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Daily fecal sex steroid hormonal changes and mating success in captive female cheetahs (*Acinonyx jubatus*) in Japan

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ABSTRACT

Daily fecal estrogen and progestin concentrations were measured by enzyme immunoassay in five female cheetahs (*Acinonyx jubatus*) for 4–6 months. The animals were housed under different conditions: (1) a female always housed in a group including one or more males; (2) two females isolated individually for short or long periods; (3) the other two females housed together. These females were separately housed with males for mating around the time of the estrogen peaks. The hormone profiles were similar in all five females regardless of the housing conditions. However, only the female that had been isolated from other cheetahs for over a year mated and reproduce cubs successfully, whereas the remaining four did not (one was isolated for only 6 weeks, another was always housed with males and the other two were housed together). In all females, the estrogen peaks were obtained at regular intervals of approximately 8–15 days. Unlike estrogen, the progestin concentrations were always low in all females except during pregnancy and they did not increase following the estrogen surges. These results showed that female cheetahs are typically reflex ovulators and female receptiveness may not be reflected to her hormonal states. It was also suspected that individual housing and long-term separation are advantageous for breeding this wild cat in captivity, mimicking the ecological/behavioral patterns in the wild, though housing condition might have no effect on the estrous cycle.

1. Introduction

In the 1900s, approximately 100,000 wild cheetahs (*Acinonyx jubatus*) were estimated to inhabit at least in 44 countries throughout Africa and Asia (Myers, 1975). However, recent surveys suggest that the population has decreased to not much more than 7,000 individuals (Marker, 2002). A further concern is that cheetahs breed poorly in captivity and the estimated percentage of the population contributing to the gene pool through reproductive success could be less than a half of the total population (Kelly, 2001). Consequently the cheetah is highly endangered due to the losses of habitats in the wild and the failure to reproduce in captivity.

In Japan, 94 cheetahs have been kept at nine zoological institutions in the last decade (Ito, 2007). This number is relatively large world wide and is the largest in Asia (China, Indonesia, Japan, Singapore, South Korea and Thailand) (Marker, 2010). Therefore, the role of Japan in maintaining the population of this species is important and in 1988, the Japanese Association of Zoological Gardens and Aquariums (JAZGA) formed the Species Survival Committee of Japan (SSCJ) and initiated a cooperative population management and conservation program for selected rare species including cheetahs. However, in the captive population of Japan, fewer than 20% of cheetahs have reproduced naturally, and the number of deaths has exceeded that of captive births (Ito, 2007).

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Table 1
Housing conditions of five cheetahs at Himeji Central Park (Hyogo, Japan).

Animal	IS no[a]	Housing conditions	
		Male encounter	Female encounter
A	#5794	Yes	No
B	#5871	No[b]	No[b]
C	#3958	No[c]	No[c]
D	#6085	No[d]	Yes
E	#6106	No[d]	Yes

[a] International Studbook.
[b] During the study, female B is isolated from both males and females.
[c] Female C is isolated from both males and females for more than a year.
[d] Females D and E could see males through the fence in the daytime before and during this study.

Assisted reproductive techniques, such as artificial insemination (Howard et al., 1992, 1997; Wildt et al., 1986), *in vitro* fertilization (and embryo transfer) (Donoghue et al., 1992), and cloning (Gómez et al., 2004), remain the most powerful potential ways to improve the poor reproductive rates in endangered animals including cheetahs but when applying such techniques, an accurate understanding of the ovarian cycle in the species is both a precondition and a key to a successful breeding. The ovarian or estrous cycles of captive cheetahs have been examined by a number of researchers but results are equivocal with estrous cycles reported to be short (10–14 days) (Eaton and Craig, 1970; Asa et al., 1992; Brown et al., 1996; Borque et al., 2005) and long cycles (about 26 days) (Czekala et al., 1994). In this study, we examined the change of fecal steroid concentration in five captive female cheetahs reared under various housing conditions. We discuss the hormonal changes of female cheetahs and copulation success. This study describes progestin and estrogen changes in a small group of cheetahs under captivity.

