Validation of Fecal Progesterone Analysis for Predicting Pregnancy in Siberian Flying Squirrels（Pteromys volans）

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INTRODUCTION

The Siberian flying squirrel (Pteromys volans), which is a mammal belonging to the Family Sciuridae, has evolved gliding locomotion. Its distribution is from western Finland to Hokkaido in Japan. These flying squirrels most spend of their lives on trees, resting in tree cavities [1], foraging for tree seeds, buds, catkins, and leaves [2, 3], and moving between trees by gliding [4, 5]. They therefore depend to an extreme degree on forests, but forest fragmentation has caused a decrease in forest area. As a result, populations of Siberian flying squirrels have decreased in Finland, Estonia, and South Korea [6–8]. Because the forest area in the city of Obihiro, in Hokkaido, has been decreased to 4% for 100 years [9], the population of Siberian flying squirrels in Obihiro has likely decreased in the same way as in foreign countries.

Previous studies have shown that animals living in...
fragmented forests are stressed by human activity [10, 11] and that the stress suppresses ovulation and the estrus cycle and reduces litter size and growth rates [12, 13]. Furthermore, reproductive physiology is influenced by body condition [14–16]: resources such as food are reduced by clear-cutting [17, 18], and consequently reproductive success is likely to decline because of poor body condition in animals living in fragmented forests. Consequently, forest fragmentation negatively affects reproductive success and population dynamics [19]. To conserve this squirrel’s population in fragmented landscapes, knowledge of not only its ecology but also its physiology is required [20]. The following is known about the reproductive pattern of Siberian flying squirrels. In Hokkaido, females enter estrus from the end of February to July and have a 10-day estrus cycle [21]. After copulation of the female with a few males, the gestation period lasts 40 days [22]. Females produce an average of three neonates per litter [21, 22], and the pups are weaned at 60 days old [3]. After the first reproduction up to 30% of females produce second litters [3]. However, we have little information on the animal’s basic reproductive physiology, including its estrus cycle and pregnancy. Progesterone concentrations are generally measured to study reproductive physiology, and for this purpose plasma samples are frequently used in mammals [23, 24]. However, repeated blood sampling of Siberian flying squirrels is considered unsuitable because of its impracticality. In contrast, recently, a fecal progesterone analysis, which is non-invasive and uses enzyme immunoassay (EIA), was developed and applied to a variety of wild and zoo animals [25]. Therefore, fecal progesterone analysis could be applicable to Siberian flying squirrels. We conducted two tests to determine whether fecal progesterone analysis with this commercial EIA kit could be used to evaluate reproductive status in Siberian flying squirrels. The first experiment was a serial dilution test to evaluate whether the EIA kit would react properly. The second was a comparison of progesterone concentrations among four groups—pregnant females, adult females in non-breeding season, juvenile females, and adult males—to determine whether fecal progesterone measurement was able to evaluate reproductive status.

MATERIALS AND METHODS

Animals and sample collection

Thirty-three flying squirrels were captured from April 2013 to September 2014 in nest boxes set in forests in the city of Obihiro (42°46′–42°53′ N, 143°4′–143°11′ E). Eighteen fecal samples were collected from six pregnant females, 11 from 10 adult females in non-breeding season, 10 from eight juvenile females, and 10 from nine adult males. Females captured in the breeding season were kept over the gestation period in cages 46.5 × 46.5 × 56.5 cm or 41 × 37 × 73 cm in the laboratory; females that gave birth within the period of captivity were classified as “pregnant female”. Namely, feces collected from females that did not give birth within this period of captivity were not used in our study. The body mass was measured twice a week to check body condition. The primary diet consisted of sunflower seeds and apples. Foods foraged by flying squirrels in the field [2] were also provided, e.g., the Japanese white birch Betula platyphylla var. japonica, the Japanese elm Ulmus davidiana var. japonica, willow Salix spp., and maple Acer ginnala var. aidzuense. Water was provided ad libitum. Adults were classified as having a body mass of >80 g [26] or by the development of nipples (females) or testes (males). Adult females in non-breeding season were classified as those captured in the non-breeding season, i.e. from August to February [21]. Juveniles were classified by the following: body mass <80 g, or individuals found with breeding adult females in nest boxes or born in the laboratory. The body mass of juveniles born in the laboratory was measured twice a week for check of body condition.

