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Quality of cauda epididymal sperm immediately after collection and after freezing-thawing from Amur leopard cats (*Prionailurus bengalensis euptilurus*) and a local population of the subspecies Tsushima leopard cats

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ABSTRACT. In this study, cauda epididymal sperm were collected from Amur leopard cats with various causes of death as well as Tsushima leopard cats that underwent castration surgery, and sperm quality was compared with that in domestic cats. A sufficient number of sperm similar to those in domestic cats could be collected from the cauda epididymis of Amur leopard cats. However, in old leopard cats, no or very few cauda epididymal sperm were recovered, although there were no differences in sperm motility and sperm abnormality. There were no significant differences in sperm quality immediately after collection and after freezing-thawing of cauda epididymal sperm compared with corresponding estimates in domestic cats.

KEYWORDS: Amur leopard cat, epididymal sperm, frozen semen, semen quality, Tsushima leopard cat

Tsushima leopard cats (*Prionailurus bengalensis euptilurus*), small wild felids endemic to Japan, are in danger of extinction. They are known as a local population of the Amur leopard cat [9], and the two belong to the same subspecies of the Bengal leopard cat. Several Bengal leopard cat subspecies, including the Amur leopard cat, are currently classified as LC (Least Concern) on the IUCN Red List and are still considered to be at low risk of extinction [8]. The risk of extinction is expected to increase in the near future if conservation efforts are not started soon.

Despite various conservation activities in the habitat of Tsushima leopard cat in response to the threat of extinction, various accidents, including traffic accidents and fatal attacks by stray dogs, occur annually. During these events, sperm may be collected from the cauda epididymis (the site of sperm are storage), cryopreserved, and utilized for artificial insemination, in vitro fertilization, or intracytoplasmic sperm injection if the animal is male, as conducted in other animal species [2–4, 7, 11, 13, 17, 18, 22]. If this is achieved, even an animal that died unexpectedly and suddenly may be useful for increasing the population size while maintaining genetic diversity. Although some studies of domestic cats have been published [3, 4, 11, 17, 18, 22], the quality of cauda epididymal sperm and effectiveness of sperm cryopreservation in wild small felids, such as Tsushima leopard cats and Amur leopard cats, have not been reported to date.

In the present study, we collected sperm from the cauda epididymis of Amur leopard cats that died of different causes and Tsushima leopard cats in which castration surgery was performed. We treated Amur leopard cats and Tsushima leopard cats as the same species

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and compared the testicular/epididymal size and sperm qualitiy with those of domestic cats, a different felid species with a similar size. Furthermore, some sperm were cryopreserved, and the quality of the cryopreserved cauda epididymal sperm was evaluated after thawing.

Five male Amur leopard cats (LC-1 to -5) and two male Tsushima leopard cats (LC-6 and -7) maintained in the Inokashira Park Zoo, Tokyo, Japan were included. All Amur leopard cats died of different causes. The Tsushima leopard cats underwent castration surgery. The age and body weight of each leopard cat at the time of death and cause of death as well as the age and body weight at the time of castration surgery are presented in Tables 1 and 2. This study was conducted in conformity with the animal study guidelines of Japanese Association of Zoos and Aquariums.

Two leopard cats (LC-4 and -6) were frequently used for breeding before death, and the coitus ability and fertility were confirmed. However, the other animals were not used for breeding; fertility were unclear. In one of these animals (LC-3), semen was collected by urethral catheterization and transrectal electric stimulation under general anesthesia before death as described below (this animal died immediately after the final session of semen collection). In the other animals, semen was not collected. These animals were maintained in an individual cages in an animal barn and fed chicken meat, chicken heads, horsemeat, and mice, with no difference in food composition among the seasons.

As a control group, five male domestic cats (DC-1 to -5) were used (Table 2). These cats were all after pubertal age, and were brought to an animal hospital for castration.

The Amur leopard cats were stored in a refrigerator (5°C) immediately after death, except one cat (LC-3). Subsequently, the testis and epididymis covered by the tunica vaginalis communis were removed at different times depending on the individual (3 to 12 hr after death), immersed in sterile physiological saline, and transported at room temperature to a laboratory room (about 30 min) to collect sperm from the cauda epididymis. In the castrated Tsushima leopard cats and domestic cats, sperm were promptly collected from the cauda epididymis after the removal of the testis and epididymis following standard methods.

