

A trial of semen collection by transrectal electroejaculation method from Amur leopard cat (*Prionailurus bengalensis euptilurus*)

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ABSTRACT. We collected semen from a male Amur leopard cat using the transrectal electroejaculation method and investigated the semen qualities for about four years. In addition, the influence of the season on the spermatogenic function of the Amur leopard cat was investigated with regard to the semen qualities, testicular volume and serum testosterone level. As a result, we could collect semen with good sperm qualities that would be useable for artificial insemination. Some seasonality was noted in the testicular volume and serum testosterone level. We clarified that the semen qualities were favorable before and during the female breeding season compared with those after the breeding season.

KEY WORDS: Amur leopard cat, electroejaculation, seasonality, semen collection, semen quality

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The Amur leopard cats (*Prionailurus bengalensis euptilurus*) belong to the small wild felids and inhabit only the Korean Peninsula, Northeast China and East Siberia. They are a subspecies of leopard cat (*Prionailurus bengalensis*), to which Iriomote leopard cats (*Prionailurus bengalensis iriomotensis*) and Tsushima leopard cats (*Prionailurus bengalensis euptilurus*) indigenous to Japan also belong [11]. Amur leopard cats are classified into the category of Least Concern (LC) in the IUCN Red List [10], and their extinction risk is currently considered low, unlike the two small wild felids indigenous to Japan. However, their risk of extinction may increase in the near future, as is the case for so many of the wild felids.

To increase the populations of these wild felid animals, many zoos in Japan are attempting reproduction through *ex situ* conservation, but natural reproduction is difficult because males and females are socially incompatible, and the timing for housing them together is unclear due to less noticeable estrous signs of females. To succeed in their reproduction, the introduction of assisted reproductive technologies (ART) is considered necessary [14], but no study

on ART of these small wild felids has been reported. For the actual introduction of these techniques, methods applied to domestic cats may be a starting point, but the reproductive characteristics are slightly different between domestic cats and small wild felids, so the existing methods cannot be applied without modification.

For ART, it is necessary to collect sufficient quantities of semen with good sperm qualities from male animals. The collected semen can be used for ART, such as artificial insemination (AI), *in vitro* fertilization and intracytoplasmic sperm injection [1, 9]. General semen collection methods used for domestic cats include the artificial vagina (AV) [5, 15] and transrectal electroejaculation (TE) [6, 12, 13, 19, 21] methods. When collection using these methods is difficult, sperm collection from the caudal epididymis is selected [17]. Dooley and Pineda [5] compared semen collection from domestic cats between the TE and AV methods. The total ejaculated sperm count was higher using the AV method (mean: 61.0×10^6) than using the TE method (mean: 42.7×10^6). In addition, the AV method is less stressful to the animal, because it does not require general anesthesia, which is necessary for the TE method; namely, ejaculation close to natural conditions can be induced using the AV method, suggesting its superiority as a semen collection method. However, training of the animal is necessary to collect semen using an AV, and its application is difficult because animals of the wild felid cannot be directly touched. Moreover, sperm collection from the caudal epididymis is only selected when an animal dies due to an unexpected event, such as a car accident or

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the occasion of an orchiectomy in a living animal, and this technique is thus inappropriate for reproduction of healthy animals. Accordingly, the TE method under general anesthesia is generally used to collect semen from wild felids [3, 6–8, 16, 20].

In the TE method, the voltage, frequency of stimulation, and the size of probe and shape of electrode vary among species, and these conditions influence the qualities of the collected semen. To collect sufficient semen with high sperm qualities, it may be necessary to establish the optimum electric stimulation conditions while minimizing stress on the animal. However, there have been no reports on semen collection from Amur leopard cats using the TE method, and the semen qualities of Amur leopard cats have not been investigated.

In this study, we collected semen from a male Amur leopard cat using the TE method and investigated the semen qualities. In addition, the influence of the season on the spermatogenic function of the Amur leopard cat was investigated with regard to the semen qualities, testicular volume and serum testosterone level.

The animal was a male Amur leopard cat born in April 2003 and maintained at Inokashira Park Zoo, Tokyo, Japan. The mating ability and fertility of this male animal had been confirmed, because a female housed together with it became pregnant in May 2004. Normally, the animal is maintained in an individual cage in an animal barn and fed chicken heads, horsemeat and mice, with no difference among the seasons. The study period was about four years, from April 2006 to June 2010. Semen was collected 19 times in total with approximately 2-month or longer intervals in consideration of the stressful influences of capture, general anesthesia and electric stimulation. This study was conducted in conformity with the animal study guidelines of Japanese Association of Zoos and Aquariums.