2. Materials and methods

2.1. Females

Five healthy female cheetahs, A (International Studbook #5794), B (#5871), C (#3958), D (#6085) and E (#6106) kept at Himeji Central Park (Hyogo, Japan) were monitored in this study. Females A, B, D and E were 3 years old at the start of the study and nulliparous, female C was 9 years old and parous.

Before and during this study, the females were reared under different housing conditions (Table 1). Females A and B had been housed with one or more males until the start of this study in the exhibition space (Fig. 1). Female C had been isolated from other individuals for a long time (more than a year) excluding her cubs born in 2006 in the outdoor enclosure #1 and #2 (Fig. 1). After starting this study, female A was housed with males during the day in the same space, while female B was isolated from others including males in the outdoor enclosure #3 (Fig. 1). No changes were made to the housing conditions of female C who was kept isolated. Females D and E had been divided by a fence in the same place (Fig. 1). These females could also see males through the fence in the daytime before and during this study. Meanwhile, individuals isolated from others were within the range of audio and olfactory interactions. At night (around 5:00 PM–9:00 AM), all individuals were separated from other cheetahs in each camp (Fig. 1A–E).

All cheetahs were fed horseflesh supplemented with vitamins and minerals, but were fasted 1 day a week. Water was available *ad libitum*.

2.2. Fecal steroid analysis

Fecal samples were collected almost every day from June 28 to October 20, 2007, from females A ($n = 98$) and B ($n = 93$), from November 11, 2007 to May 8, 2008, from female C ($n = 168$), and from November 1, 2008 to May 27, 2009, from D ($n = 139$) and E ($n = 115$). These samples were stored at −35 °C until analysis.

Feces were dried in an electric drying oven (DRA330DA, Advantec Toyo Kaisya Ltd., Tokyo, Japan) at 50 °C for 24 h and pulverized. Then, 0.06 g of powdered feces was vortexed for 30 min in 3 ml of 80% methanol and centrifuged at 2500 rpm for 10 min. The extracts of estrogen and progestin metabolites in the supernatant were analyzed by enzyme immunoassay (EIA) according to the method described elsewhere (Kinoshita et al., 2009). Both steroid metabolites are indicated as μg/g dry fecal weight (DFW).

The estradiol-17β antibody (FKA236E; Cosmo Bio Co., Ltd., Tokyo, Japan) cross-reacted 100% with estradiol-17β, 56.30% with estradiol-3-glucuronide, 26.80% with estradiol-3-sulfate, 1.20% with estrone-3-glucuronide, 0.86% with estrone-3-sulfate, 0.80% with estrone, 0.50% with estriol, and 0.05% with testosterone. The progesterone antibody (FKA301; Cosmo Bio Co., Ltd.) was cross-reacted 100% with progesterone, 12.50% with 5α-pregnanedione, 5.30% with 11α-OH-progesterone, 2.00% with pregnenolone, 0.20% with 20α-OH-progesterone, and 0.01% with deoxycorticosterone, 17α-OH-progesterone, corticosterone, cortisol, and aldosterone.

The intra- and inter-assay coefficients of variation (intra and inter-assay CVs, respectively) were evaluated from the concentrations obtained in control samples. The intra-assay CV was calculated from 8-wells within a plate, and the inter-assay CV was calculated from 26 (estrogen) or 28 plates (progestin). The intra- and inter-assay CVs were 6.5% and 10.2% for estrogen, 6.9% and 11.9% for progestin, respectively. The sensitivities of the assay were 0.2 and 1.2 pg/well for estrogen and progestin, respectively. Female D was used to evaluate parallelism between serially diluted standards and fecal samples and to determine recovery of known amounts of hormone from sample based on Hama et al. (2009) and Larson et al. (2003). Samples collected on January 20 and 21, 2009 were used for parallelism to measure concentrations at the serial dilutions (2×, 4×, 8×, 16×, 32×, 64× and 128×) in progestin and estrogen, respectively. The standard linear line and the sample linear line were parallel (estrogen; $F = 3.356$, $P = 0.097$, progestin; $F = 0.014$, $P = 0.907$). Samples collected on January 15 and March 24, 2009 were used for the recovery test of estrogen and progestin, respectively. Steroid standards (1,000 ng/ml) were added to each 1-ml sample extract in increasing amounts: 12.5, 25, 50, 100 and 200 μl estradiol-17β and 25.5, 50, 100, 200 and 400 μl proges-