Fecal samples from pregnant females were collected at the time when the body mass was checked or when feces were found in the cage tray within 3 hr after the cages had been checked. Fecal samples from adult females in non-breeding season and adult males were collected at the time of capture. Fecal samples from juvenile females were collected at the time of capture and during body mass checks. Each fecal sample was placed in a 2-ml micro-tube and immediately stored at −30°C until analysis.

This study followed the guidelines of the Mammal Society of Japan published in 2009 and was approved by the Hokkaido Government Tokachi General Subprefectural Bureau.

EIA validation and reproductive status profiling

To validate the enzyme immunoassay, parallelism between serially diluted fecal progesterone and a standard curve was determined by using methods described in a previous study [20, 27]. Fecal progesterone of Siberian flying squirrels was serially diluted 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, 1:2048, and 1:4096, and feces collected from pregnant females, which
were considered to have high progesterone concentrations, were used for the parallelism test.

Fecal progesterone concentrations were compared among pregnant females, adult females in the non-breeding season, juvenile females, and adult males to determine whether the concentrations were appropriate indicators of reproductive status in flying squirrels.

**Fecal progesterone extraction and enzyme immunoassay**

Feces were dried in a drying oven at 60°C for 2 h and then pulverized. Each sample was weighed out to 0.02 ± 0.0009 g, placed in a 2-ml micro-tube into which 1 ml of 100% methanol had been added to, vortexed for 15 min, and centrifuged at 2500 × g for 15 min. Next, the supernatant was poured into a 2-ml glass vial and stored at −30°C until EIA.

Fecal progesterone concentrations were determined with a Progesterone EIA Kit (item no. 582601, Cayman Chemical, Michigan, USA). Supernatant extracted from each fecal sample was diluted in EIA buffer (1 M phosphate solution containing 1% BSA, 4 M sodium chloride, 10 mM EDTA, and 0.1% sodium azide) at a concentration of 1:200. The cross-reactivities of the antiserum were 100% for progesterone, 7.2% for 17β-estradiol, 6.7% for 5β-pregnane-3α-ol-20-one, 2.5% for pregnenolone, 0.5% for 17-hydroxyprogesterone, <0.05% for testosterone, <0.01% for 5α-pregnane-3α,20α-diol, <0.01% for 5β-pregnane-3α,20α-diol glucuronide, <0.01% for 17α-estradiol, <0.01% for estriol, and <0.01% for estrone described in the kit manual.

Before the fecal progesterone analysis the recovery rate was examined in the following way. The extraction procedure was performed five times on each fecal sample collected from a pregnant female, and five supernatants were obtained. The fecal progesterone concentrations of the five supernatants were then measured. The sum of the concentrations in the five supernatants was considered to be the total progesterone concentration that could be extracted from the fecal sample; progesterone in each of the supernatant of the fifth extraction was not detected or less than 1% of the total progesterone concentrations. The percentage of progesterone concentrations of the first supernatant to the sum of the five concentrations was considered to be the recovery rate. The recovery rate was 77 ± 7% (mean ± SE; n = 4). The sensitivity of the assay was 10 pg/ml. The intra-assay and inter-assay coefficients of variation were 5.1% (n = 8) and 12.7% (n = 6), respectively.

**Statistical analysis**

To determine parallelism between serially diluted fecal progesterone and the standard curve we compared the slopes by using an analysis of covariance (ANCOVA) test. Furthermore, we compared the 95% confidence interval of progesterone concentrations in pregnant females with those in animals of different reproductive status by using general linear mixed-effect models with data of progesterone concentrations as the objective variable, with categorical data of reproductive status as the explanatory variable, and with categorical data of individual ID as a random effect implemented in the R package “lme4” because of pseudo replication. All statistical analyses followed the software R version 3.0.1 (R Development Core Team 2013, URL: http://www.r-project.org/).