The Amur leopard cat was placed in a squeeze cage to immobilize it at the place of maintenance before semen collection, and anesthesia was induced by intramuscular administration of a mixture of 0.05–0.08 mg/kg medetomidine hydrochloride (Domitor[®], Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan) and 1.7–2.6 mg/kg ketamine hydrochloride (Ketalar[®]50, Sankyo Co., Ltd., Tokyo, Japan). The dosage of the anesthetic was calculated based on the estimated body weight. The animal was then precisely weighed at the animal hospital in Inokashira Park Zoo followed by the insertion of a tracheal tube (5 Fr), and anesthesia was maintained with isoflurane (Escain[®], Mylan Seiyaku Ltd., Tokyo, Japan) until the completion of semen collection.

After the induction of general anesthesia, blood was collected from the cephalic vein of the forearm or lateral saphenous vein. Serum was separated by centrifugation (x 600 g, 10 min) at 4°C and stored at –40°C until hormone measurement. All blood samples were collected at the same time of day (1:00 p.m.–2:00 p.m.). The serum testosterone level was measured using an automatic immunofluorescence measurement device (SPOTCHEM VIDAS SV-5010, Arklay Inc., Kyoto, Japan). Serum samples were simultaneously subjected to hormone measurement in order to reduce

measurement errors of the kit.

The testicular size—major and minor axes, and thickness—were measured on the scrotal wall using calipers, and the testicular volume (cm³) was calculated using the following formula: $4/3\pi \times \text{major axis}/2 \times \text{short axis}/2 \times \text{thickness}/2$. All measurements of testicular size were performed by the same person in order to reduce procedural errors.

The Amur leopard cat was placed in a lateral position. To reduce contamination of semen with urine, urine was collected using a 3 Fr indwelling feeding tube (Atom Medical Co., Tokyo, Japan) immediately before electric stimulation. A small amount of white Vaseline was applied to a rectal probe, which was inserted via the anus and fixed. The exposed penis was covered with a sterile conical tube, and electric stimulation was applied. The intensity and duration were adjusted while observing erection and ejaculation. The conical tube was changed to a new one when ejaculation was observed. The devices used for the total of 19 semen collections (Table 1) were an electric stimulator (NF-01, Fujihira Industry Inc., Tokyo, Japan; Fig. 1a) and four types of rectal probes (diameter: 1.0–1.5 cm) as follows: 1) a probe equipped with 4-stranded ring-shaped electrodes (F-4, Fujihira Industry Inc.; Fig. 1b), 2) F-4 modified to 3-stranded ring-shaped electrodes by insulating the electrode at the root with vinyl tape (F-3; Fig. 1c), 3) a probe with 3 parallel electrodes (O-3, custom-made; Fig. 1d) and 4) a probe with 1 parallel electrode and stainless steel at backend (O-1, custom-made; Fig. 1e). The rectal probe insertion distance, voltage and frequency of stimulation were changed in accordance with the conditions of the Amur leopard cat and semen collection. The voltage was elevated in 3 steps from low to high level (0.5–6.0 V) applied for 3 sec each, which was regarded as one set of stimulation. A 10-min rest was carried out after applying 10 sets. Electric stimulation was completed when sperm was hardly seen in the collected semen.

Since the collected semen volume was small, 50 μ l of a semen extender, egg yolk Tris-fructose citric acid solution [15], was immediately added to prevent drying. The sperm motility, viability and abnormality and total sperm count were then measured as we previously reported [15, 19].

Since estrus has been observed in late January–April in a female Amur leopard cat maintained in Inokashira Park Zoo, all collected data were classified into three subgroups, i.e., those collected in January–April [breeding season (BS) group, n=6], May–August [post-breeding season (post-BS) group, n=7] and September–December [pre-breeding season (pre-BS) group, n=6], to observe the influence of the season on the semen qualities. The data for the subgroups were compared using multiple comparisons. When a significant difference was noted, it was analyzed using the Tukey–Kramer test. In addition, correlations among the body weight, mean volume of the bilateral testes, serum testosterone level and the semen qualities were analyzed using Spearman's rank correlation coefficient. A significance level lower than 5% was regarded as significant.