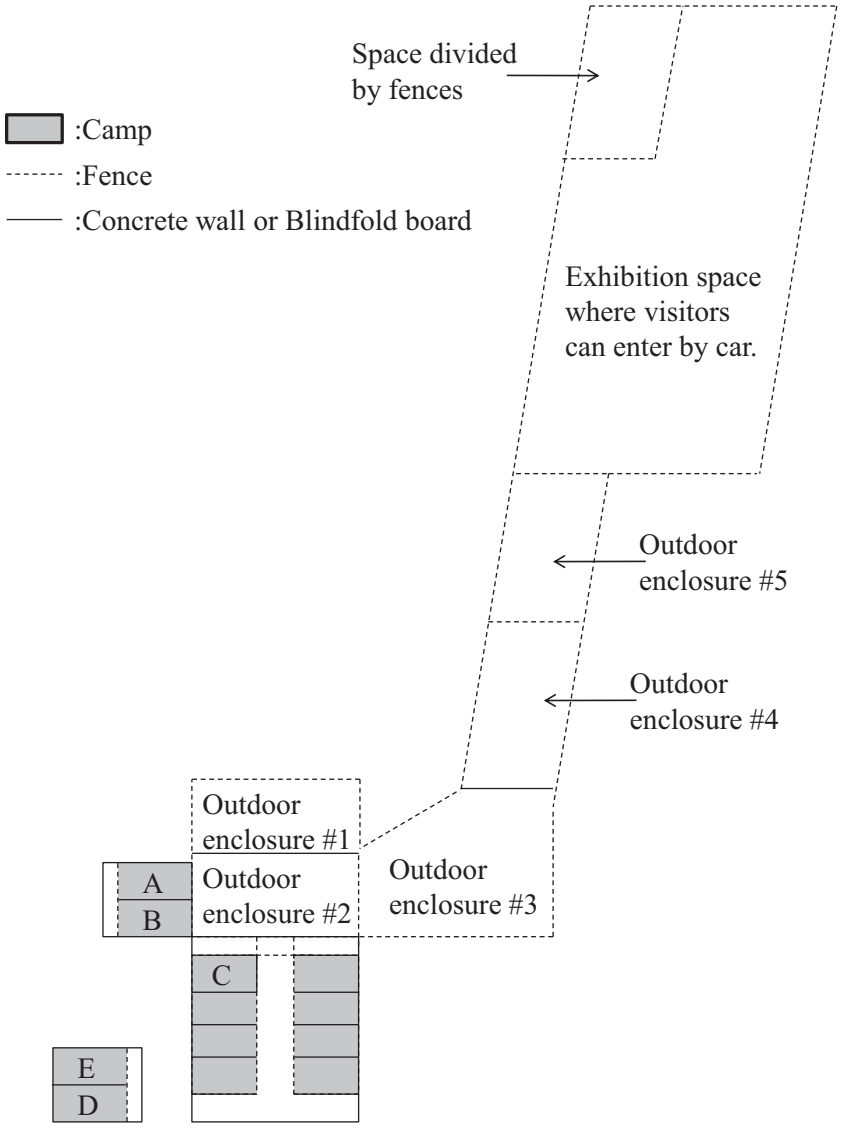

Fig. 1. Layout of the indoor and outdoor enclosures and the exhibition space. A, B, C, D and E indicate each camp for female cheetahs A, B, C, D and E in Himeji Central Park.

terone. Recovery tests produced lines with average slopes of 1.297 and 0.9504 for estrogen and progestin, respectively.

