Animal biology from feces: ecological and endocrinological research in Siberian flying squirrels *Pteromys volans*

2016. 3

Course of Biotic Environment Science,

The United Graduate School of Agricultural Science

Iwate University, Japan

(Obihiro University of Agriculture and Veterinary Medicine)

Tatsuki Shimamoto

Animal biology from feces: ecological and endocrinological research in Siberian flying squirrels *Pteromys volans*

Tatsuki Shimamoto

General Introduction

Animal research methods can be broadly divided into two styles, namely direct observation and indirect observation. Direct observation is a fundamental approach that have been used for many years (Latta et al. 1967; Koprowski 1993; Uchida et al. in press). However, with some exceptions, it is often difficult to directly observe mammals in the field. Indirect observation is an alternative that can be used for difficult-to-observe animals; with this method for focus is frequently on the animal's sign (Sponheimer et al. 2003; Kendall et al. 2009; Mastromonaco et al. 2014).

Feces are among the most common signs used in research. Putman (1984) reviewed the potential for research using feces to reveal animal ecology. Feces are used as indicators of animals' presence, nest sites, and population density (Reunanen et al. 2002; Hamasaki et al. 2001; Suzuki et al. 2011). Feces have been used for a long time to investigate the diets of many animals (Kunz et al. 1983; Dickman and Huang 1988; Hewitt and Robbins 1996; Orr and Harvey 2001; Deagle et al. 2007). Furthermore, thanks to the development of molecular scatology, we can investigate behavioral biology, census population size, home and territory range, and genetic variation by purifying DNA from feces (see review by Kohn and Wayne 1997). Not only ecological research, but also endocrinological research, now often used feces. Fecal steroid hormone analysis (oestrogen, androgen, progestogen, and glucocorticoid metabolites) has been developed over the past two decades (Schwarzenberger 2007), and the validation of fecal steroid hormone analysis to profile endocrine patterns and to use reliable indicator of stress has been demonstrated in mammals, birds, reptiles, amphibian, and fish species (Graham et al. 2002; Millspaugh and Washburn 2004; Pettitt et al. 2007; Sheriff et al. 2009); therefore this analysis used in a range of zoo and wild animals (see review by Schwarzenberger 2007). Although feces can be obtained non-invasively, they contain diverse information. They can therefore be used as a tool for studying difficult-to-observe animals, and the results obtained can help to elucidate various aspects of animal biology, such as life history, genetic structure, and endocrine patterns.

The Siberian flying squirrel, *Pteromys volans*, a member of the Family Sciuridae, is difficult to observe, because it is nocturnal and small mammal (Yamaguchi and Yanagawa 1995; Yanagawa 1999; Fig. 1). However, its feces are distinctive and can be easily identified (Fig. 2). According to Kadosaki (2001), they are riziform in shape and greenish yellow or brownish yellow when fresh. With time, the color changes to burnt umber or copper. The scats are 4 to 15 mm long and 2 to 5 mm in diameter. Nest trees can be detected by collecting feces in an umbrella placed at the base of the tree (Suzuki et al. 2011). Feces are also used to identify occupied forests (Reunanen et al. 2002; Hurme et al. 2007). However, despite the diversity of information offered by fecal analysis there has been little research on feces in Siberian flying squirrels. Research focusing on fecal characteristics could help to elucidate the ecology and endocrinology of this species.

Here, the aim of study was to demonstrate the validation of fecal sample in Siberian flying squirrels on the field and the laboratory research. With a focus on the benefits of using feces, author studied the ecology and endocrinology of Siberian flying squirrels. First, author established the confirmation method for presence of Siberian flying squirrels by using feces (Section 1). Then, author validated the use of fecal progesterone analysis for predicting pregnancy (Section 2). Finally, author investigated progesterone concentrations during lactation and the progesterone dynamics of lactating females to estimate the presence of postpartum estrus in Siberian flying squirrels (Section 3).

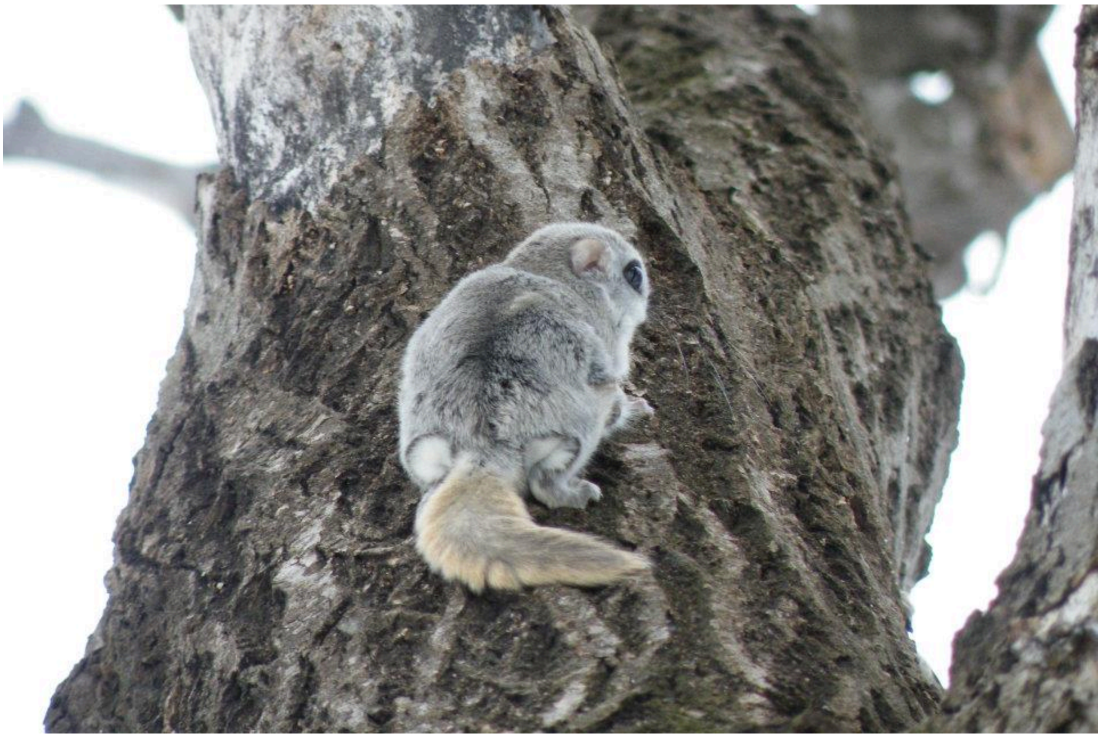

Fig. 1. The Siberian flying squirrel *Pteromys volans*.

Fig. 2. Feces of Siberian flying squirrels.

Study area

Study areas were the fragmented forests located in Obihiro city, Hokkaido, Japan (42°46'–42 °53'N, 143°4'–143°11'E). The forests that author surveyed were comprised of conifers, including the Korean white pine *Pinus koraiensis*, the Eastern white pine *Pinus strobus*, the Japanese larch *Larix leptolepis*, and broad-leaved trees, including the Japanese elm *Ulmus davidiana* var. *japonica*, the Manchurian ash *Fraxinus mandshurica*, the white birch *Betula platyphylla*, the Japanese walnut *Juglans mandshurica*, and the Daimyo oak *Quercus dentata*.

1. A confirmation method for the presence of the Siberian flying squirrel via feces

Introduction

The population of Siberian flying squirrels in worldwide is declining because of forest fragmentation (Hokkanen et al. 1982; Timm and Kiristaja 2002; Jackson 2012). To conserve this species, long-term monitoring is necessary to evaluate the effect of forest fragmentation on the population (Debinski and Holt 2000; Koskimäki et al. 2013). For long-term monitoring author needs information on the species' presence. To date, the method used to confirm the presence of squirrels has been to search whole forest for feces (Reunanen et al. 2002; Hurme et al. 2007). However, this method is labor intensive and is inefficient if the forest size huge. Moreover, the details of using this method—such as how to search feces and enough research effort—are not clear. Author, therefore, needs to establish an efficient and quantitative method to confirm presence of Siberian flying squirrels.

Direct observation is insufficient, because the squirrels are small and nocturnal (Yamaguchi and Yanagawa 1995; Yanagawa 1999). However, it is easy to identify their feces (Kadosaki 2001), and Suzuki et al. (2011) reported that by using an umbrella to trap and collect feces they were able to find nest trees. Author, therefore, considered that feces could be similarly used to confirm the animals' presence. However, because it is inefficient to search whole forest for feces, to simplify the research it would be necessary to clarify the characteristics of places in which feces were often found. In addition, author needed a method that was readily available to anyone and in which the research effort required to determine the animals' presence was easily quantifiable. The goal of this research was therefore to elucidate the characteristics of points that should be searched for feces and the research effort required to confirm the presence of Siberian flying squirrels.

Materials and Methods

Study area

Author searched for the squirrels' feces in 11 fragmented forests in the city of Obihiro from April to May 2013. The size of the forest fragments ranged from 0.42 to 13.69 ha. Ten of the forests were deciduous broad-leaved forest, and one was a mixed forest.

Search for feces and characterization of places where feces were found

Author randomly set up 12 transects (10 m long × 4 m wide) in each forest and searched for feces within each transect. The time taken for the feces to disintegrate was unknown, but author targeted feces that could be identified even if they seemed old. To ensure uniformity of the research effort, search time was less than 15 min per transect.

First, to characterize the places where feces were found, author measured the distance between the feces and the closest tree, along with the diameter at breast height (DBH) of that tree. Author hypothesized that the feces were often found close to trees with large DBHs, because Siberian flying squirrels prefer old-growth forests containing high trees (Reunanen et al. 2002) and selectively land in high trees when gliding (Suzuki et al. 2012). To test this hypothesis, author compared the DBHs of the trees with and without feces. And, author did not examine thin trees (DBH less than 10 cm), because Northern flying squirrels *Glaucomys sabrinus* do not land on trees with a DBH of less than 10 cm (Vernes 2001). Author used general linear mixed-effect model to compare the DBHs of the trees with and without feces, with data on the presence of Siberian flying squirrels as the objective variable, categorical data on tree DBH as the explanatory variable, and categorical data on forest ID as a random effect implemented in the R package "lme4" because of pseudo replication. The significance of the analysis was tested by using a χ^2 test.

Research effort

Second, to reveal whether or not the research effort would change with forest size, author investigated the relationship between the number of transects on which author found feces and forest size. Author hypothesized that the research effort would increase with forest size. To test this hypothesis, author used a general linear model with data on the number of transects where feces were found (feces-positive transects) as the objective variable and categorical data on the forest size as the explanatory variable. The significance of this analysis was tested by using a χ^2 test. Furthermore, author used the formula below to determine research effort required to search for feces in each forest to confirmation the animal's presence. The discovery rate of feces (R) was calculated by using the number of feces-positive transects as a proportion of the 12 transects. The number of research transects needed to confirm the presence of the flying squirrels was then determined with a probability of more than 95%.

R = 1－{(12－n)/12}m

R: The discovery rate of feces

n: Number of feces-positive transects

m: Number of research transects

Results

Feces were found in all forests. The average number of feces-positive transects was 6.2±1.7 (mean ± SD, n = 11).

Feces were found 22.46 ± 4.25 cm (mean ± SE, n = 11) from the closest tree; more than 80% of feces were within 20 cm (Fig. 2). The DBH of trees with feces was 34.87 ± 1.29 cm (mean ± SD, n = 11); that of trees without feces was 26.96 ± 0.66 cm (n = 11). Seventy-five percent of the former had a DBH of more than 25 cm, and more than 80% of the latter had a DBH of less than 35 cm (Fig. 2). The DBH of trees with feces was significantly larger than that of trees without feces (estimate = 0.07453, SE = 0.01574, P = <0.001). Feces were therefore often found close to large DBH trees.