Changes in the body weight and mean volume of the bilateral testes over the study period and the means (\pm S.E.)

Table 1. The electric stimulation conditions at each semen collection

No.	Date	Rectal probe	Insertion length of probe	Voltage	Frequency of stimulation
1	2006/04/24	F-4	10 cm	2–6 V	13
2	2006/06/29	F-4	10 cm	1–3 V	11
3	2006/08/17	F-4	10 cm	1–3 V	13
4	2006/11/18	F-4	8, 10 cm	1–3 V	13
5	2007/05/28	F-4	10 cm	1–3 V	18
6	2007/07/30	F-4	8 cm	1–3 V	7
7	2007/10/02	F-4	8, 10 cm	1–3 V	9
8	2008/01/28	F-3	6.5 cm	1–4 V	13
9	2008/03/24	F-3	6.5 cm	1–4 V	15
10	2008/06/05	F-3	6.5 cm	1–3 V	10
11	2008/10/07	F-3	6.5 cm	1–3 V	9
12	2008/12/11	F-3	6.5 cm	1–4 V	9
13	2009/02/24	O-3	6.5 cm	1–3 V	8
14	2009/04/23	O-3	6.5 cm	0.5–3 V	9
15	2009/06/25	O-3	6.5 cm	1–3 V	9
16	2009/10/13	O-1	6.5 cm	0.5–2 V	16
17	2009/12/09	O-1	6.5 cm	0.5–2 V	16
18	2010/03/04	O-1	6.5 cm	0.5–2 V	11
19	2010/06/11	O-1	6.5 cm	0.5–2 V	8

in the season-based subgroups are shown in Fig. 2. Body weight showed some variation during the study period; it was highest in the pre-BS group and significantly different between the BS and post-BS groups ($P < 0.05$ and $P < 0.01$). The testicular volume was slightly different between the bilateral testes, but there was no significant correlation. Thus, the mean volume of the bilateral testes was adopted. The mean volume of the bilateral testes varied during the study period, being lowest in the post-BS group and highest in the BS group, showing a significant difference between the 2 groups ($P < 0.05$). No significant correlation was noted between the body weight and mean volume of the bilateral testes.

The electric stimulation conditions are shown in Table 1, and the data on the semen qualities immediately after collection and the means in the subgroups are shown in Fig. 3. The semen qualities varied slightly among the electric stimulation conditions, but no significant difference due to the rectal probe type or strength or frequency of stimulation was observed. However, when F-4 probe was placed at a site 8–10 cm from the anus, Sample Nos. 1 and 7 were contaminated with urine, and No. 6 was contaminated with urine and slightly with blood. The sperm motility and viability were low in these samples, and the level of sperm abnormality was slightly increased. Because these samples were judged to have been artificially degraded by urine and blood, their data were excluded from statistical analysis. Among the 19 semen collections, ejaculation was observed, but almost no sperm was contained in semen in the sample collected in August (No.3). Sperm was contained in ejaculated samples at all the other time points, but the total sperm count varied among the periods. It was lower in the post-BS than in the BS and pre-BS groups, but the differences were not significant. No significant correlation was observed between the total sperm

count and body weight or mean volume of the bilateral testes. The sperm motility and viability were slightly lower in the post-BS group and sperm abnormality was highest in the post-BS group, but the differences from those in the 2 other groups were not significant. The observed abnormality was malformation of the tail in many abnormal sperm.

Changes in the serum testosterone level measured at the semen collection time points and the means in the subgroups are shown in Fig. 2. The serum testosterone level varied markedly among the time points. The mean level was the highest in the pre-BS group and lowest in the post-BS group, and a significant difference was noted between these two groups ($P < 0.05$). No correlation was noted between the serum testosterone level and body weight or mean volume of the bilateral testes, nor was there any correlation between the serum testosterone level and total sperm count, sperm motility, viability or abnormality.

Initially, semen collection from the male Amur leopard cat using the TE method was performed using the procedure generally employed for domestic cats and wild felids [4, 5, 20]. The rectal probe tip insertion length for domestic cats is 6–7 cm [4], but it was made slightly deeper because wild-cats have a slightly longer trunk and larger physique, and a 4-stranded ring-type rectal probe (F-4) was placed 8–10 cm from the anus. However, semen was frequently contaminated with urine after electric stimulation. The insertion length was then adjusted to about 6.5 cm from the anus by using the F-3 probe, and urine contamination did not occur. We assumed that electric stimulation was transmitted to the urinary bladder and urine excretion was promoted when the rectal probe was excessively advanced, and that the appropriate rectal probe placement site for Amur leopard cats is about 6.5 cm from the anus.