2.3. Mating test

Recently, a high rate of multiple paternities was demonstrated in wild cheetahs (Gottelli et al., 2007). This finding also demonstrated that female fidelity was low, and explained that females chose to mate with several unrelated males within an estrous cycle. Bertschinger et al. (2008) reported that cheetahs could be bred successfully in captivity. He conducted the breeding management under which males in groups of three to five were allowed to meet with a female. We adopted this unique multiple male mating system. In the current study, all females were given opportunities to meet with a total of eight adult males. These males were one 2-year-old, four 4-year-old, two 9-year-old and an 11-year-old male. Most male cheetahs reach sexual maturity at 2.5 years (Kelly et al., 1998) and the presence of viable spermatozoa was also confirmed in 3-year old males by Bertschinger et al. (2006). These mating timings were set around the days immediately before or during the fecal estrogen peak based on the results of fecal steroid analysis sampled almost every day. Only female A had unlimited opportunities for coitus, as she was always housed with males during the day.

2.4. Statistical analysis

Data are presented as mean ± SEM. For each female, baseline estrogen concentrations were calculated using an iterative process in which values that exceeded 2 standard deviations (SD) above the mean were excluded; the average

was recalculated and the elimination process was repeated until no values exceeded 2SD above the mean (Brown et al., 1994; Graham et al., 2000). Based on the method reported by Pelican et al. (2004), the average of the remaining values was considered the "baseline" level for the animals. Values greater than 3 times the baseline were considered "elevated". The Kruskal–Wallis test was carried out to evaluate whether the estrogen cyclicity was significantly different among all females. Regression of both of the standards and diluted samples was adapted to linear lines, respectively, and the parallelism among the lines was also examined by one-way analysis of covariance. A P value of <0.05 was deemed significant. These analyses were performed with SPSS Ver. 10 (SPSS Japan Inc., Tokyo, Japan).

3. Results

3.1. Daily fecal estrogen changes

Fig. 2 depicts the fecal estrogen and progestin profiles in the five female cheetahs. The range of estrogen concentrations (Table 2) and the mean peak levels (asterisks in Fig. 2, female A: 7.15 ± 1.02 μg/g DFW, $n = 11$; female B: 9.34 ± 1.47 μg/g DFW, $n = 9$; female C: 4.02 ± 0.45 μg/g DFW, $n = 8$; female D: 6.41 ± 0.98 μg/g DFW, $n = 18$; female E: 3.16 ± 0.46 μg/g DFW, $n = 13$) during this study varied among individuals. However, in all females, these peaks were appeared at regular intervals and there was no significant difference among these individuals (Table 2, $P = 0.15$), regardless of their housing conditions. Brown et al. (1996) reported that the cyclicity was apt to interrupt by periods of anoestrus in captive female cheetahs. Fortunately no such interruption had been occurred in all females during this study.

3.2. Mating test

Among all five females, female C only achieved successful copulation. Female C was introduced to four males individually from 6 days prior to 3 days after the eighth estrogen peak. She was mated with a 4-year-old male (International Studbook #5700). A total of 94 days after coitus (January 27, 2008), she gave birth to three healthy cubs. Female A did not mate with any males, even though she was always housed with eight males including #5700. Female B was housed with two males including #5700 for a limited period, from several days before to the day after the fifth estrogen peak, and then kept permanently with eight males including #5700 after the seventh peak. However, no coupling was observed in female B. Similarly, females D and E were housed with a total of four males including #5700 from 1 to 9 days after the second estrogen peak (female D) or from the day of the first estrogen peak to 5 days after the peak (female E), but copulation never occurred.