**RESULTS**

Comparison of the slope of the regression lines between sample progesterone concentrations and the standard curve revealed no significant difference (df = 1, SS = 73, MS = 73, F = 1.48, P = 0.245; Fig. 1). This result showed that the two regression lines were parallel.

Mean progesterone concentrations were 9.494 ± 2.184 µg/g (mean ± SE; 18 fecal samples from 6 females) in pregnant females, 0.404 ± 2.181 µg/g in adult females in the non-breeding season (11 fecal samples from 10 females), 0.600 ± 2.241 µg/g in juvenile females (10 fecal samples from 8 females), and 0.394 ± 2.300 µg/g in adult males (10 fecal samples from 9 males). The 95% confidence interval of progesterone concentrations (mean ± 2 SE) in pregnant females did not overlap with those in the other groups (Fig. 2). Thus, fecal progesterone concentrations were significantly higher in pregnant females than in adult females in the non-breeding season, juvenile females and adult males.

**DISCUSSION**

Assays of antibody show parallelism only if the sample corresponds exactly with those in the assay [28]. Here, the parallelism showed that our measurements of progesterone concentrations in the fecal samples of Siberian flying squirrels were exactly even though we used only tiny amounts of feces. Previous studies have shown that stress affects progesterone secretion [29], and our handling of the animals during body condition checking may have been a stressor. However, the lag time of 1 or 2 days between a stressful event and fecal steroid
Fig. 1  Serial dilution results from fecal progesterone (A) and a standard curve (B). Fecal progesterone was serially diluted 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, 1:2048, and 1:4096. Standards were serially diluted from 7.8 to 1000 pg/ml. The slopes of the two regression lines did not differ significantly ($P = 0.245$).

Fig. 2  Ninety-five percent confidential intervals of fecal progesterone concentrations among pregnant females (18 fecal samples from 6 females), adult females in non-breeding season (11 fecal samples from 10 females), juvenile females (10 fecal samples from 8 females), and adult males (10 fecal samples from 9 males), shown as means ± 2 SE. Crosses represent means; 95% confidential intervals are not shown as less than zero, because the fecal progesterone was necessarily more than zero.
hormone was observed [30]; therefore, the stress of handling would not have affected fecal progesterone analysis.

This is the first report about enzyme immunoassay for fecal progesterone analysis in Siberian flying squirrels. Fecal progesterone concentrations were significantly higher in pregnant females than in adult females in the non-breeding season, juvenile females, and adult males. In general, fecal progesterone concentrations are significantly higher during pregnancy or luteal phase than during non-pregnancy or non-luteal phase [20, 27, 31, 32], because progesterone is secreted by the corpus luteum or the placenta, or both, for maintenance of pregnancy [33]. As in previous studies, in our study fecal progesterone analysis could be used to predict pregnancy. Therefore, our findings suggest that fecal progesterone analysis, which is non-invasive, could be useful for predicting pregnancy. Progesterone concentrations during the luteal phase are highly similar to those in the gestation period [20]. Therefore, fecal progesterone analysis could also be used to detect the luteal phase, and fecal progesterone analysis has great potential in research into the reproductive physiology of Siberian flying squirrels. However, we need to investigate the difference of fecal progesterone concentrations between pregnant females and non-pregnant females during luteal phase for more precise prediction of pregnancy.

Steroid hormone titers in feces increase with storage time because of the action of fecal bacteria [34, 35]. We may need to investigate how storage time influences fecal progesterone analysis in flying squirrels to apply in the field without the need for capture. Nevertheless, in our study we successfully used feces that had been defecated within 3 h, and could detect pregnancy in the Siberian flying squirrels. Suzuki et al. [36] reported that feces of this species can be collected by an umbrella set at the base of a cavity tree. Because Siberian flying squirrels leave their nest cavities 20 to 40 min after sunset [37], progesterone analysis of feces collected by an umbrella within 3 h of sunset should be accurate in detecting pregnancy.