Then, for the rectal probe, we used a probe with parallel

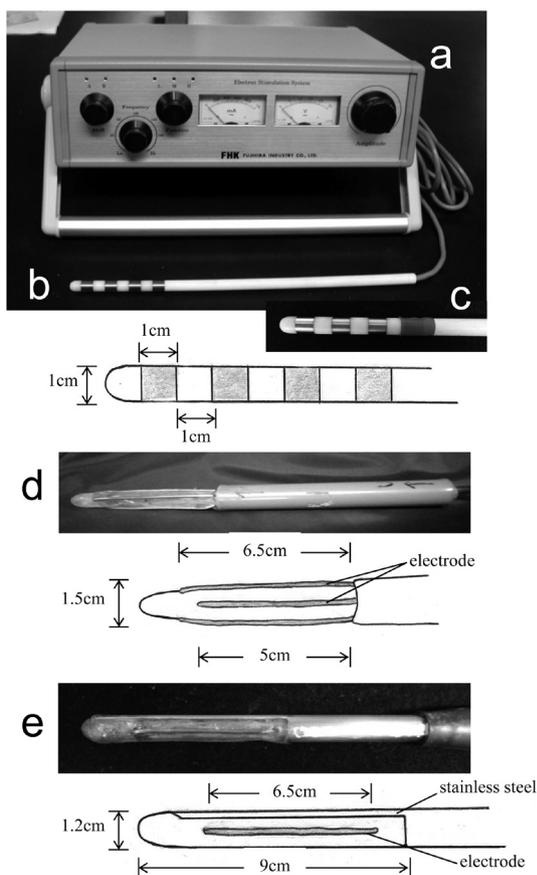


Fig. 1. An electric stimulator and rectal probes used in this study. a) An electric stimulator (Fujihira Industry Inc.), b) A probe equipped with 4-stranded ring-shaped electrodes (F-4, Fujihira Industry Inc.), c) F-4 modified to 3-stranded ring-shaped electrodes by insulating the electrode at the root with vinyl tape (F-3), d) A probe with 3 parallel electrodes (O-3, custom-made), e) A probe with 1 parallel electrode and stainless steel at the back (O-1, custom-made).

electrodes (O-3 and O-1), because parallel electrodes had previously been used in domestic cats [4]. However, no difference was noted in the physical reaction of the Amur leopard cat to the stimulation and the sperm qualities among the 4 type probes. Moreover, no significant difference was observed in the number of electric stimulations required to exhaust sperm in ejaculated semen. In general, electric stimulation to the ventral side alone was sufficient to induce ejaculation. Thus, because the O-1 probe has only one electrode, we thought that this probe would minimize stress to the body of the Amur leopard cat, and we suggest that a probe with 1 parallel electrode and stainless steel on the back is the most appropriate rectal probe for transrectal electric stimulation in Amur leopard cats.

Regarding the voltage of transrectal electric stimulation applied to domestic cats, many researchers start at 2 V and gradually increase to 5 V [5, 12, 20, 21]. According to these reports, stimulation at a high voltage may increase the pos-

sibility of contamination with urine, but the collected sperm count increases. We did not apply a high voltage in order to minimize stress on the animal, because the physical reaction to stimulation at 2 V was sufficient in the first trial, and contamination with urine was noted when a higher voltage was applied. However, it is also possible that the insertion site of the rectal probe was inappropriate and that this was the only cause of contamination with urine. It may be necessary to attempt electric stimulation at a higher voltage, as reported for domestic cats, in order to investigate whether or not the ejaculated sperm count can be increased in Amur leopard cats.

The anesthesia conditions and the type of anesthetic may also influence semen collection and semen qualities when employing the TE method. For example, deep general anesthesia may inhibit electric stimulation-induced ejaculation. Also, when an α -adrenergic drug, medetomidine hydrochloride, is used to induce anesthesia in felids, spontaneous ejaculation occurs after introducing general anesthesia [22]. Since we used the same anesthetic and methods throughout this study, the influence of the employed anesthesia method on the ejaculated sperm count could not be clarified. It may be necessary to investigate the optimum anesthetic and depth of anesthesia for the electric stimulation of Amur leopard cats.