3.3. Daily fecal progestin changes

After copulation, female C succeeded in becoming pregnant. The progestin level was rapidly increased after mating from 1.34 to 152.99 μg/g DFW. The heightened progestin levels were maintained for about 90 days (Figs. 2C and 3).

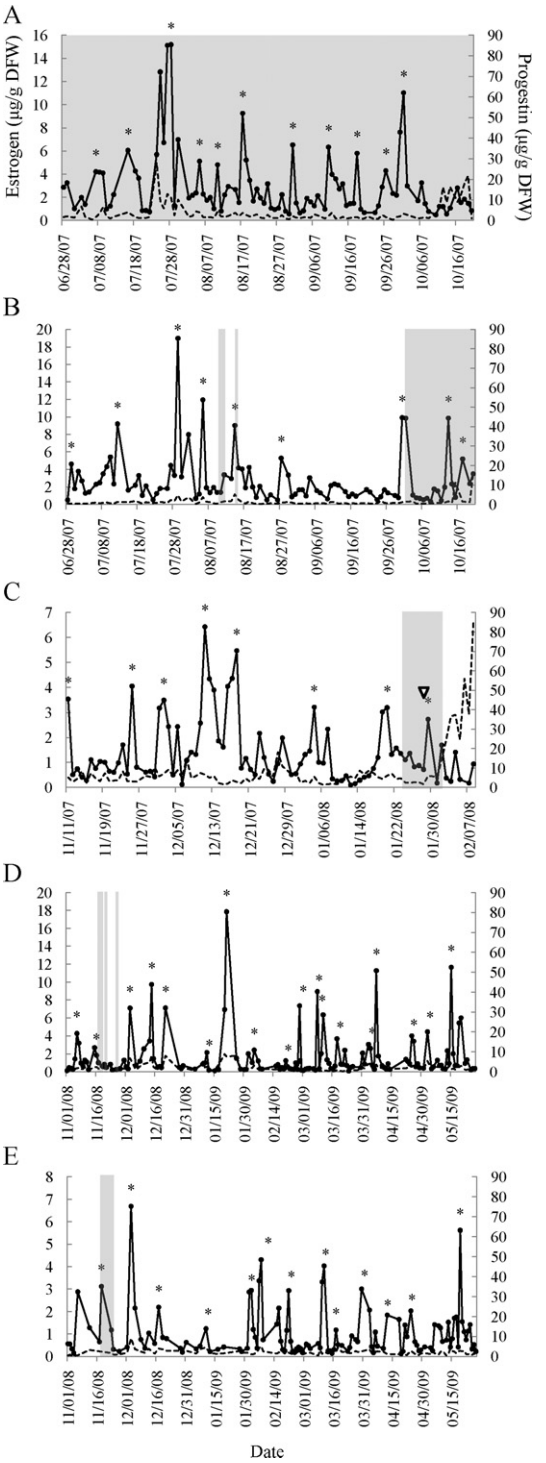

Fig. 2. Daily fecal estrogen and progestin profiles of five female cheetahs (A–E). Continuous line is estrogen and dotted line is progestin. Shaded areas represent the period when they were housed with males. Asterisks indicate the estrogen peaks. Arrowhead indicates the time coitus occurred.

Table 2
Sex steroid hormonal changes in five cheetahs under captivity.

Animal	Sample size	Estrogen value ranges[a] (estrogen peak intervals)	Progestin value ranges[a]
A	$n = 98$	0.48–15.19 (8.60 ± 0.90 days, $n = 10$)	0.33–27.99
B	$n = 93$	0.33–18.98 (13.75 ± 3.23 days, $n = 8$)	0.29–15.82
C	$n = 86$	0.09–6.43 (11.29 ± 1.61 days, $n = 7$)	1.34–152.99[b]
D	$n = 139$	0.07–17.86 (11.18 ± 1.27 days, $n = 17$)	0.05–8.76
E	$n = 115$	0.12–6.69 (15.17 ± 1.85 days, $n = 12$)	0.21–8.98

[a] μg/g DFW.
[b] Pregnant.