Little is known about how forest fragmentation influences reproduction in the flying squirrels. To demonstrate its effects on reproduction we need to know more about the animal’s basic reproductive physiology. Previous studies have investigated the reproductive physiology of ground and arboreal squirrels [38-42]. The most striking feature of reproductive physiology is the increase in progesterone concentrations during lactation. In ground squirrels, such as the Cape ground squirrel Xerus inauris, the California ground squirrel Spermophilus beecheyi, the European ground squirrel Spermophilus citellus and the woodchuck Marmota monax, high progesterone concentrations have been detected during lactation, although these species produce only one litter per year [38-41]. The estrus cycle is reinitiated during lactation period, because examination has revealed the presence of neither corpus luteum nor their remains during early lactation [23]. In arboreal squirrels, Tait et al. [42] investigated progesterone concentrations of the gray squirrel Sciurus carolinensis during pregnancy and lactation. They found that, unlike in ground squirrels, progesterone concentrations after parturition were low. However, their investigation lasted for only 2 weeks after parturition. Therefore, the analytical period was not enough to reveal whether or not the estrus cycle had been reinitiated. In contrast, the American red squirrel Tamiasciurus hudsonicus enters estrus during lactation, because mating occurs during lactation [43]. An increase in progesterone levels should therefore be detectable. The females of some arboreal squirrels produce a second litter after a successful first reproduction [43]. Although the reproductive trial per year differs between ground and arboreal squirrels, in both types of squirrel the estrus cycle is reinitiated after parturition. Therefore, estrus cycling during lactation seems common in the Family Sciuridae. Like other squirrels, flying squirrels may enter estrus and mate during lactation.

We hope that fecal progesterone analysis will be used to study the reproductive physiology of Siberian flying squirrels. Because this analysis is non-invasive, it should be possible to study the reproductive physiology of Siberian flying squirrels in fragmented forests without subjecting the animals to additional stress. To conserve populations of Siberian flying squirrels in fragmented forests, it is important to their monitor population dynamics. Demographic parameters such as pregnancy rate could be estimated by measuring progesterone [44]. Therefore, fecal progesterone analysis could help to evaluate population dynamics in the flying squirrels, although data on other parameters such as age at maturation and mortality rate are also needed.

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原著 繁殖学

タイリクモモンガの妊娠状態の推定における糞中プロジェステロン測定の有効性

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要 約

タイリクモモンガ Pteromys volans は森林分断化の影響によって、個体数の減少や地域個体群の絶滅が危惧されている。森林分断化は動物に対してストレスやボディコンディションの低下を引き起こし、繁殖生理にも大きく影響するため、分断林に生息するタイリクモモンガにおける保全対策においては生態学的な視点のみではなく生理学的な評価も重要である。そこで、本研究では市販の酵素免疫測定法（EIA）キットを用いて、タイリクモモンガにおいて糞中プロジェステロン（P4）測定が妊娠状態の評価に有効かを検証した。まず、EIA の実験が正常に反応しているのかを評価するために、糞中 P4 を 2 倍段階希釈したものと標準曲線の平行性を検定した。その結果、階段希釈した糞中 P4 と標準曲線の 2 つの回帰直線の傾きに有意な差はなかったため、平行性が示された。つまり、本種の糞中 P4 測定は正確に行われたことがわかった。また、繁殖状態の推定が可能かを明らかにするために、P4 濃度が高いため妊娠期のメスと P4 濃度が低いため非繁殖期のメス、幼獣および成獣オスの間で P4 濃度を一般化線形混合モデルにより比較した。測定結果では、妊娠期のメスの P4 濃度は非繁殖期のメスや幼獣メス、成獣オスよりも有意に高かった。したがって、微量な糞からでも繁殖状態の変化に伴う P4 の変動を検出でき、市販の EIA キットを用いた糞中 P4 測定によりタイリクモモンガの妊娠を推定し、生理学的な研究に応用することができるだろう。

キーワード：タイリクモモンガ、妊娠、繁殖状態、糞中プロジェステロン

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