The number of feces-positive transects was unrelated to forest size (estimate = −0.02079, SE = 0.04906, P = 0.6720: Fig. 3). From the formula, five transects (95% CI, 4 to 6 transects) were required to confirm the presence of Siberian flying squirrels with a probability of more than 95%.

Discussion

It was efficient to search for feces within 20 cm of trees with a DBH of more than 25 cm. Feces were often found close to large DBH trees, because Siberian flying squirrels defecate arboreally (Kadosaki 2001), prefer old-growth forests (Reunanen et al. 2002), and selectively land in high trees (Suzuki et al. 2012).

In this study, the number of feces-positive transects was unrelated to forest size. This result indicated that forest size did not affect the discovery rate of feces, and the research effort did not need to change to suit the forest size. However, this finding is applicable only to forests no bigger than this study areas. If author examine in a forest bigger than 14 ha, then author need to re-examine the relationship between forest size and research effort. This result suggested that, to confirm the presence of squirrels with a probability of more than 95%, five transects needed to be set per forest. In summary, for maximum efficiency, five transects could be set close to trees with a DBH of more than 25 cm and a search conducted for the animal's feces within 20 cm of the trees.

The population density of Siberian flying squirrels would affect the discovery rate of feces: the discovery rate would increase with increasing density. In general, it is difficult to predict the population density of mammals, and in this study author was unable to consider the effect of population density on the discovery rate of feces. If the relationship between population density and fecal discovery rate was clear, then this method might be useful as an indicator of population density. In future, consideration of the effect of population density on fecal discovery rate will be needed to develop a more precise method of confirming squirrel presence and population density.

Forest structure might also influence the recovery rate of feces and change the manner to search feces. Fig. 2 showed that although the histograms of the DBHs differed between the trees with feces and without feces, DBHs with 24 to 30 cm dominated in our study area. But, if dominated DBHs are smaller or bigger, the characteristic of the place where feces are often found will change. Therefore, the manner that feces are searched close to trees with a DBH of more than 25 cm is just indication, and we need to reveal it again when we research at the different study area.

Season might affect the discovery rate of feces and the research effort. This study was performed in April and May, when vegetation in the study area was poor. If research were conducted in summer, when the vegetation is abundant, more careful research would be required. In winter the discovery rate of feces would be higher than in this study period, because it is easy to find the feces on snow cover.

Because almost all of this study area was deciduous broad-leaved forest, author was unable to consider the effect of forest type on discovery rate of feces. However, the vegetation in coniferous forests, which Siberian flying squirrels use as breeding sites and for foraging, is poorer than that in broad-leaved forest (Messier et al. 1998). Therefore, the confirmation method could be adapted for use in coniferous forests.

In addition, the other environmental factors of fragmented forests, such as the number of tree cavities, would affect our results. However, in present study we did not research the relationships between the factors and our results; therefore, established method can be used only in our study area. We will need to reveal this relationship and to confirm more generalized method in order to research other areas.

There are the evidence that the confirmation method can be simpler resulting from the present study. This study demonstrated that feces were often found close to trees, indicating that the number of trees included in one transect would affect the discovery rate of feces. That is, the number of trees investigated could determine whether or not the presence of Siberian flying squirrels were confirmed. To simplify the confirmation method, author needs to determine the optimum number of trees that needs to be investigated to determine the presence.

Figures

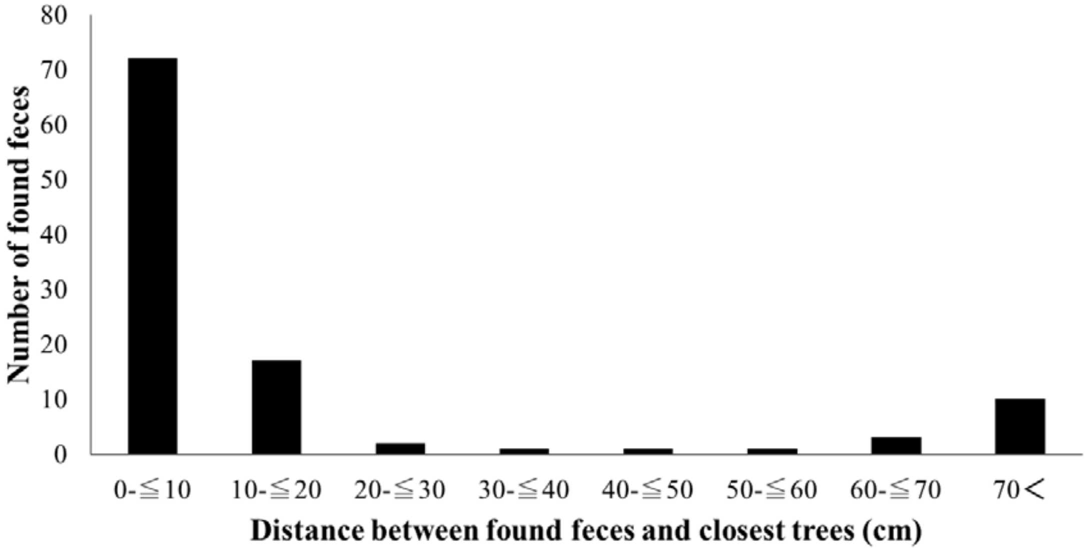

Fig. 1. Histogram of the distance between feces and closest trees

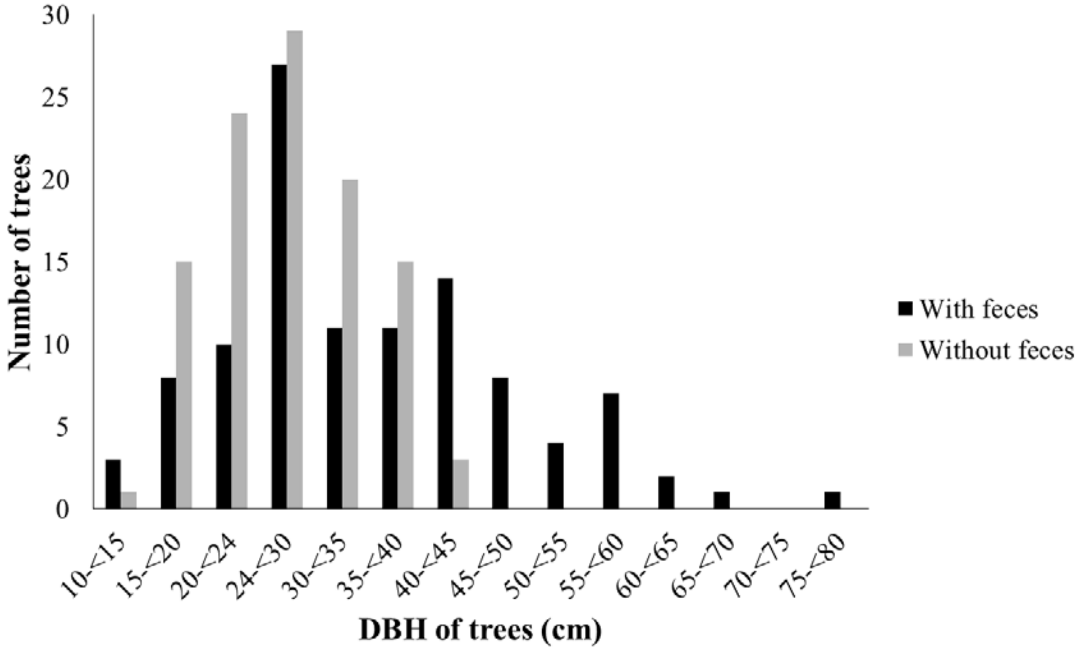

Fig. 2. Histogram of DBHs of trees with and without feces

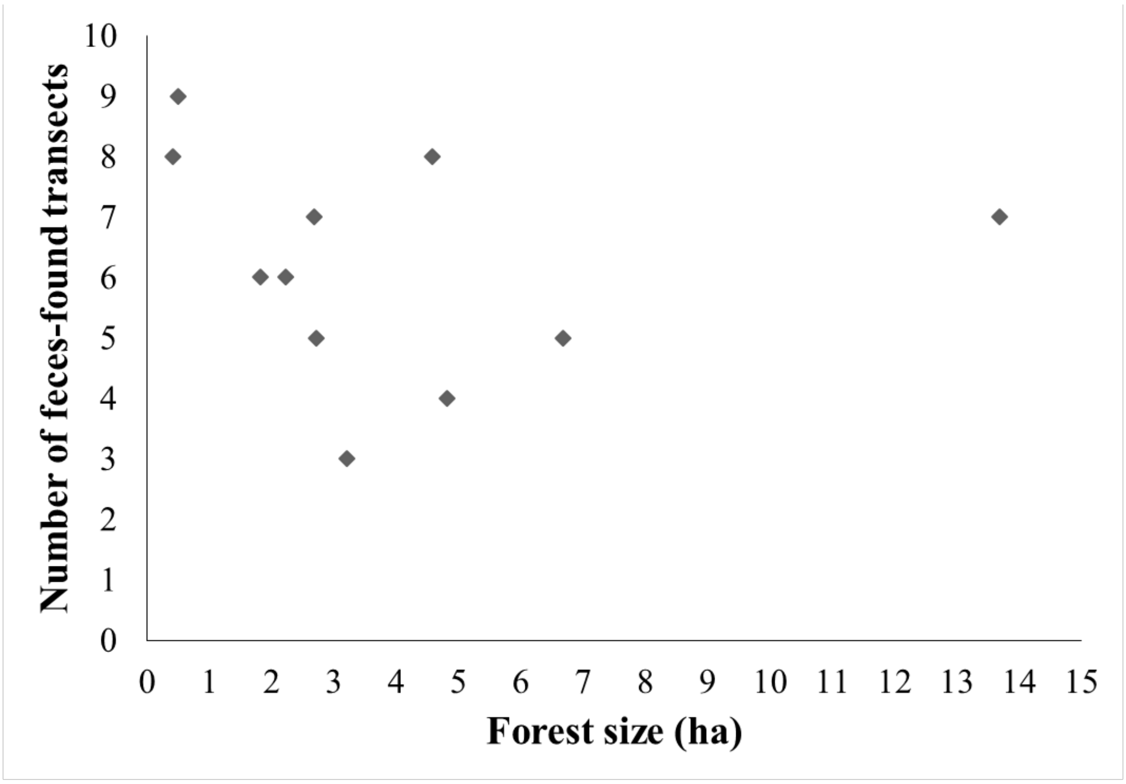

Fig. 3. Interaction between the number of feces-positive transects and forest size

2. Validation of fecal progesterone analysis for predicting pregnancy in Siberian flying squirrels *Pteromys volans*

Introduction

Steroid hormone concentrations are generally measured to study reproductive physiology, and for this purpose plasma samples are frequently used in birds, mammals, reptiles, and amphibians (Seal et al. 1979; Wood et al. 1986; Whittier et al. 1987; Orchinik et al. 1988; Tsubota et al. 1998: Van Duyse et al. 2002; Millesi et al. 2008). However, repeated blood sampling of small mammals is considered unsuitable because of its impracticality. In contrast, recently steroid hormone analysis using feces have been developed during past twenty years. This method is non-invasive and capable of repeatedly obtaining fecal samples; hence fecal steroid hormone analysis is applied to a variety of wild and zoo animals (see review by Schwarzenberger 2007).

Siberian flying squirrels are one of the Family Sciuridae, and the following is known about the reproductive pattern. In Hokkaido, females enter estrus from the end of February to July and have a 10-day estrous cycle (Yanagawa 1999). After copulation of the female with a few males, the gestation period lasts 40 days (Selonen et al. 2013). Females produce an average of three neonates per litter (Yanagawa 1999; Airapetyants and Fokin 2003; Selonen et al. 2013), and the pups are weaned at 60 days old (Hanski et al. 2000). After the first reproduction up to 30% of females produce second litters (Hanski et al. 2000). However, author have little information on the animal's basic reproductive physiology, including its estrous cycle and pregnancy. Repeated blood sampling is unsuitable for Siberian flying squirrels, because they are small mammals.