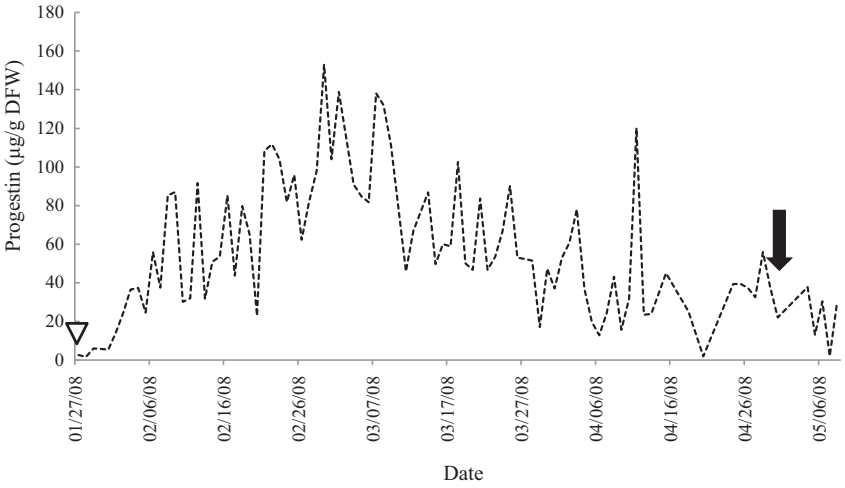

Fig. 3. Daily fecal progestin profiles in female cheetah C during pregnancy. Arrowhead and arrow indicate coitus and delivery, respectively.

In contrast, progestin levels in the other four females were always low (Table 2 and Fig. 2).

4. Discussion

Felines are usually characterized as induced ovulators (Brown et al., 1994; Brown, 2006; Concannon, 1991; Kinoshita et al., 2009; Lawler et al., 1993). The cheetah is also generally considered a typical induced ovulator (Bertschinger et al., 1998; Brown, 2006). However, spontaneous ovulations without mating are widely known to occur sometimes in various felines including cheetahs (Brown et al., 1996), domestic cats (Graham et al., 2000; Concannon, 1991; Lawler et al., 1993; Gudermuth et al., 1997), lions (Schmidt et al., 1979; Schramm et al., 1994), clouded leopards (Brown et al., 1995), leopards (Schmidt et al., 1988), pallas' cats (Brown et al., 2002), fishing cats (Moreland et al., 2002) and margays (Moreira et al., 2001). In this study, concentrations of fecal progestin did not increase following the estrogen surges in all females except in the case of the successful copulation (female C). Therefore, our results support the hypothesis that cheetahs are induced ovulators.

Many studies have been published concerning the estrous cycle in the cheetah. In these studies, the lengths of the cycles varied considerably from about 10 days to 26 days (e.g., Brown et al., 1996; Czekala et al., 1994). In a report on vaginal cytology, anuclear superficial cells appeared in a high proportion in vaginal smears at an interval of approximately 10–12 days (Asa et al., 1992). In another study on ethology, reproductive behaviors were observed frequently at the same short intervals (ca. 2 weeks) (Eaton and Craig, 1970). Furthermore, Brown et al. (1996) and Borque et al. (2005) reported that fecal estrogen varied regularly at an interval of approximately 13 days. However, using the same hormone analysis, a longer cycle (about 26 days) was also reported by Czekala et al. (1994).

In the current study, the estrogen peaks showed regularly at intervals of about 8–15 days in all five females. We should note that the frequencies of fecal sampling were relatively high (i.e., almost every day), and fortunately the interruptions of estrus shown by Brown et al. (1996) were never observed in all five females. Our results were also very similar to many other studies (see e.g., Eaton and Craig, 1970; Asa et al., 1992; Brown et al., 1996; Borque et al., 2005).