There have been no previous reports on the semen qualities of male Amur leopard cats. The sperm motility and viability were very high. The sperm abnormality was high when the sample was contaminated with urine and blood, but it was generally low, and abnormal sperm as observed in other animals of the wild felids was not frequently noted [9]. These semen qualities were similar to those of domestic cats apart from the ejaculated sperm count [15, 19]. The total ejaculated sperm count ranged widely depending on the time points, but the mean was 19×10^6 , being much lower than those collected in domestic cats using the TE method (mean 42.7×10^6) [13] and an AV (50–80 $\times 10^6$) method [15]. It is unclear whether this sperm count is typical of Amur leopard cats in general or specific to this individual, because the experiment was performed with only one animal. Although the number of sperm that would be necessary for AI of Amur leopard cats has not been clarified, based on the count necessary for AI of domestic cats [18], we assume that the observed sperm count would be insufficient for intravaginal AI, but conception by intrauterine or intratubal AI may be possible.

Although semen collection was investigated in only one male Amur leopard cat, the influence of season on the spermatogenic function of male Amur leopard cats could be investigated because semen was collected 3–5 times a year for four years. The seasonal influence was investigated by dividing the data into three subgroups based on the collection time points referring to the breeding season of female Amur leopard cats. No significant difference was noted in any semen quality among the three subgroups, but the total sperm count and sperm motility and viability were lowest in the post-BS group, and sperm abnormality was highest in the post-BS group, showing some differences. The post-BS in-

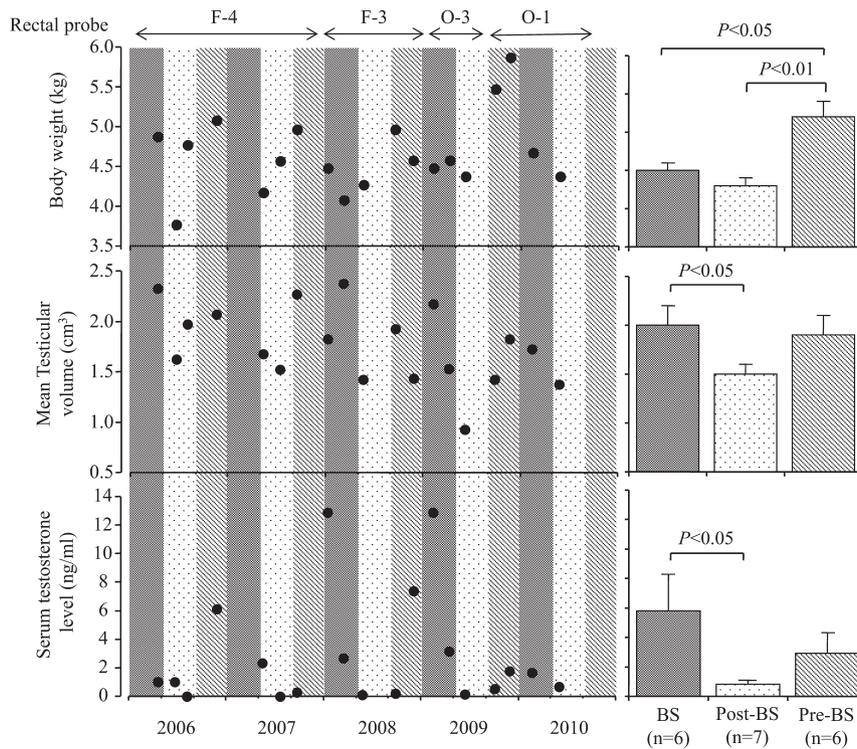


Fig. 2. Seasonal changes in the body weight, mean volume of the bilateral testes and serum testosterone level of a male Amur leopard cat over the study period. The four types of rectal probes are listed in Fig. 1. Season-based subgroups: BS (breeding season, Jan-Apr), post-BS (May-Aug), pre-BS (Sep-Dec).

cluded midsummer, in which the semen qualities decrease in many animal species. Sperm was absent only in semen collected in August. The body weight showed marked seasonal changes and was significantly higher in the pre-BS group. The mean volume of the bilateral testes significantly decreased after the breeding season compared with that in the breeding season, and a significant difference was also noted in the serum testosterone level. Since the content and amount of food given to the Amur leopard cat were not changed throughout the year in our zoo, this body weight gain may have been due to the suppression of metabolism and the accumulation of energy to prepare for the coldness starting in fall, with the energy being used from the breeding season. However, no significant correlation was noted between the body weight, testicular volume, and serum testosterone level and each semen quality. Some seasonality in the spermatogenic function of domestic cats has been reported [2, 19]. When seasonal changes in the semen qualities were investigated in the same male domestic cats, spermatogenesis and hormone secretion were observed during the non-breeding season of female domestic cats, but significant differences from those in the breeding season were noted, clarifying that the reproductive function of male domestic cats is also influenced by the season (hours of sunlight). Although the variation in spermatogenic function was small in the Amur leopard cat, there was some seasonal influence: the level was

low after the breeding season and high before and during the breeding season.