Considering the characteristic of Siberian flying squirrels, fecal progesterone analysis seemed to be applied to this species to reveal the reproductive physiology.

Here, author 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 this 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 (Asari et al. 2008) were also provided, e.g., the Japanese white birch *Betula platyphylla* var. *japonica*, the Japanese elm *Ulmus davidiana* var. *japonica*, withy Salix spp., and maple *Acer ginnala* var. *aidzuense*. Water was provided ad libitum. Adults were classified as having a body mass of >80 g (Yanagawa 2009) 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 (Yanagawa 1999). 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 (Graham et al. 2002; Pettitt et al. 2007). 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 2500g 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,

<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 author compared the slopes by using an ANCOVA test. Furthermore, author 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).

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 9494 ± 2184 ng/g (mean ± SE; 18 fecal samples from 6 females) in pregnant females, 404 ± 2181 in adult females in the non-breeding season (11 fecal samples from 10 females), 600 ± 2241 in juvenile females (10 fecal samples from 8 females), and 394 ± 2300 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 (Davies 2013). Here, the parallelism showed that the measurements of progesterone concentrations in the fecal samples of Siberian flying squirrels were exactly even though author used only tiny amounts of feces. Previous studies have shown that stress affects progesterone secretion (Asher et al. 1989), and the 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 hormone was observed (Goymann et al. 1999; Peel et al. 2005); 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 (Gudermuth et al. 1998; Hamasaki et al. 2001; Graham et al. 2002; Pettitt et al. 2007), because progesterone is secreted by the corpus luteum or the placenta, or both, for maintenance of pregnancy (Norris and Lopez 2011). As in previous studies, fecal progesterone analysis could be used to predict pregnancy in present study. Therefore, the 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 (Graham 2002; Mohammed et al. 2011). 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, author 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 (Möstl et al. 1999; Yamauchi et al. 1999; Khan et al. 2002; Muren et al. 2014). Author 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 this study author successfully used feces that had been defecated within 3 hr, and could detect pregnancy in the Siberian flying squirrels. Suzuki et al. (2011) 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 (Yamaguchi and Yanagawa 1995), progesterone analysis of feces collected by an umbrella within 3 hr of sunset should be accurate in detecting pregnancy.

Little is known about basic reproductive physiology of Siberian flying squirrels. Previous studies have investigated the reproductive physiology of ground and arboreal squirrels (Tait et al. 1981; Concannon et al. 1983; Holekamp et al. 1988; Pettitt et al. 2008; Strauss et al. 2009). 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 (Concannon et al. 1983; Holekamp et al. 1988; Strauss et al. 2009). The estrous cycle is reinitiated during lactation period, because examination has revealed the presence of neither corpus luteum nor their remains during early lactation (Millesi et al. 2008). In arboreal squirrels, Tait et al. (1981) 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 estrous cycle had been reinitiated. In contrast, the American red squirrel *Tamiasciurus* *hudsonicus* enters estrus during lactation, because mating occurs during lactation (Boutin et al. 2006). An increase in progesterone levels should therefore be detectable. The females of some arboreal squirrels produce a second litter after a successful first reproduction (Boutin et al. 2006). Although the reproductive trial per year differs between ground and arboreal squirrels, in both types of squirrel the estrous 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.

Author 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 (McKenzie et al. 2005). 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.

Figures

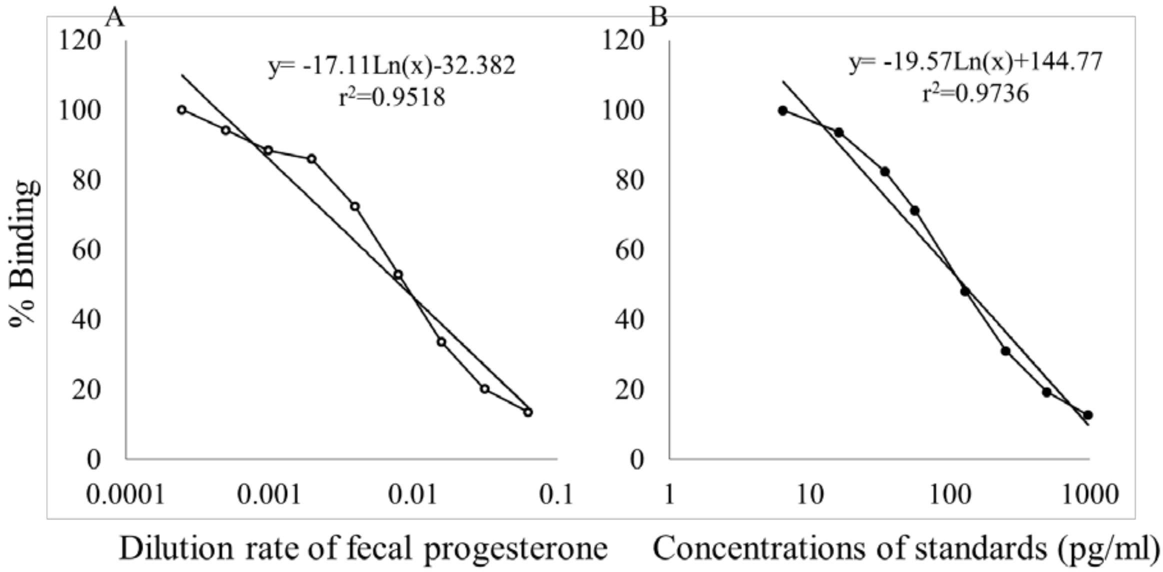

Fig. 1. Serial dilution results from fecal progesterone (A) and a standard curve (B). Fecal progesterone was serially diluted 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, 1:2048, and 1:4096. Standards were serially diluted from 7.8 to 1000 pg/ml. The slopes of the two regression lines did not differ significantly (P = 0.245).

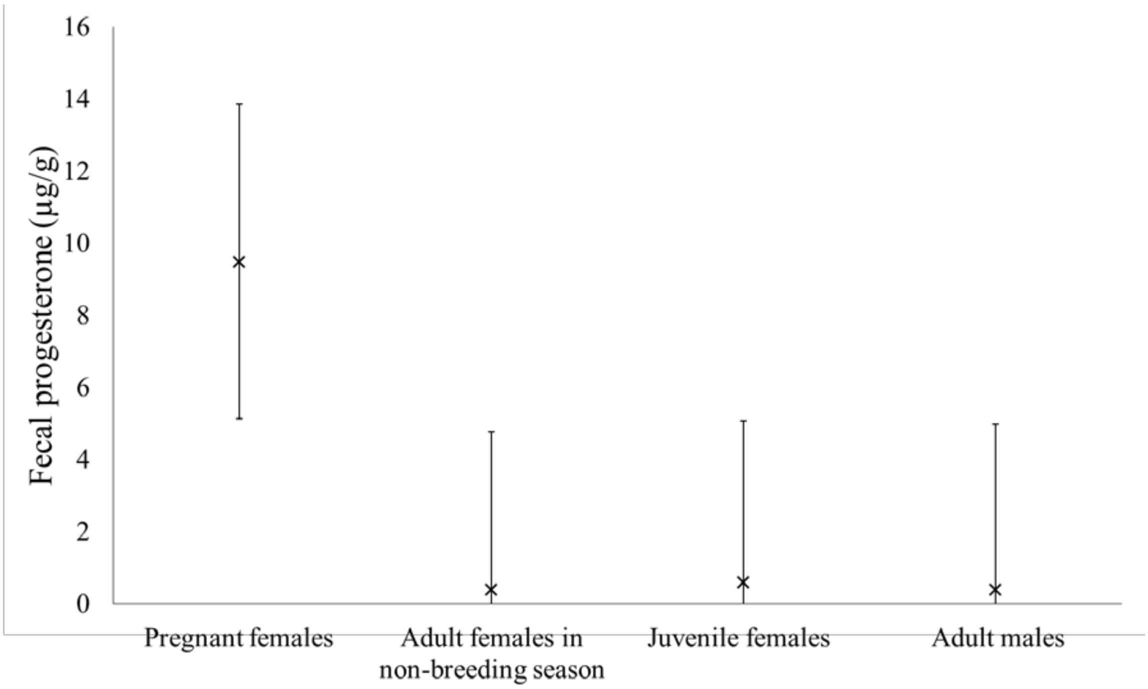

Fig. 2. Ninety-five percent confidential intervals of fecal progesterone concentrations among pregnant females (18 fecal samples from 6 females), adult females in non-breeding season (11 fecal samples from 10 females), juvenile females (10 fecal samples from 8 females), and adult males (10 fecal samples 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.

3. Fecal progesterone concentrations and dynamics during lactation in Siberian flying squirrels *Pteromys volans*

Introduction

The reproductive strategy is a key concept of species survival. The Siberian flying squirrel *Pteromys volans* is a seasonal breeder. Females enter estrus from the end of February to July in Hokkaido, Japan and have a 10-day estrous cycle (Yanagawa 1999). *Pteromys volans* has a mean gestation length of 40 days (Selonen et al. 2013) and a mean lactation length of 60 days (Hanski et al. 2000). Females produce up to two litters a year (Hanski et al. 2000); *P. volans* inhabiting locations with severe winter is imposed to deliver two litters in a limited breeding season.

In seasonally breeding mammals, reproductive behavior often underlies such time constraints, because cessation or reduction in lactation is needed to start the next reproduction (McNeilly 1997). Multiple breeders such as *P. volans* are particularly susceptible to the effects of time constraints on reproduction. To circumvent time constraints, many small mammals, including squirrels, have a postpartum estrus to produce a second litter or more (Gilbert 1984; Boutin et al. 2006; Franceschini et al. 2007; Kawamichi 2010; Smith et al. 2011). Postpartum estrus is considered a time-saving strategy, because concurrent pregnancy and lactation reduces the interval between litters (Gilbert 1984; Franceschini-Zink and Millesi 2008). Therefore, similarly to these small mammals, *P. volans* may have a postpartum estrus to maximize reproductive output. However, it is not known whether this occurs.

Postpartum ovulation is inhibited by many factors. Suckling stimulus suppresses lutenizing hormone (LH) secretion from pituitary gland (Brogan et al. 1999) and the cooperation action of suckling stimulus and prolactin has suppressive effect on LH secretion (Maeda et al. 1990). Also, metabolic drain of milk production is contributed to the suppression of leptin during lactation period, which leads to inhibit LH secretion and ovulation (Brogan et al. 1999). Consequently, these mechanism suppresses ovulation during lactation and progesterone concentrations are low during lactation (McNeilly et al. 1994; McNeilly 1997; Graham et al. 2002). However, in mammals with a postpartum estrus, follicular development can be detected after parturition, and postpartum ovulation occurs (Mossman and Duke 1973; Bravo et al. 1994; Dadarwal et al. 2004). Progesterone levels are is known to be high after postpartum estrus because of the formation of the corpus luteum (CL), which is the main source of progesterone (Franceschini et al. 2007; Krepschi et al. 2013). If *P. volans* has a postpartum estrus, ovulation should occur after parturition and high progesterone concentrations should be detected during lactation. However, the progesterone secretion patterns of this species during lactation are unknown.

Our goal was to elucidate whether or not *P. volans* has the physiological potential to mate during lactation. Therefore, we investigated progesterone secretion patterns during lactation. Progesterone concentrations were measured by fecal progesterone analysis, because this method is non-invasive and fecal sampling can easily be repeated; moreover, this method has been validated in *P. volans* (Shimamoto et al. 2015).