Before sperm collection, the testis and epididymis were measured. Briefly, after the epididymis was separated from the testis, the major axis, minor axis, and thickness of the testis were measured using a caliper. Subsequently, its weight was measured. The testicular volume was calculated using the following formula: $4/3\pi \times$ major axis/2 $\times$ minor axis/2 $\times$ thickness/2. Furthermore, the width and thickness of the caput and cauda epididymis as well as the entire length were measured using a caliper. Subsequently, the weight of whole epididymis was measured.

Sperm from the cauda epididymis in all individuals was recovered by the mincing method [17]. First, the blood vessels on the surface of the epididymis were removed as much as possible in order to suppress blood contamination in the recovered sperm. After cutting at the corpus part near the cauda epididymis, the cauda epididymis and vas deferens were minced using a surgical scalpel blade on a plastic dish with a small amount of egg yolk tris-fructose citrate solution (EYT-FC) [21]. The solution to transmigrate sperm was filtered through a metal mesh (80 µm) to remove tissue fragments, and sperm were recovered at room temperature (22–23°C). The time from the excision of the epididymis to transmigration of sperm was about 20 min. Then, the quality of cauda epididymal sperm was examined as described below.

Concerning LC-3, semen was collected using urethral catheterization and transrectal electric stimulation under general anesthesia four times during the year before death. Therefore, data for collected semen were used for quality comparisons with cauda epididymal sperm. Semen was collected using a previously reported transrectal electric stimulation method [14]. Furthermore, urethral catheterization was performed before transrectal electric stimulation. Briefly, the penis was exposed by retracting the foreskin of the Amur leopard

Table 1. Information of Amur leopard cats at the death

Animal No.	LC-1	LC-2	LC-3	LC-4	LC-5
Age at death (years)	8	8	8	14	16
Body weight at death (kg)	4.8	3.1	3.6	5.2	5.4
Cause of death	Pneumonia	Kidney failure	Pneumothorax	Kidney failure	Senility
Month at death	December	April	April	March	November
Time from death to sperm collection (hour)	13	4	1	5	7
Freezing of collected sperm	+	+	+	-	-

LC: leopard cat.

Table 2. Information of Tsushima leopard cats and domestic cats at the time of castration

Animal No.	LC-6	LC-7	DC-1	DC-2	DC-3	DC-4	DC-5
Breed	Tsushima leopard cat		British Shorthair	Mix	Mix	Mix	Mix
Age at the castration (years)	>15 *	>16 *	1	1	1	2	3
Body weight at the castration (kg)	4.0	3.4	4.5	5.0	4.5	5.2	4.7
Month at the castration	January	January	December	October	June	November	September
Freezing of collected sperm	-	-	-	-	+	+	+

LC: leopard cat, DC: domestic cat. *Age is shown as a breeding period in zoo because it is an individual captured in the wild.

cat immobilized under sedation with medetomidine hydrochloride (Domitor®, 60 µg/kg; Nippon Zenyaku Kogyo Co., Ltd., Tokyo, Japan). A 3 Fr. feeding catheter (Atom Medical Co., Tokyo, Japan) was inserted from the urethral tip to the depth at which the prostatic seminal duct may be open (approximately 8 to 9 cm from the urethral opening). Semen that entered the catheter was collected into a sterile plastic 1.5-mL conical tube. Subsequently, quality was evaluated, as described below.

Sperm recovered from the cauda epididymis and ejaculated sperm were examined with respect to motility, viability, abnormalities, the rate of immature sperm, and total sperm counts, as reported previously [14, 22]. In brief, the sperm concentration was determined using a hematocytometer and the total sperm count was calculated by multiplying the semen volume by the sperm concentration. The total number of sperm collected from one pair of epididymides was considered the total sperm count. Sperm motility was subjectively defined as the percentage of progressively motile sperm on a semen quality examination plate and a warm-plate, and the percentages of viable, morphologically abnormal, and immature sperm were assessed by eosin-nigrosin staining. Sperm abnormality was estimated as the percentage of total sperm with morphological abnormalities. Sperm exhibiting cytoplasmic droplets on the mid-piece were judged as immature.

