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Technical note

Fecal steroid metabolites and reproductive monitoring in a female Tsushima leopard cat (*Prionailurus bengalensis euptilurus*)

I. Adachi[a], S. Kusuda[b,*], E. Nagao[c], Y. Taira[b], M. Asano[b], T. Tsubota[d], O. Doi[b]

[a] *The Graduate School of Agricultural Science, Gifu University, Gifu 501-1193, Japan*
[b] *Faculty of Applied Biological Sciences, Gifu University, Gifu 501-1193, Japan*
[c] *Fukuoka City Zoological Garden, Fukuoka 810-0037, Japan*
[d] *Graduate School of Veterinary Medicine, Hokkaido University, Sapporo Hokkaido 060-0818, Japan*

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Abstract

Although the Tsushima leopard cat (*Prionailurus bengalensis euptilurus*) is one of the most endangered mammals in Japan, its reproductive physiology and endocrinology have been not elucidated. The objective was to establish the non-invasive monitoring of reproductive endocrinology in a female Tsushima leopard cat and to identify the types of fecal reproductive steroid metabolites in this species. Fecal concentrations of estrogen and progestin were determined by enzyme immunoassays, from 60 d before to 60 d after the last copulation, during three pregnancies. Fecal estrogen metabolite concentrations were increased before/around the mating period and after mid-pregnancy. Fecal progestin metabolite concentrations increased after the last copulation and remained high during pregnancy. The gestation period was 65.0 ± 0.6 d (mean ± SD). Fecal extracts were separated by high-performance liquid chromatography for identification of fecal metabolites. Fecal estrogens were identified as estradiol-17β and estrone. Fecal progestins during pregnancy contained 5α-reduced pregnanes: 5α-pregnan-3α-ol-20-one, 5α-pregnan-3β-ol-20-one and 5α-pregnan-3,20-dione, and nonmetabolized progesterone was barely detected in feces. In conclusion, measurement of fecal estrogen and progestin metabolites was effective for noninvasive reproductive monitoring in the Tsushima leopard cat. An immunoassay for fecal estradiol-17β concentrations seemed useful to monitor follicular activity, whereas an immunoassay with high cross reactivity for 5α-reduced pregnanes was useful to monitor ovarian luteal activity and pregnancy.

Keywords: Estrogen; Fecal hormone; HPLC; Progestin; Steroid metabolite; Tsushima leopard cat

1. Introduction

The Tsushima leopard cat (*Prionailurus bengalensis euptilurus*) is a wild feline inhabiting Tsushima island, Nagasaki, Japan. Based on mitochondrial DNA sequence analysis, it is very closely related to the leopard cat (*Felis bengalensis*) of Southeast Asia [1]. The Tsushima leopard cat is one of the most endangered mammals in Japan, and was designated as a Natural Monument of Japan in 1971, and as a National Endangered Species in the Law for the Conservation of Endangered Species of Wild Fauna and Flora in 1994. Furthermore, in the Red Data Book of Japan's Ministry of Environment (MOE), the Tsushima leopard cat is classified as a Critically Endangered species. A population census by the MOE from 2002 to 2004 estimated 80–110 individuals, with their distribution concentrated in the northern area of Tsushima island. The causes of the population decline are fewer fallow fields, deforesta-

* Corresponding author. Tel.:/fax: +81 58 293 2862.
E-mail address: kusuda@gifu-u.ac.jp (S. Kusuda).

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tion, road kills, trapping as harmful animals attacking domestic fowl, and killing by feral dogs [2]. Furthermore, feline immunodeficiency virus (FIV) has been identified in some Tsushima leopard cats since 1996; the source of infection was domestic cats [3,4].

The MOE Tsushima Wildlife Conservation Center (TWCC) was established on Tsushima island in 1997. The roles of this institution are *in-situ* conservation of wild animals and plants on Tsushima island. Since 1996, the MOE has been collaborating with the Fukuoka City Zoological Garden (FZG), Fukuoka, Japan in conducting captive breeding for this species, as a part of the Programs for Rehabilitation of Natural Habitats and Maintenance of Viable Populations. The first successful breeding in captivity was in 2000 [5,6]. Currently, Tsushima leopard cats are also kept at several Japanese zoos to reduce the risk of extinction, promote reproduction, maintain the captive population, collect/analyze biological information, and disseminate information nationwide on captive populations.