Materials and methods

Animals and sample collection

A total of six female *P. volans* were captured in April between 2013 and 2015 by nest boxes set in forests in the city of Obihiro (42°46′–42°53′N, 143°4′–143°11′E). The captured females were kept temporarily in cages 46.5 × 46.5 × 56.5 cm or 41 × 37 × 73 cm each individual per cage. The primary diet consisted of sunflower seeds and apples. Foods foraged by *P. volans* in the field (Asari et al. 2008) were also provided (e.g., Japanese white birch, *Betula platyphylla* var. *japonica*; Japanese elm *Ulmus davidiana* var. *japonica*; withy, *Salix* spp.; and maple, *Acer ginnala* var. *aidzuense*). Water was provided ad libitum. We had intended that if a female had not given birth within the period of captivity, which was at least 40 days (the length of gestation), we would release the female. But, all six females were used in present study because they were pregnant.

To assess progesterone dynamics during lactation, fecal samples were collected from the six females during the latter part of pregnancy and during lactation at the time when body condition was checked or when feces were found in the cage tray within 3 h after the cages had last been checked. During the latter part of pregnancy, the frequency of fecal collection depended on the individual squirrel. In most cases, feces were collected six or seven times a week; in the case of a few females, the frequency of collection was only once or twice a week. During lactation, feces were collected three or four times a week until or just before the offspring began to eat a solid diet. 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.

Fecal progesterone extraction

Fecal progesterone was extracted in accordance with the method described by Shimamoto et al. (2015). Briefly, 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 to which 1 ml of 100% methanol had been added, 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 enzyme immunoassay (EIA).

Enzyme immunoassay

Fecal progesterone concentrations were determined by using the EIA procedure described by Miyamoto et al. (1992). Standards and supernatants extracted from fecal samples were diluted in EIA buffer (0.04 M Na_2HPO_4, 0.145 M NaCl, 0.1% bovine serum albumin; pH 7.2). The standard curve for progesterone ranged from 48 to 50000 pg/ml. The recovery rate was 84% ± 2% (mean ± SE). The intra-assay and inter-assay coefficients of variation were 4.7% and 10.2%, respectively.

Before the analysis, we tested for parallelism between serially diluted fecal progesterone and a standard curve (Fig. 1) and compared progesterone concentrations among different reproductive status females (Fig. 2); the result of this test was similar to that of the previous study (Shimamoto et al. 2015).

Statistical analysis

The duration of the luteal phase during lactation was determined by using the process described by Graham et al. (2002). Briefly, the mean and SD were calculated, and values above the mean + 1.96 SD were removed from the data set. The mean and SD of the new data set were recalculated, and the elimination process was repeated until no value exceeded the mean + 1.96 SD. Removed values were significantly higher than those that were not removed and were considered to represent the luteal phase. For this analysis, only feces collected during lactation were used.

Results

The fecal progesterone profiles of the six females are shown in Figure 3. Progesterone concentration ranges were 171.9 to 2303.9 in female A (n = 39), 133.9 to 1011.7 in female B (n = 23), 277.3 to 959.8 in female C (n = 22), 7.2 to 5389.6 in female D (n = 33), 202.8 to 2417.7 in female E (n = 40), and 26.4 to 1575.6 ng/g in female F (n = 39). Progesterone concentrations stayed high during the latter part of pregnancy in all females, although sharp fluctuations were observed throughout this phase in most females; at parturition the levels dramatically declined. Although one of the six females showed low levels of progesterone concentrations throughout lactation, the other five showed periods of elevated progesterone concentrations considered as luteal phases. Spikes began to appear 2 to 18 days after parturition. In addition, the same five females showed cycling patterns of progesterone secretion. There were four luteal phases and 6, 6, and 10 days between luteal phases in female A; three phases and intervals of 13 and 10 days in female B; three phases and intervals of 10 and 9 days in female C; two phases and an interval of 15 days in female D; and two phases and an intervals of 18 days in female E. The average number of phases in these females was 2.6 ± 1.0 (mean ± SD), and the average interval was 10.8 ± 3.7 (mean ± SD) days.

Discussions

Fecal progesterone analysis revealed that periods of elevated progesterone concentrations in five of the six females were observed from early lactation periods. This provides evidence that the existence of CLs during these periods, although whether or not an estrous cycle was reinitiated after parturition is unknown. However, in *P. volans*, regression of CLs would have coincided with a sharp decline in the progesterone concentration at parturition, because the CLs regress at parturition in some squirrels—the European ground squirrel *Spermophilus citellus* and the gray squirrel *Sciurus carolinensis*—and other mammals (Tait et al. 1981; Boyd 1984; O'Shea and Wright 1985; Millesi et al. 2008). Therefore, our result suggests that ovarian activity was reinitiated after parturition, ovulation occurred, and the CLs that formed began secreting progesterone.

There are further findings to support the hypothesis of resumption of ovarian activity after parturition. Fecal progesterone analysis during lactation showed that luteal phases occurred at least twice and the interval between luteal phases was approximately 11 days. The estrous cycle of *P. volans* has been reported to last for 10 days (Yanagawa 1999), so the intervals observed were mostly consistent with the length of estrous cycle. The pattern of cycling of progesterone secretion would therefore have supported postpartum ovulation and resumption of ovarian activity.

However, some of the six females seemed to have abnormal length of estrous cycles, although the precise range of normal estrous length is unknown in *P. volans*. Female A had two estrous cycles with durations of 6 days, and female E had one of 18 days; these lengths seem abnormal. Previous studies have reported that the incidence of abnormal ovarian cycles in high-producing females is increased and that such females have greater loss of body condition than those with normal ovarian activity (Shrestha et al. 2005; Kafi et al. 2012). Uterine infection —which prolongs the estrous cycle—also causes abnormality of the cycle (Fonseca et al. 1983). These factors may therefore have been associated with estrous cycle abnormality documented in our study.

All except one of the female showed cycling pattern of progesterone secretion during lactation; in the other progesterone concentrations remained low. Thus postpartum ovarian activity is likely suppressed in some females. The duration of postpartum anestrus is influenced mainly by maternal body condition and suckling intensity (Montiel and Ahuja 2005; Hultén et al. 2006). As previously reported, poor maternal body condition or a strong suckling stimulus, or both might cause low progesterone concentrations. More information on the effects of these factors on progesterone secretion patterns during lactation is needed to reveal more details of postpartum ovarian activity in this squirrel.

In addition, the number of luteal phases during lactation was different among the females. De Santiago-Miramontes et al. (2009) showed that does in greater body condition had greater ovulation rate than those in lower body condition. This difference seems be due to a change in concentrations of leptin (Zhang et al. 2005), which is secreted from adipose tissue and plays the central role to regulate the GnRH–LH system (Barb and Kraeling 2004). Low body condition, thus, negatively influences ovulation rate and reproductive behavior via a reduction in leptin secretion. In present study, five of the six females had different number of luteal phases, which would be dependent on the degree of body reserve. In order to reveal this hypothesis, we will need to investigate the relationship among ovulation rate, body reserve, a frequency of LH surge, and the concentrations of leptin.

Unlike a number of mammals, many, but not all, *P. volans* seem to circumvent the lactation-associated physiological inhibition of postpartum ovulation. Thus, as hypothesized, many females have the physiological potential to mate during lactation. The periods of luteal phases during lactation overlap the potential mating period for second litters, because in our study area *P. volans* enters estrus between the end of February and July (Yanagawa 1999). These findings, therefore, mean that *P. volans* likely has a postpartum estrus. However, we could not identify whether or not estrus followed postpartum ovulation in this animal, because behavioral monitoring was not performed. To confirm postpartum mating, this will need to be done.

Postpartum estrus can provide some benefits to *P. volans*. This species is obligated to reproduce in a limited breeding season because of the presence of severe winter, but this time constraint can be circumvented if the species has a postpartum estrus. In addition to this benefit, early birth after a postpartum estrus could prolong the growing time of the offspring before the next winter, which could thus gain more weight than those born late in the season. The ratio of surface area to body mass is related to thermoregulation (Louw 1993). Heavier individuals are less likely to be threatened by heat loss, because heat loss is positively related to surface area, and the ratio of surface area to body mass decreases with increasing volume. Survival rate is strongly positively related to body mass to overwinter (Loison et al. 1999). Therefore, offspring delivered earlier because of conception during a postpartum estrus are more likely to survive. In this way, postpartum estrus could be important reproductive strategy to improve the survival rate of litters of *P. volans*.

General endocrine pattern during lactation seems to be observed among different species of squirrels. Previous studies have shown that some species of tree squirrels and flying squirrels have a postpartum estrus (Boutin et al. 2006; Smith et al. 2011; Kawamichi 2010); therefore postpartum ovulation likely occur. In addition, in many species of ground squirrel, such as the California ground squirrel *Spermophilus beecheyi*, the European ground squirrel *Spermophilus citellus*, and the woodchuck *Marmota monax*, postpartum ovulation or high progesterone concentrations have been detected during lactation (Concannon et al. 1983; Holekamp et al. 1988; Strauss et al. 2009), although these ground squirrels reproduce only once a year and never mate after their first reproduction of the year owing to hibernation. Thus, the presence of postpartum ovulation in squirrels does not necessarily depend on the possibility of a second reproduction after a successful reproduction. This indicates that postpartum ovulation may be strong phylogenetic constraint pattern in the Family Sciuridae, although its function, and that of progesterone secretion, in the non-breeding season has been not yet been clarified. In *P. volans,* high progesterone concentrations following postpartum ovulation may be detected, whether females mate during the second lactation or not.

In conclusion, we detected periods of elevated progesterone concentrations and cycling pattern of progesterone secretion from early lactation in some *P. volans*. This indicated that postpartum ovarian activity was reinitiated and that progesterone was secreted from the CLs formed. Therefore, female *P. volans* have the physiological potential to mate during lactation.

Figures

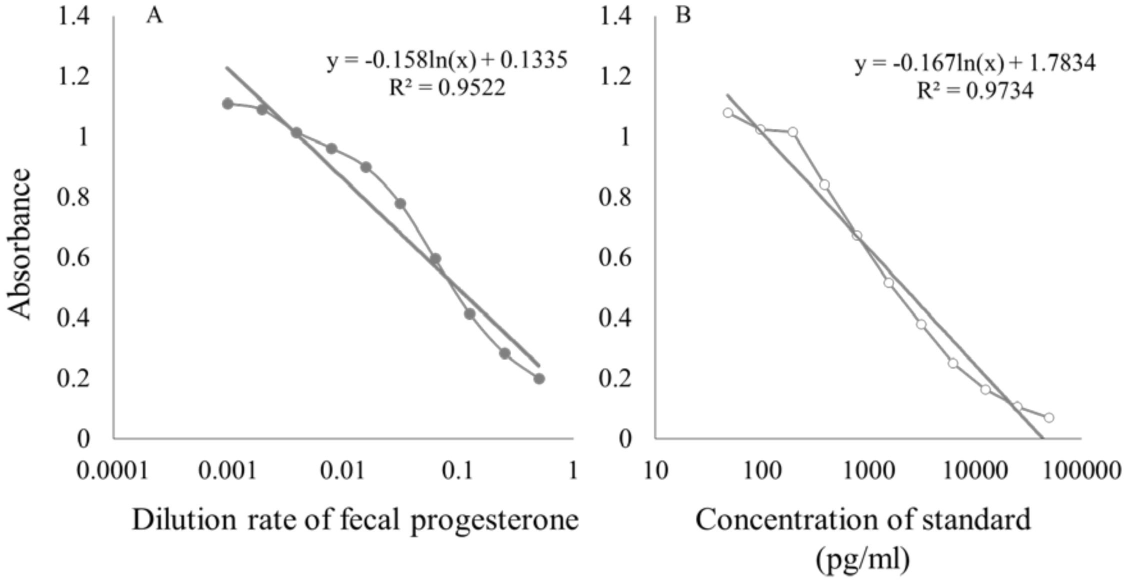

Fig. 1. Serial dilution results for fecal progesterone (A) and a standard curve (B). Fecal progesterone was serially diluted at 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, and 1:1024. Standards were serially diluted from 48 to 50,000 pg/ml. The slopes of the two regression lines did not differ significantly ($P = 0.144$).