Cauda epididymal sperm from three Amur leopard cats (LC-1 to -3) and three domestic cats (DC-3 to -5) were cryopreserved following the previously reported plunging-in method for domestic cats [17, 18, 22]. Semen was suspended in EYT-FC as a semen extender prior to freezing. After the initial assessments described above, semen was subjected to an initial dilution at 20°C to adjust the sperm concentration to 1×10^8/mL and maintained to a first refrigeration at 4°C for 1 hr using a programmable cooling system (UH-JF, Chino Ltd., Tokyo, Japan). The second dilution was performed by dripping an equal volume of the secondary extender, EYT-FC supplemented with 14% glycerol (Sigma-Aldrich, St. Louis, MO, USA) and 2% Orvus ES Paste (Nova Chemical Sales, Inc., Scituate, MA, USA) at 4°C. After this second and final dilution, the concentrations of sperm, glycerol, and OEP were 5×10^7/mL, 7%, and 1%, respectively. Then, 200 µL of semen was placed in a 0.25 mL straw and equilibrated with glycerol for 1 hr at 4°C. Straws were frozen by pouring liquid nitrogen (LN_2) in a Styrofoam box. After exposure to LN_2 vapor for 10 min at 7-cm heights from the LN_2 surface, straws were plunged into LN_2 for final freezing and storage.

For the examination of semen quality after thawing, the straw was thawed by putting it in warm water at 37–38°C for 30 sec one week or more after freezing. Immediately after thawing, sperm motility, sperm viability, and sperm abnormality were examined. In addition, the semen was kept at 20°C in a constant temperature bath (e-ThermoBucket, TAITEC Co., Ltd., Saitama, Japan) and the time-course of sperm motility and sperm viability were examined at 1, 2, 4 and 6 hr after thawing.

Data are shown as mean ± SE. For analyses, animals were divided into three groups: LC-1 to -3 (middle-aged), LC group; LC-4 to -7 (old-aged), old-LC group; and DC-1 to -5, DC group. Differences in sperm parameters among groups were analyzed using one-way ANOVA and post hoc Tukey-Kramer tests using STATVIEW 5.0 (Abacus Concepts Inc., Berkely, CA, USA). Values of $P<0.05$ were regarded as significant.

The testicular and epididymal sizes and weights in each group are presented in Table 3. There was a slight variation among individuals in these parameters. The testicular weight in the old-LC group was significantly lower than that in the LC group ($P<0.01$). The testicular volume in the old-LC group was significantly lower than that in the DC group ($P<0.05$); however, there were no significant differences between the old-LC and LC groups. Furthermore, the entire length of the epididymis in the old-LC group was shorter than those in the other two groups, however there was no significant difference among groups. In addition, there was no significant difference in epididymal weight among groups.

The qualities of cauda epididymal sperm immediately after collection in each group are presented in Table 4. LC-4 in the old-LC group was not included in analyses of sperm quality owing to a lack of sperm. In LC-7, a small number of sperm were collected from the left cauda epididymis; however, there were no sperm in the right cauda epididymis. Therefore, only the left cauda epididymal sperm from this animal were included in analyses. In the other animals, sperm quality was similar on the left and right sides.

Sperm progressive motility and viability immediately after collection varied among the animals. However, there were no significant differences between groups. The rate of sperm abnormalities in the old-LC group was slightly higher than those in the other groups; however, there was no significant difference between groups. The rate of immature sperm in the DC group was significantly higher than that in the old-LC group ($P<0.05$). In the LC and DC groups, the total number of sperm on the left and right sides varied among individuals. However, there was no significant difference between the two groups. The total number of sperm in the old-LC group was extremely small; however, there were no significant differences among groups.

Qualities of sperm collected by four times by urethral catheterization and transrectal electric stimulation and cauda epididymal sperm collected from an Amur leopard cat (LC-3) are summarized in Table 5. Sperm motility rates were slightly higher for cauda epididymal sperm compared to sperm collected by other methods. Sperm viability and rates of sperm abnormalities were similar. The rate of immature sperm was slightly higher in epididymal sperm compared to sperm collected by other methods. The mean number of ejaculated sperm (across four time points) was very low at 5.3×10^6; however, many spermatozoa were collected from the right and left epididymis.

