evaluation in the domestic cat. *Biol. Reprod.* **29**: 1019–1025.
35. Wildt, D. E., Bush, M., O'Brien, S. J., Murray, N. D., Taylor, A. and Graves, J. A. M. 1991. Semen characteristics in free-living koalas (*Phascolarctos cinereus*). *J. Reprod. Ferti.* **92**: 99–107.
36. Yamamoto, K., Tsubota, T. and Kita, I. 1998. Observation of sexual behavior in captive Japanese black bear (*Ursus thibetanus japonicus*). *J. Reprod. Dev.* **44**: j13–j18 (in Japanese with an English summary).