Wielebnowski et al. (2002) reported that the estrous cyclicities of females were sometimes inhibited by each other when female cheetahs were kept in pairs. However, they also reported one exception in a bonded female pair who exhibited only affiliative behaviors. In this case, the suppression of follicular estrogenic activity was not seen in either female (Wielebnowski et al., 2002). In our study, females D and E had been always housed together, but no aggressive behavior had been observed between them. Therefore, the inhibition of estrus is not expected from the relationship between females D and E.

We should also note the breeding trials were carried out with multiple paternities in this study. Gottelli et al. (2007) reported that, unlike the other felines, multiple

paternities were the usual mating system in wild cheetahs. However, in zoos, this mating system has usually been avoided, because the paternity of newborn has to be identified after parturition. Recently, we reported that the cat microsatellite could be used to determine parentage of cheetahs (Yoneda et al., 2010). Therefore, in this study, all females met with more than one male in mating test. In this way, all females were provided equal opportunity to copulate with males, mimicking natural systems.

Even though all females showed the similar regular estrogen cycle, the only female to copulate was C. Several factors can be possibly relevant to the copulation success of only female C: (1) age, (2) sampling season, (3) a matured female with past breeding experiences (pregnancy history), and (4) the long-term isolated housing. First, age is negative. Female cheetahs become sexually mature at 2.5 years of age (Bertschinger et al., 2008). In Japan, almost 40% females that make the first birth were under 4 years of age (Ito, 2007). Therefore, all 3-year-old females monitored in this study should have the sufficient reproductive capability. Second, as for sampling season, it should also have no impact on the result because female cheetahs are known to be not restricted to a breeding season. However, it was reported that the most of parturition occurs during March and May or during October and December (Thompson and Vestal, 1974). In Japan, offspring were often produced in May or during September and January (Ito, 2007). From this data, coupled with a known gestation of approximately 90 days, the expected months of frequent breeding were February or between June and November. In the current study, the meeting periods were scheduled within these highly reproductive seasons. Thus, sampling season is not likely to be relevant to the copulation of female C. Third, we have the negative result with pregnancy history. Prior to this study, female C was exposed to males several times after short term separation (approximately 12 weeks). At the time she had already been parous. However, she did not copulate successfully (data not shown). Therefore, long-term solitary confinement is the only factor still valid for the successful copulation in female C, though we have only one sample with no repetition. Housing effects should be tested urgently in near future.

Behavioral ecology of cheetahs in the wild also suggests that the long-term solitary existence may be responsible for successful copulation, because female cheetahs are well known as the solitary animals for much of the time. Meltzer (1999) observed that the success of breeding was triggered by intermittent contact between females and males. If males and females are housed permanently together, the libido of both females and males is decreased markedly by habituation (Meltzer, 1999). Moreover, Wielebnowski (1999) and Wielebnowski and Brown (1998) supported the conclusion that unpaired animals were more likely to breed successfully. In this context, Bertschinger et al. (2008) introduced the effective layout of enclosure for breeding that allow only intermittent contacts with males to each female. We suggest that the long-term solitary confinement that is mimicking the wild condition may further improve breeding success in female cheetahs. However, we should note that female copulation acceptance may not be controlled by her hormonal status in cheetahs. Such physiological or endocrinological inresponsiveness may become a hardship in captive breeding success in cheetahs.

5. Conclusions

This paper is the first report on daily fecal steroid changes in female cheetahs kept captive in Japan. Regardless of the housing conditions, hormone profiles were similar in all five females, and estrogen peaks were obtained at regular intervals of 8–15 days and progestin levels did not increase following the estrogen peaks and were always low except during pregnancy. Only a female isolated for a long period of time and introduced to males at around the fecal estrogen peak successfully mated and conceived. These results support the hypothesis that cheetahs are typically a reflex ovulator and it appears that exhibition of estrus at peak estrogen and successful copulation may be dependent on social/housing issues.

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