The qualities of cauda epididymal sperm from Amur leopard and domestic cats after freezing/thawing are presented in Table 6. Sperm motility and viability immediately after thawing were similar between the two groups. There was no significant difference in sperm motility between the two groups within 6 hr after thawing. Sperm viability in the LC group 2 hr ($P<0.01$) and 6 hr ($P<0.05$) after thawing were lower in the DC group. Furthermore, there was no difference in the sperm abnormality between the two groups.

In this study, we compared cauda epididymal sperm from three Amur leopard cats of approximately 8 years of age with different causes of death with those from domestic cats. The epididymis was not obtained from age-matched domestic cats; therefore, cauda epididymal sperm were obtained from young domestic cats with a mean age of 1.6 years. As a result, there was no significant difference in sperm quality between the two groups. There was no significant difference in the total number of sperm between groups, despite the

Table 3. Testicular and epididymal sizes and weights in each individual and group

		LC group							old-LC group								
		LC-1		LC-2		LC-3		Mean ± SE	LC-4		LC-5		LC-6		LC-7		Mean ± SE
		L	R*[1]	L	R	L	R		L	R	L	R	L	R	L	R	
Testis	Major axis (cm)	1.6	1.7	1.6	1.6	1.3	1.2	1.50 ± 0.09	1.7	1.7	1.4	1.3	1.2	1.3	1.1	1.1	1.35 ± 0.09
	Minor axis (cm)	1.4	1.3	1.4	1.5	1.1	1.1	1.30 ± 0.07[a*3]	1.1	1.1	1.0	1.2	1.1	1.2	1.1	1.1	1.11 ± 0.02[b,c]
	Thickness (cm)	1.2	1.1	1.2	1.3	0.9	0.9	1.10 ± 0.07	1.1	1.3	1.2	0.9	1.1	1.1	0.8	0.8	1.04 ± 0.07
	Volume (cm^3)*[2]	1.4	1.3	1.4	1.6	0.7	0.6	1.17 ± 0.18	1.1	1.3	0.9	0.7	0.8	0.9	0.5	0.5	0.83 ± 0.10[a]
	Weight (g)	1.2	1.3	1.5	1.5	3.0	3.0	1.92 ± 0.38[c]	1.0	1.5	0.8	0.8	0.8	0.9	0.6	0.7	0.89 ± 0.11[d]
Epididymis	Entire length (cm)	3.5	3.2	3.4	3.8	2.8	3.8	3.42 ± 0.17	3.5	3.1	2.9	3.1	2.6	3.1	2.0	2.7	2.88 ± 0.17
	Weight (g)	0.4	0.3	0.4	0.4	0.8	0.8	0.52 ± 0.10	0.4	0.3	0.6	0.5	0.3	0.3	0.8	0.8	0.50 ± 0.08
	Width of the caput (cm)	0.8	0.6	0.5	0.7	0.4	0.3	0.55 ± 0.08	0.5	0.5	0.6	0.7	0.6	0.6	0.4	0.6	0.56 ± 0.03
	Thickness of the caput (cm)	0.3	0.2	0.3	0.2	0.2	0.2	0.23 ± 0.02[a]	0.4	0.2	0.2	0.4	0.3	0.2	0.2	0.2	0.26 ± 0.03[a]
	Width of the cauda (cm)	0.8	1.0	0.9	0.7	1.0	1.8	1.03 ± 0.18[a,c]	0.6	0.8	0.6	0.8	0.7	0.8	0.6	0.6	0.69 ± 0.04[b]
	Thickness of the cauda (cm)	0.4	0.4	0.5	0.4	0.4	0.4	0.42 ± 0.02	0.5	0.3	0.4	0.5	0.3	0.3	0.3	0.3	0.36 ± 0.03