Since the semen qualities were observed from 4 to 8 years old in the same animal, changes with aging were also investigated, but no marked change was noted in any semen quality throughout the study period. There have been no reports of changes with aging in domestic cats, we have seen no problem with the semen qualities until 12 years old in domestic cats maintained in our laboratory (personal data), and Tsushima leopard cats normally breed at 8 years old (in 2014, reported at Fukuoka City Zoological Garden, Fukuoka, Japan), suggesting that our findings were not contradictory. Since we are also interested in the spermatogenic function at an advanced age, we will continue to collect semen from this Amur leopard cat and investigate its function.

We could collect semen with good sperm qualities from an Amur leopard cat using the TE method. Some seasonality was noted in the semen qualities in correlation with seasonal changes in the testicular volume and serum testosterone level, and it was clarified that the semen qualities were favorable before and during the female's breeding season compared with those after the breeding season. However, since this study was performed with only one Amur leopard cat, individual differences could not be investigated. There may be no marked variation in the physique of Amur leopard cats, but investigation in another individual may be

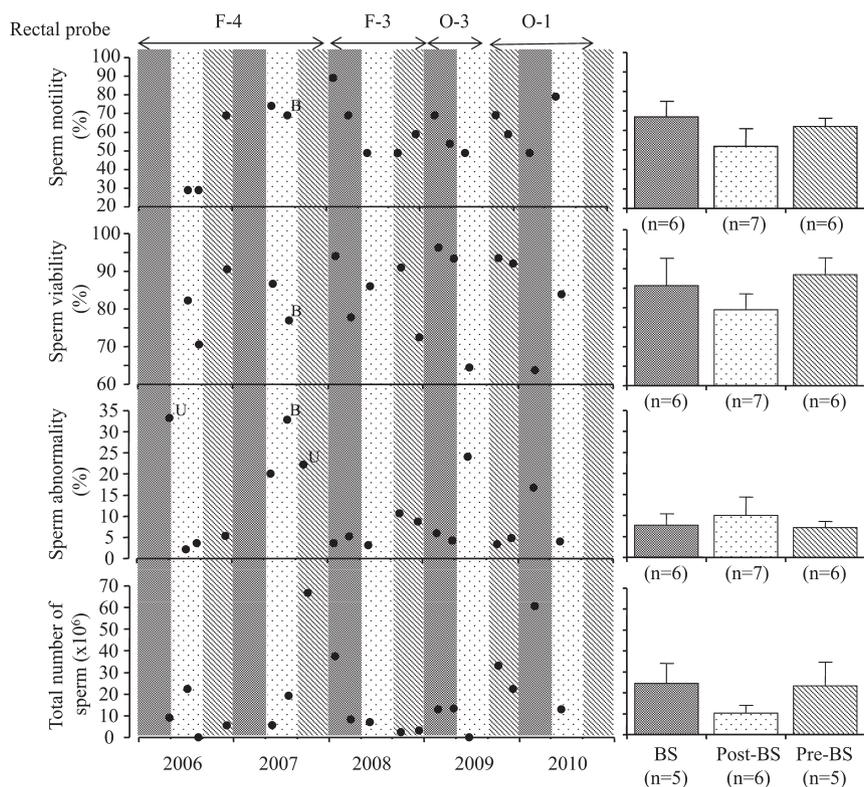


Fig. 3. Seasonal changes in sperm motility, viability and abnormality, and total number of sperm immediately after collection from a male Amur leopard cat over the study period. U: contamination of urine, B: contamination of blood.

necessary. The method used may be applicable to Tsushima leopard cats and Iriomote leopard cats, which are closely related to Amur leopard cats and are in danger of extinction in Japan. Studies on various ART for Amur leopard cats may provide useful information for the artificial reproduction of these small wild felids.

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