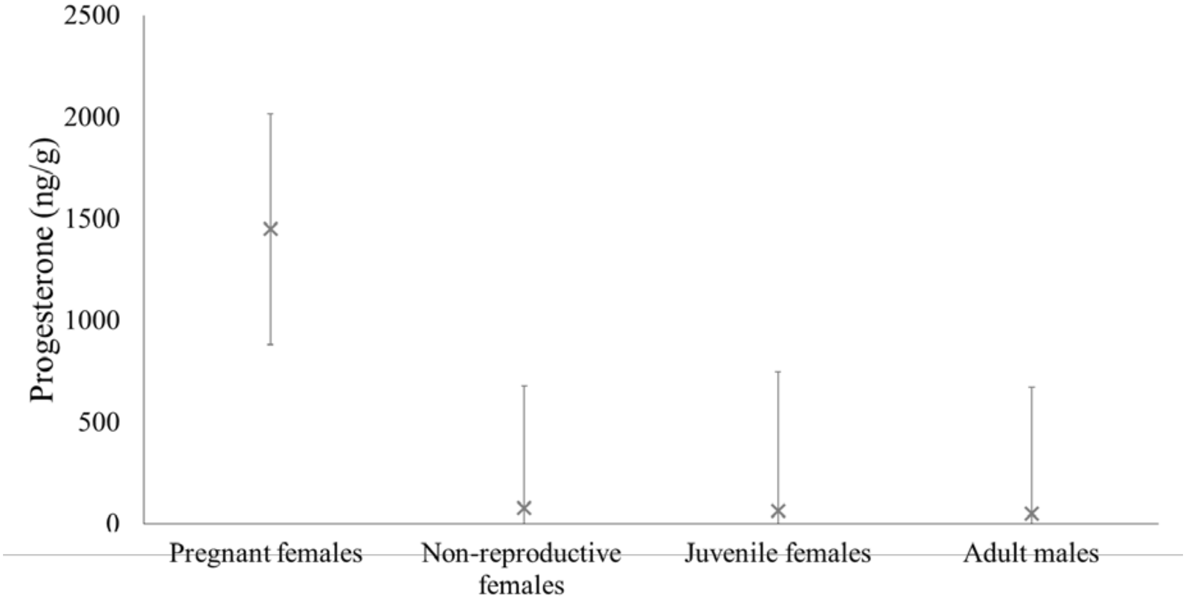

Fig. 2. Ninety-five percent confidence intervals of fecal progesterone concentrations in pregnant females, non-reproductive females, juvenile females, and adult males, shown as means ± 1.96 SD. Crosses represent means; concentrations less than zero are not shown, because the progesterone concentration was necessarily more than zero.

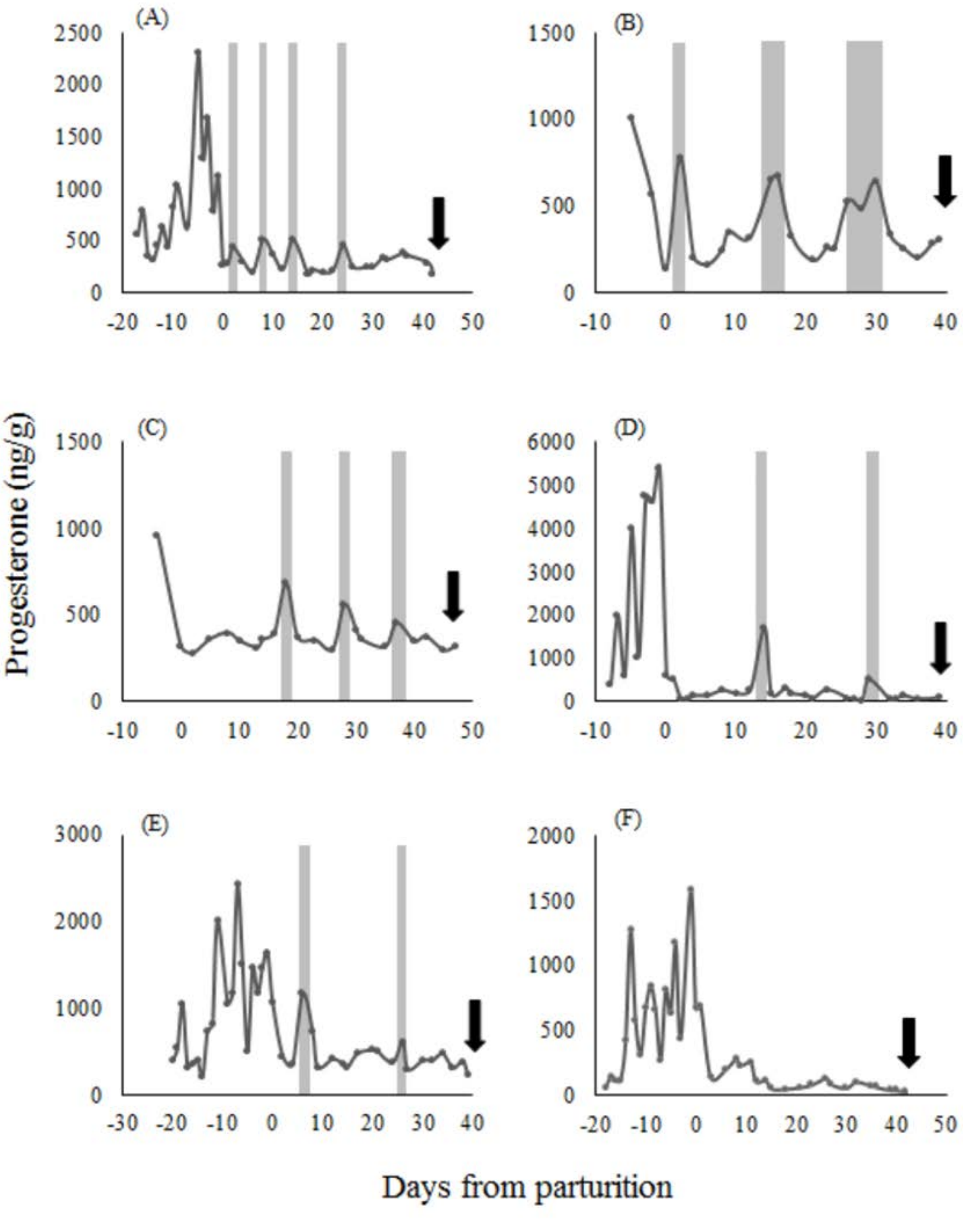

Fig. 3. Progesterone dynamics of six lactating females during the latter part of pregnancy and in lactation (A: female A; B: female B; C: female C; D: female D; E: female E; F: female F). Shaded areas are durations of the luteal phase. Arrows indicate the periods when or just before offspring began to eat solids.

General discussion

Feces are used for various purposes, including ecological, genetic, and endocrinological studies (see review by Putman 1984 and Kohn; Wayne 1997). Fecal analysis could help to elucidate the biology of Siberian flying squirrels. Here, author used feces to validate quantitative method to confirm presence and to investigate fecal progesterone levels and their patterns of variation.

Previously, presence had been determined by searching for feces throughout the whole forest, which seemed to be an inefficient method. In section 1, author found that it is more efficient to set up five transects and to search for feces 20 cm from trees with a DBH greater than 20 cm. In this way, author established a method for confirming the presence of Siberian flying squirrels. In contrast to the traditional method, this quantitative method does not require the use of professional techniques or knowledge to confirm the presence of squirrels. Presence–absence data is one of the most basic information used to investigate animal ecology—especially abundance and distribution (Royle and Nichols 2003; Engler et al. 2004; MacKenzie 2005). Therefore, use of the fecal analysis method established in section 1 could help to estimate the abundance and distribution of Siberian flying squirrels. However, determination of absence by using this method can often be uncertain, because it is generally impossible to confirm an animal's absence (MacKenzie 2005). To reduce bias, repeated surveys could improve the probability of absence, or simulated analysis using pseudo-absence data could enhance the quality of results (Engler et al. 2004; MacKenzie 2005).

Fecal progesterone analysis has been used to predict reproductive patterns (Li et al. 2001; Ghosal et al. 2012), because reproductive behavior is regulated by steroid hormones (Norris and

Lopez 2011). In section 2, author demonstrated the validity of using fecal progesterone analysis in Siberian flying squirrels to predict pregnancy. In section 3, author used fecal progesterone analysis to profile progesterone concentrations and dynamics during lactation. Because the Siberian flying squirrel is nocturnal and small, it is difficult to observe its reproductive behavior and monitor its reproductive status unless radio telemetry is used. However, the results obtained from fecal progesterone analysis indicated that lactating females had the opportunity to mate. Thus, fecal progesterone analysis is useful for investigating the reproductive biology of these animals.

Siberian flying squirrels depend to an extreme degree on forests, but forest fragmentation has caused breaking apart forest area and losing forest size in the worldwide. As a result, populations of Siberian flying squirrels have decreased in Finland, Estonia, and South Korea (Hokkanen et al. 1982; Timm and Kiristaja 2002; Jackson 2012). In the city of Obihiro, in Hokkaido, the population of Siberian flying squirrels has likely decreased in the same way as overseas, because the forest area has now decreased to 4% of that 100 years ago (Konno 2002). Many studies have been conducted in an effort to conserve this squirrel. Occupancy sites can be predicted by using estimation models (Reunanen et al. 2002; Hurme et al. 2008a; Santangeli et al. 2013), but it is unknown whether the populations at these estimated occupancy sites can be maintained in the long term, because the effects of forest fragmentation have a time lag (Kuussaari et al. 2009); the responses of Siberian flying squirrels to these time lags are unclear and need to be monitored. Therefore, long-term monitoring using the quantitative method, established from the method documented in section 1, could help more precisely to evaluate the effects of forest fragmentation; however, several questions remained unclear in order to achieve mentioned above.

To establish the quantitative methods for confirmation of the presence or for evaluation of the population density, we need to take into account pellet decay rate (Sato et al. 2005). Prugh and Krebs (2004) reported that habitat type can affect pellet decay rate, which was likely due to differences in moisture levels and substrate type. In addition, pellet decay rate is higher in the wet season than that in the dry season (Rivero et al. 2004); thus, season can also affect it. However, pellet decay rate or the effect of habitat type or season on it are unknown in *P. volans*.

The quantitative method will also require the number of defecation and pellet (Sato et al. 2005). The diet can affect these, because the inclusion of fiber in the diet increases fecal weight and number of defecations (Kelsay et al. 1978). *P. volans* shifts the diet with a change of season (Asari et al. 2008). Therefore, the number of defecation and pellet in *P. volans* will change with season. To use the method established in Section 1 as monitoring methodology, we need to reveal not only the effects of environmental factors, such as habitat type and season, on pellet decay rate, but also the variation of the number of defecation and pellet.

Previous studies have shown that animals living in fragmented forests are stressed by human activity (Wasser et al. 1997; Suorsa et al. 2003) and that the stress suppresses ovulation and the estrous cycle and reduces litter size and growth rates (Rivier and Rivest 1991; Sheriff et al. 2009). Furthermore, reproductive physiology is influenced by body condition (Walker et al. 1984; Rhind and McNeilly 1986; Romero 2004): resources such as food are reduced by clear-cutting (Zanette et al. 2000; Fahrig 2003), 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 (Faaborg

1995). Therefore, reproductive status needs to be assessed if we are to evaluate the effects of forest fragmentation on Siberian flying squirrels. Progesterone concentrations are measured to assess reproductive status and detect pregnancy in wild populations of other animals (McKenzie et al. 2005; Rolland et al. 2005). Also, in Siberian flying squirrels, fecal progesterone analysis could be used to predict pregnancy rates in populations in fragmented forests, and this could help to assess population dynamics in these fragmented areas.