		DC group										
		DC-1		DC-2		DC-3		DC-4		DC-5		Mean ± SE
		L	R	L	R	L	R	L	R	L	R	
Testis	Major axis (cm)	1.9	1.8	1.0	1.1	1.5	1.6	1.8	1.5	1.6	1.7	1.55 ± 0.11
	Minor axis (cm)	1.5	1.5	1.4	1.1	1.4	1.3	1.3	1.3	1.3	1.3	1.34 ± 0.04[d]
	Thickness (cm)	1.5	1.4	0.9	0.9	1.3	1.2	1.1	1.1	1.2	1.2	1.18 ± 0.07
	Volume (cm^3)*[2]	2.2	2.0	0.7	0.6	1.4	1.3	1.3	1.1	1.3	1.4	1.33 ± 0.19[b]
	Weight (g)	2.2	2.0	0.7	0.6	1.4	1.5	1.0	1.0	1.5	1.5	1.34 ± 0.20
Epididymis	Entire length (cm)	3.7	3.4	3.0	2.0	3.6	3.4	3.2	3.1	3.6	3.9	3.29 ± 0.20
	Weight (g)	0.4	0.5	0.3	0.3	0.5	0.5	0.2	0.2	0.3	0.3	0.35 ± 0.04
	Width of the caput (cm)	0.4	0.7	0.6	0.6	0.8	0.8	0.4	0.4	0.6	0.6	0.59 ± 0.04
	Thickness of the caput (cm)	0.4	0.5	0.3	0.3	0.5	0.4	0.2	0.3	0.4	0.4	0.37 ± 0.04[b]
	Width of the cauda (cm)	0.7	0.6	0.7	0.7	0.7	0.7	0.6	0.6	0.8	0.4	0.65 ± 0.04[d]
	Thickness of the cauda (cm)	0.6	0.6	0.3	0.4	0.4	0.3	0.3	0.4	0.4	0.3	0.40 ± 0.04

*1: L: left epididymis R: right epididymis. *2: The testicular volume was calculated from a formula of 4/3π ×major axis/2 ×minor axis/2 ×thickness/2. *3: Significantly different between different alphabets of the same row (a–b: $P<0.05$, c–d: $P<0.01$). LC: leopard cat, DC: domestic cat.

Table 4. The qualities of cauda epididymal sperm immediately after collection in each group

	LC group (n=3 animals)		Old-LC group (n=3 animals)		DC group (n=5 animals)	
Sperm progressive motility (%)	83.3 ± 1.7	(80.0–90.0)	75.0 ± 5.9	(60.0–95.0)	82.0 ± 1.1	(80.0–85.0)
Sperm viability (%)	84.9 ± 3.1	(72.0–90.9)	91.7 ± 1.4	(88.9–95.0)	90.1 ± 1.9	(81.4–99.1)
Sperm abnormality (%)	4.3 ± 1.2	(1.8–8.7)	9.7 ± 4.7	(2.0–23.0)	6.9 ± 0.9	(3.3–10.5)
Immature sperm (%)	4.9 ± 1.5	(1.2–10.8)	1.5 ± 0.9[a*1]	(0.0–4.2)	12.2 ± 3.0[b]	(1.8–33.9)
Total number of sperm ($\times 10^6$)*[2]	78.6 ± 12.7	(55.6–99.6)	2.0 ± 1.0	(0.1–3.2)	90.7 ± 33.5	(30.8–198.9)

*1: Significantly different between different alphabets of the same row ($P<0.05$). *2: Total number of sperm were collected from the right and left cauda epididymis. In one animal of old-LC group, only the left cauda epididymal sperm were included in analyses. LC: leopard cat, DC: domestic cat.

Table 5. The qualities of sperm collected by four times by urethral catheterization (UC) and transrectal electric stimulation (ES) and left and light cauda epididymal sperm (EP) from an Amur leopard cat (LC-3)

	EP (left)	EP (right)	ES	UC	Mean of ES and UC
Sperm progressive motility (%)	90.0	85.0	70.0 ± 15.1	63.3 ± 14.7	67.1 ± 9.1
Sperm viability (%)	90.1	85.9	90.6 ± 4.8	80.9 ± 0.6	86.4 ± 3.2
Sperm abnormality (%)	8.7	6.9	6.4 ± 1.8	6.7 ± 0.8	6.5 ± 0.9
Immature sperm (%)	6.6	4.2	0.6 ± 0.1	1.3 ± 0.8	0.9 ± 0.3
Total number of sperm ($\times 10^6$)	23.8	31.8	4.2 ± 3.4	1.5 ± 0.6	5.3 ± 3.5*[1]

*1: This is the mean total sperm number of four semen collected by UC and ES.