The reproductive endocrinology of the Tsushima leopard cat has not yet been reported; however, this information is essential to improve captive breeding management. Noninvasive fecal steroid metabolite analyses to monitor reproductive endocrinology have been widely used in zoo and wildlife species [7,8], and are effective for monitoring reproduction in felids [9–12]. In domestic cats, >95% of estrogen [13] and progestin [14] were excreted in the feces; however, the excretion of these metabolites varies considerably among species, even those which are closely related [7].

The objectives of the present study were to establish non-invasive monitoring of reproductive endocrinology in a female Tsushima leopard cat and to identify the types of fecal reproductive steroid metabolites in this species.

2. Materials and methods

2.1. Animals and sample collection

Fecal samples were collected from a single female Tsushima leopard cat kept at FZG. Fresh feces (except for those excreted in water) were collected 1 to 9 times a month (between 10:00 and 12:00) and analyzed from 60 d before to 60 d after the last copulation (January 24, 2004, February 26, 2005, and January 26, 2006, respectively) in three pregnancies (from 2004 to 2006). Feces were stored at −20 °C immediately after collection. This female was kept individually in an indoor pen and an outdoor paddock, separated from other Tsushima leopard cats, and was housed together with a male Tsushima leopard cat for several days (up to ~1 wk), from December to February. The female was fed mice, various kinds of meat, and fish. Drinking water was available *ad libitum*.

2.2. Fecal steroid hormone analysis

Fecal steroids were extracted using methanol, as previously reported [8]. Briefly, frozen feces were lyophilized for approximately 36 h, and 0.1 g of fecal powder was extracted with 5 mL of 80% methanol by vortex-mixing for 30 min. After centrifugation (1444 × *g* for 10 min), the supernatant methanol fraction was removed and diluted (ratios of 1:20 for the estradiol-17β assay and 1:100 for the progesterone assay) using assay buffer (phosphate buffer containing 0.1% bovine serum albumin). These dilutions were decided to be within a range of concentration of the standard curves for each hormone.

Fecal concentrations of estrogen and progestin were determined by enzyme immunoassays (EIAs), using estradiol-17β antiserum (1:3,000,000, FKA 236-E; Cosmo Bio, Tokyo, Japan) and progesterone antiserum (1:1,000,000, LC-28; Aska Pharma Medical, Kanagawa, Japan), as previously reported [8]. The main cross reactivity of estradiol-17β antiserum was estradiol-17β (100%), estrone (0.8%), estriol (0.5%), estrone-3-sulfate (26.8%), estradiol-3-glucuronide (56.3%), estrone-3-sulfate (26.8%), and estrone-3-glucuronide (1.2%). The main cross reactivity of the progesterone assay was progesterone (100%), 5α-pregnan-3,20-dione (62.2%), pregnenolone (6.3%), 11-deoxycorticosterone (3.9%), 17α-hydoroxyprogesterone (2.3%), and 11α-hydoroxyprogesterone (1.2%).

2.3. High-performance liquid chromatography (HPLC)

Steroid metabolites were separated by a modified version of HPLC, as described [15]. Estrogen and progestin metabolites were separated on a reverse-phase Nova-Pak C-18 column (3.9 × 30.0 mm; Waters, Milford MA, USA), using an isocratic solvent system of acetonitrile (ACN)/water; 40/60, v/v) at a flow rate of 0.3 mL/min for estrogen metabolites, and 1.0 mL/min for progestin metabolites. Prior to HPLC, fecal samples were lyophilized and pulverized, and, 2.0 g fecal powder was extracted with 10 mL of 80% methanol. The supernatant was added to 35 mL assay buffer, and the total volume was passed through a Sep-Pak C-18 column (Sep-Pak plus C-18 Environmental Cartridges, Waters, Milford, MA, USA). Steroid hormone metabolites were eluted with 5 mL absolute methanol. An