In addition to in situ conservation, ex situ conservation is often applied to animals subjected to population decreases, i.e. endangered species (Conde et al. 2011). Ex situ conservation focuses mainly on captive breeding in zoological parks and aquariums to increase populations, and breeding animals are reintroduced to their original habitats. Some animals, such as carnivores, have been successfully reintroduced (see review by Jule et al. 2008). Application of endocrine techniques can suggest problems in captive-breeding programs and can guide hormone therapy in endangered species (Wikelski and Cooke 2006), and steroid hormone measurements have been conducted in a variety of wild and zoo animals (Heistermann et al. 1997; Tsubota et al. 1998). No stress, or low stress, is preferred for measurement of steroid hormones in zoo and wild animals; fecal steroid hormone analysis is thus useful because it is non-invasive (see review by Schwarzenberger 2007). Physiological research can elucidate the endocrine patterns of the estrous cycle and pregnancy, as well as reproductive behavior; it can also help in artificial insemination.

Siberian flying squirrel populations are declining, as mentioned above. Survival rates and population growth of populations in fragmented forests are low (Lampila et al. 2009a), indicating that fragmented forests are likely to be sinks. To conserve Siberian flying squirrels, its habitat preference, nest cavity selection, genetic variations, and ecological role in the ecosystem have been investigated (Selonen et al. 2001; Hurme et al. 2008b; Lampila et al. 2009b; Suzuki et al. 2013), but captive breeding may also be needed to maintain populations. In section 2, author validated the use of fecal progesterone analysis for predicting pregnancy; not only pregnancy but also other reproductive status could be predicted. Furthermore, in section 3 author revealed progesterone secretion patterns during lactation. High progesterone concentrations were detected despite lactation, and this seems to be the characteristic endocrine pattern of Siberian flying squirrels. If Siberian flying squirrels express postpartum estrus, facilitation of breeding during lactation may permit efficient captive breeding, although knowledge about nutrition is also needed (Wikelski and Cooke 2006). Nevertheless, for efficient captive breeding author needs to establish a more precise diagnosis of pregnancy and will need to reveal in more detail the dynamics of progesterone during pregnancy.

In conclusion, author's research validated this quantitative method of confirming the presence of Siberian flying squirrels and also the use of fecal progesterone analysis to predict pregnancy and examine progesterone secretion patterns during lactation. Research focusing on feces can both help to reveal basic ecology and facilitate conservation in Siberian flying squirrels, as in other animals. The use of feces for investigating the biology of Siberian flying squirrels seems effective.

Acknowledgements

I would like to express my deepest gratitude to Professor Hisashi Yanagawa, my supervisor, who offered continuing support and constant encouragement. I am also thank to Professor Masafumi Tetsuka and Professor Toshiki Aoi who provided technical help and comments. I am grateful to thank Professor Tatsuo Oshida, Professor Motozumi Matsui, and Assistant professor Takumi Akasaka for their comments on my studies. I thank to Dr. Kei Suzuki, Kenta Uchida, Ryuji Furukawa, Mizuho Hamada, and the students of the Laboratory of Wildlife Ecology at Obihiro University of Agriculture and Veterinary Medicine for helpful comments and their support with the field work. I would also like to express my gratitude to my family for their moral support and warm encouragements.

Abstract

The animal's sign is often focused on when difficult-to-observe animals are researched. Feces are among the most common for animal research, and are used to reveal the basic ecology in various studies. Furthermore, recently fecal steroid hormone analysis that is non-invasive has been developed over the past two decades to profile the endocrine patterns. Feces can therefore be used as a tool for studying difficult-to-observe animals, and the results obtained can help to elucidate various aspects of animal biology, such as life history, genetic structure, and endocrine patterns.

The Siberian flying squirrel, *Pteromys volans*, a member of the Family Sciuridae, is difficult to observe. However, its feces are distinctive and can be easily identified. Despite the diversity of information offered by fecal analysis, there has been little research on feces in Siberian flying squirrels. Research focusing on fecal characteristics could help to elucidate the ecology and endocrinology of this species.

Here, with a focus on the benefits of using feces, author studied the ecology and endocrinology of Siberian flying squirrels. First, author established the confirmation method for presence of Siberian flying squirrels by using feces (Section 1). Then, author validated the use of fecal progesterone analysis for predicting pregnancy (Section 2). Finally, author investigated progesterone concentrations during lactation and the progesterone dynamics of lactating females to estimate the presence of postpartum estrus in Siberian flying squirrels (Section 3).

1) A confirmation method for the presence of the Siberian flying squirrel via feces

There is concern about population decline and local population extinction of the Siberian flying squirrel *Pteromys volans* because of forest fragmentation. The goal of this study was to confirm a simple and efficient method of determining the presence of the squirrels to monitor the effects of forest fragmentation. Author searched for their feces in 11 fragmented forests. Author set 12 transects, each 10 m long and 4 m wide, randomly in each forest and searched for feces within each transects. First, to characterize the places where feces were found, author measured the distance between the feces and the closest tree, along with the diameter at breast height (DBH) of that tree. The feces that author found were close to large trees; author therefore found that it was efficient to mainly search for feces within 20 cm of such trees. Second, to reveal the efforts to search feces in each forest, author evaluated the relationship between the number of transects on which author found feces and forest size. The number was unrelated to forest size. Therefore, author did not need to change the research effort according to forest size. Furthermore, author found that five transects per forest gave valid results for squirrel presence.

2) Validation of fecal progesterone analysis for predicting pregnancy in Siberian flying squirrels *Pteromys volans*

Recently steroid hormone analysis using feces have been developed during past twenty years. Development of fecal steroid hormone analysis has facilitated to elucidate a variety of wild and zoo animals. However, there is a paucity of information on the Siberian flying squirrel's basic reproductive physiology, and there is no established method for studying it. The purpose of this study was to validate fecal progesterone analysis in this animal using a commercial enzyme immunoassay (EIA) kit for endocrine profiles in Siberian flying squirrels. First, author tested parallelism between serially diluted fecal progesterone and a standard curve to validate the EIA. Comparison of the slopes of the two regression lines to test for parallelism revealed no significant difference. Therefore, progesterone concentrations in the fecal samples of the Siberian flying squirrels were exactly measured. Second, author compared progesterone concentrations among four groups—pregnant females, adult females in the non-breeding season, juvenile females, and adult males—to determine whether fecal progesterone analysis was useful for evaluating reproductive status using GLMM. Fecal progesterone concentrations were significantly higher in pregnant females than in other groups. These results indicated that fecal progesterone analysis in Siberian flying squirrels was valid for predicting pregnancy.

3) Fecal progesterone concentrations and dynamics during lactation in Siberian flying squirrels *Pteromys volans*

The reproductive strategy is a key concept of species survival. *Pteromys volans*, which is a seasonal breeder, produces up to two litters per year. But, *P. volans* is imposed to reproduce in a breeding season because of severe winter. To circumvent time constraints, *P. volans* may have a postpartum estrus similarly to a variety of small mammals. If *P. volans* has a postpartum estrus, progesterone would be secreted from formed corpus luteum (CL) after postpartum ovulation. Therefore, we investigated progesterone concentrations and dynamics during lactation in this species by using an enzyme immunoassay to test this hypothesis. To compare fecal progesterone in lactating females with pregnant females and non-reproductive females, fecal samples were collected from each individuals with different reproductive status. As a result, the 95% confidence interval of fecal progesterone concentrations in lactating females overlapped with that in pregnant and non-reproductive females. This result indicated that a part of used fecal samples included luteal phase. Furthermore, author captured six pregnant females in spring and kept them temporarily to monitor progesterone dynamics during lactation. The durations with high progesterone concentration were detected in 4 of 6 lactating females, which indicated that follicular development was reinitiated after parturition, ovulation occurred, and the formed CLs began secreting progesterone. Thus, author showed that *P. volans* has the physiological potential to mate during lactation.

These studies showed fecal analysis can help to mainly elucidate the basic biology of Siberian flying squirrels. However, the research using feces could also help to conserve this squirrels of which the population have decreased due to forest fragmentation in the worldwide. For instance, the research in section 1 showed that feces can be used as the indicator of presence, and might be useful to monitor the lag time effect of forest fragmentation on the population of the squirrel. Furthermore, forest fragmentation has deleterious effect on the reproductive physiology of living animals in fragmented forests. Therefore, reproductive status needs to be assessed if we are to evaluate the effects of forest fragmentation on Siberian flying squirrels. Fecal progesterone analysis is used to assess reproductive status and detect pregnancy in wild populations of other animals. Also, in Siberian flying squirrels, fecal progesterone analysis could be used to predict pregnancy rates in populations in fragmented forests, and this could help to assess population dynamics in these fragmented areas.

In addition to in situ conservation, ex situ conservation is often applied to animals subjected to population decreases, i.e. endangered species. Ex situ conservation focuses mainly on captive breeding in zoological parks and aquariums to increase populations. Application of endocrine techniques can suggest problems in captive-breeding programs and can guide hormone therapy in endangered species. In section 2, author validated the use of fecal progesterone analysis for predicting pregnancy; not only pregnancy but also other reproductive status could be predicted. Furthermore, in section 3 author showed that high progesterone concentrations were detected despite lactation, and this seems to be the characteristic endocrine pattern of Siberian flying squirrels. If Siberian flying squirrels express postpartum estrus, facilitation of breeding during lactation may permit efficient captive breeding. Nevertheless, for efficient captive breeding author needs to establish a more precise diagnosis of pregnancy and will need to reveal in more detail the dynamics of progesterone during pregnancy.

References

Airapetyants, A.E. and Fokin, I.M. 2003. Biology of European flying squirrel *Pteromys volans* L.(Rodentia: Pteromyidae) in the north-west of Russia. *Russ. J. Theriol.* 2: 105-113.

Asari, Y., Yamaguchi, Y. and Yanagawa, H. 2008. Field observations of the food items of the Siberian flying squirrel, *Pteromys volans orii*. *J. Jpn. Wildl. Res. Soc.* 33: 7-11 (in Japanese).

Asher, G.W., Peterson, A.J. and Duganzich, D. 1989. Adrenal and ovarian sources of progesterone secretion in young female fallow deer, Dama dama. *J. Reprod. Fertil.* 85: 667-675.

Barb, C.R. and Kraeling, R.R. 2004. Role of leptin in the regulation of gonadotropin secretion in farm animals. *Anim. Reprod. Sci.* 82: 155-167.

Boutin, S., Wauters, L.A., McAdam, A.G., Humphries, M.M., Tosi, G. and Dhondt A.A. 2006. Anticipatory reproduction and population growth in seed predators. *Science* 314: 1928-1930.

Boyd, I.L. 1984. Development and regression of the corpus luteum in grey seal (*Halichoerus grypus*) ovaries and its use in determining fertility rates. *Can. J. Zool.* 62: 1095-1100.

Bravo, P.W., Fowler, M.E. and Lasley, B.L. 1994. The postpartum llama: fertility after parturition. *Bio. Reprod.* 51: 1084-1087.

Brogan, R.S., Mitchell, S.E., Trayhurn, P. and Smith, M.S. 1999. Suppression of Leptin During Lactation: Contribution of the Suckling Stimulus Versus Milk Production 1. *Endocrinology* 140: 2621-2627.

Concannon, P., Baldwin, B., Lawless, J., Hornbuckle, W. and Tennant, B. 1983. Corpora lutea of pregnancy and elevated serum progesterone during pregnancy and postpartum anestrus in woodchucks (*Marmota monax*). *Biol. Reprod.* 29: 1128-1134.

Conde, D.A., Flesness, N., Colchero, F., Jones, O.R. and Scheuerlein, A. 2011. An emerging role of zoos to conserve biodiversity. *Science* 331: 1390-1391.