Table 6. The qualities of cauda epididymal sperm from Amur leopard cats and domestic cats before and after freezing-thawing

		LC group (n=3 animals)	DC group (n=3 animals)
Sperm progressive motility (%)	Immediately after collection	83.3 ± 1.7 (80.0–90.0)	80.8 ± 0.9 (80.0–85.0)
	0 hr after thawing	26.7 ± 6.0 (15.0–35.0)	28.3 ± 4.4 (20.0–35.0)
	1 hr after thawing	26.7 ± 6.0 (15.0–35.0)	28.3 ± 4.4 (20.0–35.0)
	2 hr after thawing	21.7 ± 6.0 (10.0–30.0)	25.0 ± 2.9 (20.0–30.0)
	4 hr after thawing	15.0 ± 2.9 (10.0–20.0)	15.0 ± 7.6 (5.0–30.0)
	6 hr after thawing	6.7 ± 1.7 (5.0–10.0)	10.0 ± 7.6 (0.0–25.0)
Sperm viability (%)	Immediately after collection	84.9 ± 3.1 (72.0–90.9)	89.3 ± 2.5 (83.5–97.9)
	0 hr after thawing	55.4 ± 3.7 (48.3–60.7)	59.8 ± 1.4 (57.1–61.9)
	1 hr after thawing	43.4 ± 5.7 (34.2–53.8)	51.7 ± 1.1* (50.2–53.8)
	2 hr after thawing	41.3 ± 2.5 (37.3–40.5)[a]	54.8 ± 2.0 (52.5–58.8)[b]
	4 hr after thawing	38.0 ± 3.1 (32.8–33.6)	49.6 ± 5.5 (39.9–59.1)
	6 hr after thawing	29.5 ± 1.6 (26.5–32.1)[c]	47.0 ± 5.3 (38.3–56.5)[d]
Sperm abnormality (%)	Immediately after collection	4.3 ± 1.2 (1.8–8.7)	7.8 ± 1.1 (3.6–10.3)
	0 hr after thawing	10.8 ± 4.4 (6.0–19.5)	7.0 ± 1.0 (5.1–7.8)

* Significantly different between different alphabets of the same row (a–b: $P<0.01$, c–d: $P<0.05$). LC: leopard cat DC: domestic cat.

presence of diseases before the death of some Amur leopard cats (most cats exhibited rapid deterioration, rather than chronic disease). Of note, a large number of sperm could be collected from such cats.

In this study, cauda epididymal sperm were collected from old Amur and Tsushima leopard cats aged ≥14 years. However, no sperm were found in one Amur leopard cat (LC-4) and in the unilateral epididymis of one Tsushima leopard cat (LC-7). There were no differences in sperm motility, viability, and abnormalities between the three old leopard cats, younger Amur leopard cats, and domestic cats. The mean number of sperm collected was extremely small. This could be explained by an aging-related reduction in spermatogenic function. These results suggest that it is necessary to perform castration surgery before old age or the onset of diseases—that is, before spermatogenic hypofunction—and to collect sperm from the cauda epididymis for storage in order to effectively utilize this resource. A specific age theshold for castration surgery cannot be determined based on the results of this study alone. In a previous study [14], semen was also collected from 3 to 7 years of age in Amur leopard cats. There was no reduction in semen quality; however, the influence of aging may appear at a later timepoint. It may be more feasible to collect a large number of sperm from younger animals; therefore, if there is no breeding plan for the specific male line because of his pedigree, castration should be performed after repeated natural breeding to collect cauda epididymal sperm for freezing and storage as young as possible.