Dadarwal, D., Tandon, S.N., Purohit, G.N., and Pareek, P.K. 2004. Ultrasonographic evaluation of uterine involution and postpartum follicular dynamics in French Jennies (*Equus asinus*). *Theriogenology* 62: 257-264.

Davies, C. 2013. Immunoassay performance measures, in: Wild, D. (Eds.), The Immunoassay Handbook. Elsevier, Oxford, pp. 11-26.

Debinski, D.M. and Holt, R.D. 2000. A survey and overview of habitat fragmentation experiments. *Conserv. Biol.* 14: 342-355.

Dickman, C.R. and Huang, C. 1988. The reliability of fecal analysis as a method for determining the diet of insectivorous mammals. *J. Mammal.* 69: 108-113.

Engler, R., Guisan, A. and Rechsteiner, L. 2004. An improved approach for predicting the distribution of rare and endangered species from occurrence and pseudo‐absence data. *J. Appl. Ecol.* 41: 263-274.

Faaborg, J. 1995. Regional forest fragmentation and the nesting success of migratory birds. *Science* 267: 93-102.

Fahrig, L. 2003. Effects of habitat fragmentation on biodiversity. *Annu. Rev. Ecol. Evol. Syst.* 34: 487-515.

Fonseca, F.A., Britt, J.H., McDaniel, B.T., Wilk, J.C. and Rakes, A.H. 1983. Reproductive traits of Holsteins and Jerseys. Effects of age, milk yield, and clinical abnormalities on involution of cervix and uterus, ovulation, estrous cycles, detection of estrus, conception rate, and days open. *J Dairy Sci* 66: 1128-1147.

Franceschini, C., Siutz, C., Palme, R. and Millesi, E. 2007. Seasonal changes in cortisol and progesterone secretion in Common hamsters. *Gen. Comp. Endocr.* 152: 14-21.

Franceschini-Zink, C. and Millesi, E. 2008. Reproductive performance in female common hamsters. *Zoology* 111: 76-83.

Ghosal, R., Kalaivanan, N., Sukumar, R. and Seshagiri, P.B. 2012. Assessment of estrus cyclicity in the Asian elephant (*Elephas maximus*) by measurement of fecal progesterone metabolite 5α-P-3OH, using a non-invasive assay. *Gen. Comp. Endocr.* 175: 100-108.

Gilbert, A.N. 1984. Postpartum and lactational estrus: a comparative analysis in rodentia. *J. Comp. Psychol.* 98, 232-245.

Goymann, W., Möstl, E., Van't Hof, T., East, M.L. and Hofer, H. 1999. Noninvasive fecal monitoring of glucocorticoids in spotted hyenas, *Crocuta crocuta*. *Gen. Comp. Endocr.* 114: 340-348.

Graham, L.H., Reid, K., Webster, T., Richards, M. and Joseph, S. 2002. Endocrine patterns associated with reproduction in the Nile hippopotamus (*Hippopotamus amphibius*) as assessed by fecal progestagen analysis. *Gen. Comp. Endocr.* 128: 74-81.

Gudermuth, D.F., Concannon, P.W., Daels, P.F. and Lasley, B.L. 1998. Pregnancy-specific elevations in fecal concentrations of estradiol, testosterone and progesterone in the domestic dog (*Canis familiaris*). *Theriogenology* 50: 237-248.

Hamasaki, S.I., Yamauchi, K., Ohki, T., Murakami, M., Takahara, Y., Takeuchi, Y. and Mori, Y. 2001. Comparison of various reproductive status in Sika deer (*Cervus nippon*) using fecal steroid analysis. *J. Vet. Med. Sci.* 63: 195-198.

Hanski, I.K., Mönkkönen, M., Reunanen, P. and Stevens, P. 2000. Ecology of the Eurasian flying squirrel (*Pteromys volans*) in Finland, in Goldingay, R. and Scheibe, J. (Eds.), Biology of Gliding Mammals. Filander Verlag, Fürth, pp. 67-86.

Heistermann, M., Trohorsch, B. and Hodges, J.K. 1997. Assessment of ovarian function in the African elephant (*Loxodonta africana*) by measurement of 5α‐reduced progesterone metabolites in serum and urine. *Zoo Biol.* 16: 273-284.

Hewitt, D.G. and Robbins, C.T. 1996. Estimating grizzly bear food habits from fecal analysis. *Wildlife Soc. B.* 24: 547-550.

Hokkanen, H., Törmälä, T. and Vuorinen, H. 1982. Decline of the flying squirrel *Pteromys volans* l. populations in Finland. *Biol. Conserv.* 23: 273-284.

Holekamp, K.E., Nunes, S. and Talamantes, F. 1988. Patterns of progesterone secretion in free-living California ground squirrels (*Spermophilus beecheyi*). *Biol. Reprod.* 39: 1051-1059.

Hultén, F., Wallenbeck, A. and Rydhmer, L. 2006. Ovarian Activity and Oestrous Signs among Group‐Housed, Lactating Sows: Influence of Behaviour, Environment and Production. *Reprod. Domest. Anim.* 41: 448-454.

Hurme, E., Reunanen, P., Mönkkönen, M., Nikula, A., Nivala, V. and Oksanen, J. 2007. Local habitat patch pattern of the Siberian flying squirrel in a managed boreal forest landscape. *Ecography* 30: 277-287.

Hurme, E., Mönkkönen, M., Reunanen, P., Nikula, A. and Nivala, V. 2008a. Temporal patch occupancy dynamics of the Siberian flying squirrel in a boreal forest landscape. *Ecography* 31: 469-476.

Hurme, E., Mönkkönen, M., Sippola, A.L., Ylinen, H. and Pentinsaari, M. 2008b. Role of the Siberian flying squirrel as an umbrella species for biodiversity in northern boreal forests. *Ecol. Indic.* 8: 246-255.

Jackson, S. 2012. Gliding Mammals of the World. CSIRO Publishing, Melbourne.

Jule, K.R., Leaver, L.A. and Lea, S.E. 2008. The effects of captive experience on reintroduction survival in carnivores: a review and analysis. *Biol. Conser.* 141: 355-363.

Kadosaki, M. 2001. Field signs of *Pteromys volans* in Hokkaido. *J. Jpn. Wildl. Res. Soc.* 27: 27–33 (in Japanese with English abstract).

Kawamichi, T. 2010. Biannual reproductive cycles in the Japanese giant flying squirrel (*Petaurista leucogenys*). *J. Mammal.* 91: 905-913.

Kelsay, J.L., Behall, K.M. and Prather, E.S. 1978. Effect of fiber from fruits and vegetables on metabolic responses of human subjects I. Bowel transit time, number of defecations, fecal weight, urinary excretions of energy and nitrogen and apparent digestibilities of energy, nitrogen, and fat. *Am. J. Clin. Nutr.* 31: 1149-1153.

Kendall, K.C., Stetz, J.B., Boulanger, J., Macleod, A.C., Paetkau, D. and White, G.C. 2009. Demography and genetic structure of a recovering grizzly bear population. *J. Wildl. Manage.* 73: 3-17.

Khan, M.Z., Altmann, J., Isani, S.S. and Yu, J. 2002. A matter of time: evaluating the storage of fecal samples for steroid analysis. *Gen. Com. Endocr.* 128: 57-64.

Konno, Y. 2002. Present status of remnant forests in Obihro, eastern Hokkaido, Japan. *Obihiro Asia and the Pacific Seminar on Education for Rural Development (OASERD)*: 39-46.

Koprowski, J.L. 1993. Alternative reproductive tactics in male eastern gray squirrels:“making the best of a bad job”. *Behav. Ecol.* 4: 165-171.

Koskimäki, J., Huitu, O., Kotiaho, J.S., Lampila, S., Mäkelä, A., Sulkava, R. and Mönkkönen, M. 2013. Are habitat loss, predation risk and climate related to the drastic decline in a Siberian flying squirrel population? A 15-year study. *Popul. Ecol.* 56: 341-348.

Krepschi, V.G., Polegato, B.F., Zanetti, E.S. and Duarte, J.M.B. 2013. Fecal progestins during pregnancy and postpartum periods of captive red brocket deer (*Mazama americana*). *Anim. Reprod. Sci.* 137: 62-68.

Lampila, S., Wistbacka, R., Mäkelä, A. and Orell, M. 2009a. Survival and population growth rate of the threatened Siberian flying squirrel (*Pteromys volans*) in a fragmented forest landscape. *Ecoscience* 16: 66-74.

Lampila, S., Kvist, L., Wistbacka, R. and Orell, M. 2009b. Genetic diversity and population differentiation in the endangered Siberian flying squirrel (*Pteromys volans*) in a fragmented landscape. *Eur. J. Wildl. Res.* 55: 397-406.

Latta, J., Hopf, S. and Ploog, D. 1967. Observation on mating behavior and sexual play in the squirrel monkey (*Saimiri sciureus*). *Primates* 8: 229-245.

Li, C., Jiang, Z., Jiang, G. and Fang, J. 2001. Seasonal changes of reproductive behavior and fecal steroid concentrations in Père David's deer. *Horm. Behav.* 40: 518-525.

Loison, A., Langvatn, R. and Solberg, E.J. 1999. Body mass and winter mortality in red deer calves: disentangling sex and climate effects. *Ecography* 22: 20-30.

Louw, G. 1993. Physiological animal ecology. Addison Wesley Longman Limited, Edinburgh.

MacKenzie, D.I. 2005. What are the issues with presence-absence data for wildlife managers?. *J. Wildl. Manage.* 69: 849-860.

Maeda, K.I., Uchida, E., Tsukamura, H., Ohkura, N., Ohkura, S. and Yokoyama, A. 1990. Prolactin does not mediate the suppressive effect of the suckling stimulus on luteinizing hormone secretion in ovariectomized lactating rats. *Endocrinol. Jpn.* 37: 405-411.

Martínez-Gómez, M., Juárez, M., Distel, H. and Hudson, R. 2004. Overlapping litters and reproductive performance in the domestic rabbit. *Physiol. Behav.* 82: 629-636.

Mastromonaco, G.F., Gunn, K., McCurdy-Adams, H., Edwards, D.B. and Schulte-Hostedde, A.I. 2014. Validation and use of hair cortisol as a measure of chronic stress in eastern chipmunks (*Tamias striatus*). *Conserv. Physiol.* 2: cou055.

McKenzie, J., Parry, L.J., Page, B. and Goldsworthy, S.D. 2005. Estimation of pregnancy rates and reproductive failure in New Zealand fur seals (*Arctocephalus forsteri*). *J. Mammal.* 86: 1237-1246.

McNeilly, A.S., Tay, C.C. and Glasier, A. 1994. Physiological mechanisms underlying lactational amenorrhea. *Ann. N. Y. Acad. Sci.* 709: 145-155.

McNeilly, A.S. 1997. Lactation and fertility. *J. Mammary. Gland. Biol.* 2: 291-298.

Messier, C., Parent, S. and Bergeron, Y. 1998. Effects of overstory and understory vegetation on the understory light environment in mixed boreal forests. *J. Veg. Sci.* 9: 511-520.

Millesi, E., Strauss, A., Burger, T., Hoffmann, I.E. and Walzl, M. 2008. Follicular development in European ground squirrels (*Spermophilus citellus*) in different phases of the annual cycle. *Reproduction* 136: 205-210.

Miyamoto, A., Okuda, K., Schweigert, F.J. and Schams, D. 1992. Effects of basic fibroblast growth factor, transforming growth factor-β and nerve growth factor on the secretory function of the bovine corpus luteum in vitro. *J. Endocrinol.* 135: 103-114.

Mohammed, O.B., Green, D.I. and Holt, W.V. 2011. Fecal progesterone metabolites and ovarian activity in cycling and pregnant mountain gazelles (*Gazella gazella*). *Theriogenology* 75: 542-548.