The cat LC-3 died immediately after semen collection, and the number of sperm collected was approximately 14×10^6 (data not shown). However, approximately 55×10^6 sperm were collected from the cauda epididymis about 1 hr later, indicating that many sperm remained in the cauda epididymis even immediately after semen collection. In addition, the mean number of sperm collected from the four semen samples obtained before death was 5.3×10^6, indicating that the cauda epididymis stores approximately 10 times more sperm. It has been reported that fewer sperm can be collected from Amur leopard cats by transrectal electric stimulation and urethral catheterization than from domestic cats, and effective sperm collection methods are lacking [10, 14, 23]. Thus, it has been estimated that the number of sperm stored in the cauda epididymis is smaller than that in domestic cats. However, the results of present study showed that there was no difference in the number of sperm stored in the cauda epididymis between the leopard and domestic cats. Therefore, a larger number of sperm can be collected by modifying the semen collection method.

A previous study [14] indicated that the semen collected from Amur leopard cats varies slightly among seasons and that the quality of semen is best before the female breeding season. The breeding season for females of both the Amur and Tsushima leopard cats is February to March [12, 15]. In this study, three cats in the LC group died around the breeding season. Therefore, seasonal factors likely had little influence on sperm qualities. In a previous study [14], it was sometimes impossible to collect semen in the summer after the breeding season. Furthermore, the season had a slight influence on testicular size and there was a marked decrease in serum testosterone levels after the breeding season. A decrease in this hormone may directly influence spermatogenic function, suggesting that many sperm cannot be collected from the cauda epididymis at this time of year.

In this study, sperm could be promptly collected in castrated animals, whereas the interval from the death of Amur leopard cats to sperm collection differed among animals, ranging from 0.5 to 13 hr. However, sperm motility and viability immediately after collection in each animal were high. This could be explained by the prompt storage of animals at 5°C immediately after death. In domestic cats [5, 6, 16, 17], the quality of cauda epididymis sperm may be maintained for at least 24 hr if the testis and epididymis are stored at a low temperature (4 to 5°C) immediately after castration. In the event of an accident, such as a traffic accident, animals cannot always be detected promptly. Keeping the testis and epididymis above room temperature causes deterioration of semen quality, as reported in domestic cats [16, 17]. It may be necessary to place dead animals under a low temperature (4 to 5°C) as quickly as possible to ensure a favorable sperm quality in cases when prompt sperm collection from the cauda epididymis is not possible.

In this study, cauda epididymal sperm from Amur leopard cats were frozen and stored by same method used for domestic cats [16, 17], and there was no significant difference in sperm quality (sperm motility and viability) after thawing between cauda epididymal

sperm of Amur leopard cat and domestic cat. Based on previous reports on frozen-thawed epididymal sperm in domestic cats [19, 20], if a sperm quality similar to that of domestic cat is obtained, it may be possible to obtain offspring after artificial insemination. However, recently, Jeong *et al.* [10] reported that the quality of semen after thawing at a final glycerol concentration of 2% or 4% was better than that at 6% after the freezing/storage of semen collected from Amur leopard cats using urethral catheterization. Therefore, it is necessary to further examine the optimal concentration of glycerol.

Successful cases of surgical intrauterine insemination using fresh semen in Amur leopard cats [15] and laparoscopic intratubal insemination in Tsushima leopard cats [1] have been reported. The numbers of motile spermatozoa used for artificial inseminations were 8×10^6 (21×10^6 sperm fertilizing both uterine horns, 40% sperm motility) and 3.3×10^6, respectively. Based on sperm quality, it was suggested that artificial insemination using these qualities of sperm with progressive motility could lead to conception. In the future, intra-uterine or -tubal artificial insemination with frozen/thawed cauda epididymal sperm must be performed.

In conclusion, our results indicated that a sufficient number of sperm with favorable quality characteristics, similar to those in domestic cats, could be collected from the cauda epididymis of Amur leopard cats. In addition, sufficient frozen/thawed sperm were available for artificial insemination. However, young animals are required, and it may be necessary to maintain the animal at a low temperature (about 5°C) when sperm collection immediately after death is not possible. This study provides valuable basic data on artificial assisted reproductive technologies for rare, small wild Felidae species.

CONFLICT OF INTEREST. The authors declare no potential conflicts of interest with respect to the research and authorship.

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