Montiel, F. and Ahuja, C. 2005. Body condition and suckling as factors influencing the duration of postpartum anestrus in cattle: a review. *Anim. Reprod. Sci.* 85, 1-26.

Mossman, H.W. and Duke, K.L. 1973. Comparative morphology of the mammalian ovary, The University of Wisconsin Press, London.

Möstl, E., Messmann, S., Bagu, E., Robia, C. and Palme, R. 1999. Measurement of glucocorticoid metabolite concentrations in faeces of domestic livestock. *J. Vet. Med. A* 46: 621-631.

Muren, Kusuda, S., Huricha, Takasu, M., Goto, Y., Saito, M., Kosaka, M., Hori, Y. and Doi, O. 2014. A comparison of ovarian cycle and pregnancy between Kiso Mares (*Equus caballus*) and Przewalski Mares (*Equus przewalskii*) by fecal gonadal hormones measurement. *Jpn. J. Zoo. Wildl. Med.* 19: 87-99 (in Japanese with English summary).

Norris, D.O. and Lopez, K.H. 2011. The Endocrinology of the Mammalian Ovary, in: Norris, D.O. and Lopez, K.H. (Eds.), Hormones and Reproduction of Vertebrates Mammals. Academic Press, London, pp. 59-72.

O'Shea, J.D. and Wright, P.J. 1985. Regression of the corpus luteum of pregnancy following parturition in the ewe. *Cells. Tissues. Organs.* 122: 69-76.

Orchinik, M., Licht, P. and Crews, D. 1988. Plasma steroid concentrations change in response to sexual behavior in Bufo marinus. *Horm. Behav.* 22: 338-350.

Peel, A.J., Vogelnest, L., Finnigan, M., Grossfeldt, L. and O'Brien, J.K. 2005. Non‐invasive fecal hormone analysis and behavioral observations for monitoring stress responses in captive western lowland gorillas (*Gorilla gorilla gorilla*). *Zoo Biol.* 24: 431-445.

Pettitt, B.A., Wheaton, C.J. and Waterman, J.M. 2007. Effects of storage treatment on fecal steroid hormone concentrations of a rodent, the Cape ground squirrel (*Xerus inauris*). *Gen. Comp. Endocr.* 150: 1-11.

Pettitt, B.A., Waterman, J.M. and Wheaton, C.J. 2008. Assessing the effects of resource availability and parity on reproduction in female Cape ground squirrels: resources do not matter. *J. Zool.* 276: 291-298.

Prugh, L.R. and Krebs, C.J. 2004. Snowshoe hare pellet-decay rates and aging in different habitats. *Wildlife Soc. B.* 32: 386-393.

Putman, R.J. 1984. Facts from faeces. *Mammal rev.* 14: 79-97.

Reunanen, P., Nikula, A., Mönkkönen, M., Hurme, E. and Nivala, V. 2002. Predicting occupancy for the Siberian flying squirrel in old-growth forest patches. *Ecol. Appl.* 12: 1188-1198.

Rhind, S.M. and McNeilly, A.S. 1986. Follicle populations, ovulation rates and plasma profiles of LH, FSH and prolactin in Scottish Blackface ewes in high and low levels of body condition. *Anim. Reprod. Sci.* 10: 105-115.

Rivero, K., Rumiz, D.I. and Taber, A.B. 2004. Estimating brocket deer (*Mazama gouazoubira* and *M. americana*) abundance by dung pellet counts and other indices in seasonal Chiquitano forest habitats of Santa Cruz, Bolivia. *Eur. J. Wildl. Res.* 50: 161-167.

Rivier, C. and Rivest, S. 1991. Effect of stress on the activity of the hypothalamic-pituitary-gonadal axis: peripheral and central mechanisms. *Biol. Reprod.* 45: 523-532.

Rolland, R.M., Hunt, K.E., Kraus, S.D. and Wasser, S.K. 2005. Assessing reproductive status of right whales (*Eubalaena glacialis*) using fecal hormone metabolites. *Gen. Comp. Endocr.* 142: 308-317.

Royle, J.A. and Nichols, J.D. 2003. Estimating abundance from repeated presence-absence data or point counts. *Ecology* 84: 777-790.

Romero, L.M. 2004. Physiological stress in ecology: lessons from biomedical research. *Trends. Ecol. Evol.* 19: 249-255.

Santangeli, A., Hanski, I.K. and Mäkelä, H. 2013. Integrating multisource forest inventory and anima survey data to assess nationwide distribution and habitat correlates of the Siberian flying squirrel. *Biol. Conserv.* 157: 31-38.

Sato, H., Kanda, N., Furusawa, H., Yokota, T. and Shibata, E. 2005. Problems Associated with Estimating the Population Size of Sika Deer (*Cervus Nippon*) on the Ohdaigahara Subalpine Plateau, Japan. *Jpn. J. Conserv. Ecol.* 10: 185-193 (in Japanese with English abstract).

Schwarzenberger, F. 2007. The many uses of non-invasive faecal steroid monitoring in zoo and wildlife species. *Int. Zoo Yearbk.* 41: 52-74.

Seal, U.S., Plotka, E.D., Packard, J.M. and Mech, L.D. 1979. Endocrine correlates of reproduction in the wolf. I. Serum progesterone, estradiol and LH during the estrous cycle. *Biol. Reprod.* 21: 1057-1066.

Selonen, V., Hanski, I.K. and Stevens, P.C. 2001. Space use of the Siberian flying squirrel *Ptevomys volans* in fragmented forest landscapes. *Ecography* 24: 588-600.

Selonen, V., Painter, J.N., Rantala, S. and Hanski, I.K. 2013. Mating system and reproductive success in the Siberian flying squirrel. *J. Mammal.* 94: 1266-1273.

Sheriff, M.J., Krebs, C.J. and Boonstra, R. 2009. The sensitive hare: sublethal effects of predator stress on reproduction in snowshoe hares. *J. Anim. Ecol.* 78: 1249-1258.

Shimamoto, T., Suzuki, K., Furukawa, R., Hamada, M., Tetsuka, M. and Yanagawa, H. 2015. Validation of fecal progesterone analysis for predicting pregnancy in Siberian flying squirrels (*Pteromys volans*). *Jpn. J. Zoo. Wildl. Med.*

Smith, M.J., Forbes, G.J. and Betts, M.G. 2011. Evidence of Multiple Annual Litters in *Glaucomys sabrinus* (Northern Flying Squirrel). *Northeast. Nat.* 18: 386-389.

Sponheimer, M., Robinson, T., Ayliffe, L., Passey, B., Roeder, B., Shipley, L. and Ehleringer, J. 2003. An experimental study of carbon-isotope fractionation between diet, hair, and feces of mammalian herbivores. *Can. J. Zool.* 81: 871-876.

Strauss, A., Hoffmann, I.E., Walzl, M. and Millesi, E. 2009. Vaginal oestrus during the reproductive and non-reproductive period in European ground squirrels. *Anim. Reprod. Sci*. 112: 362-370.

Suorsa, P., Huhta, E., Nikula, A., Nikinmaa, M., Jäntti, A., Helle, H. and Hakkarainen, H. 2003. Forest management is associated with physiological stress in an old–growth forest passerine. *P. Roy. Soc. Lond. B Bio.* 270: 963-969.

Suzuki, K., Mori, S. and Yanagawa, H. 2011. Detecting nesting trees of Siberian flying squirrels (*Pteromys volans*) using their feces. *Mamm. study* 36: 105-108.

Suzuki, K., Asari, Y. and Yanagawa, H. 2012. Gliding locomotion of Siberian flying squirrels in low-canopy forests: the role of energy-inefficient short-distance glides. *Acta Theriol.* 57: 131-135.

Suzuki, K., Sagawa, M. and Yanagawa, H. 2013. Nest cavity selection by the Siberian flying squirrel *Pteromys volans*. *Hystrix* 24: 187-189.

Tait, A.J., Pope, G.S. and Jonhson, E. 1981. Progesterone concentrations in peripheral plasma of non-pregnant and pregnant grey squirrels (*Sciurus carolinensis*). *J. Endocr.* 89: 107-116.

Timm, U. and Kiristaja, P. 2002. The Siberian flying squirrel (*Pteromys volans* L.) in Estonia. *Acta Zool. Lituan.* 12: 433-436.

Tsubota, T., Howell-Skalla, L. Boone, W.R., Garshelis, D.L. and Bahr, J.M. 1998. Serum progesterone, oestradiol, luteinizing hormone and prolactin profiles in the female black bear (*Ursus americanus*). *Anim. Reprod. Sci.* 53: 107-118.

Tucker, H.A. 1994. Lactation and its hormonal control, in: Knobil, E., Neill, J.D., Greenwald, G.S., Markert, C.L. and Pfaff, D.W. (Eds.), The Physiology of Reproduction, 2nd edition. Raven Press, Ltd., New York, pp. 1065-1098.

Uchida, K., Suzuki, K., Shimamoto, T., Yanagawa, H. and Koizumi, I. in press. Seasonal variation of flight initiation distance in Eurasian red squirrels in urban versus rural habitat. *J Zool*

Van Duyse, E., Pinxten, R. and Eens, M. 2002. Effects of testosterone on song, aggression, and nestling feeding behavior in male great tits, Parus major. *Horm. Behav.* 41: 178-186.

Vernes, K. 2001. Gliding performance of the northern flying squirrel (*Glaucomys sabrinus*) in mature mixed forest of eastern Canada. *J. Mammal.* 82: 1026-1033.

Walker, M.L., Wilson, M.E. and Gordon, T.P. 1984. Endocrine control of the seasonal occurrence of ovulation in rhesus monkeys housed outdoors. *Endocrinology* 114: 1074–1081.

Wasser, S.K., Bevis, K., King, G. and Hanson, E. 1997. Noninvasive physiological measures of disturbance in the northern spotted owl. *Conserv. Biol.* 11: 1019-1022.

Whittier, J.M., Mason, R.T. and Crews, D. 1987. Plasma steroid hormone levels of female red-sided garter snakes, *Thamnophis sirtalis parietalis*: relationship to mating and gestation. *Gen. Comp. Endocr.* 67: 33-43.

Wikelski, M. and Cooke, S.J. 2006. Conservation physiology. *Trends. Ecol. Evol.* 21: 38-46.

Wood, A.K., Short, R.E., Darling, A.E., Dusek, G.L., Sasser, R.G. and Ruder, C.A. 1986. Serum assays for detecting pregnancy in mule and white-tailed deer. *J. Wildlife. Manage.* 50: 684-687.

Yamaguchi, Y. and Yanagawa, H. 1995. Field observations on circadian activities of the flying squirrel, *Pteromys volans orii*. *Mammal. Sci.* 34: 139-149 (in Japanese with English summary).

Yamauchi, K., Hamasaki, S.I., Takeuchi, Y. and Mori, Y. 1999. Application of enzyme immunoassay to fecal steroid analysis in Sika Deer (*Cervus nippon*). *J. Reprod. Dev.* 45: 429-434.

Yanagawa, H. 1999. Ecological notes on the Russian flying squirrel (*Pteromys volans orii*) with a video camera. *Mammal. Sci*. 39: 181-183 (in Japanese).

Yanagawa, H. 2009. Home range and movement of Siberian flying squirrel (*Pteromys volans orii*) in windbreak forest. *The Report of Obihiro University of Agriculture and Veterinary Fund* 37: 15-17 (in Japanese).

Zanette, L., Doyle, P. and Trémont, S.M. 2000. Food shortage in small fragments: evidence from an area-sensitive passerine. *Ecology* 81: 1654-1666.

Zhang, S., Blache, D., Blackberry, M.A. and Martin, G.B. 2005. Body reserves affect the reproductive endocrine responses to an acute change in nutrition in mature male sheep. Anim. Reprod. Sci. 88: 257-269.