

# What Does the Siberian Flying Squirrel Eat?

Analysing the diet of the Siberian flying squirrel (*Pteromys volans*)  
by DNA barcoding



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<p>Ekologian peruskysymyksiä on eläinlajien ruokavalion koostumus. Lajin ruokavalio liittyy ratkaisevasti sen ympäristövaatimuksiin, ja tietoa mahdollisista ruokavalion muutoksista tarvitaan, kun tutkitaan ympäristön muuttumisen, esimerkiksi ilmastonmuutoksen, vaikutuksia lajeihin. Liito-orava (<i>Pteromys volans</i>), on Suomessa vaarantuneeksi luokiteltu laji, joka elää havu- ja sekametsissä. Sen ravinnonkäytöstä tarvitaan lisää tietoa lajin suojelun tarpeisiin.</p> <p>Tutkielmani tavoite on selvittää aikuisten liito-oravien ruokavalion koostumusta ja monimuotoisuutta käyttämällä DNA-viivakoodausta niiden ulostepapanoihin. Menetelmällä saadaan tarkkaa tietoa papanoiden lajikoostumuksesta nopeasti ja tehokkaasti. Tutkimuskysymyksiäni olivat: vaihteleeko ruokavalio sukupuolten välillä eri vuodenaikoina, onko ruokavaliolla vaikutusta oravien kuntoon ja naaraiden poikasmenestykseen, ja onko ruokavalion monimuotoisuudella yhteys lajille sopivan metsän määrään pesien ympäristössä.</p> <p>Tutkimusryhmämme keräsi papananäytteitä 51 eri oravayksilöltä kahdella tutkimusalueella Vaasan ja Pietarsaaren lähistössä kesäkuussa 2020. Lisäksi näytteitä kerättiin 8 yksilöltä marraskuussa 2020 Vaasassa. Kerätyt näytteet lähetettiin laboratorioon Turkuun, missä DNA-viivakoodisanalyysi suoritettiin. Aineiston perusteella konstruoin tilastollisia malleja testaamaan tutkimuskysymyksiä käyttäen yleisiä lineaarisia malleja (glm).</p> <p>Vaikka aineisto oli liian pieni taatakseen tilastollisesti merkitseviä tuloksia kaikista tutkimuskysymyksistä, löydökseni viittaavat siihen, että liito-oravan ruokavalio eroaa sukupuolten välillä kuten niiden elintavatkin. Koiraiden ruokavalio on monipuolisempi kuin naaraiden, joilla on kapeampi ja erikoistuneempi ruokalajivalikoima, kuten niillä on myös koiraita pienemmät elinpiirit lisääntymisaikana lähempänä pesää. Naaraiden ruokavalion erikoistuminen pesintäaikana on tärkeä tieto suunniteltaessa suojelua. DNA-viivakooditutkimuksia suuremmilla näytemäärillä tulisi jatkaa ruokavalion monimuotoisuuden ja oravien kunnon suhteen selvittämiseksi tarkemmin ja tässä työssä tehtyjen havaintojen tilastollisen merkitsevyyden varmistamiseksi.</p>		
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<p>One of the major fundamental ecological questions is the composition of a species diet. The diet of a species is crucially linked to finding out its environmental requirements, and information about the possible changes in the diet is needed when studying the impact of environmental changes such as climate change on species.</p> <p>Siberian flying squirrel (<i>Pteromys volans</i>), classified as endangered in Finland, is a species living in coniferous and mixed forests. More precise information about the dietary habits of the species is needed to support conservation.</p> <p>The aim of my thesis was to investigate the diet composition and diet diversity of adult flying squirrels using DNA barcoding of their excrement pellets, a technique that provides highly accurate information quickly and effectively. The main research questions were whether the diet varies between sexes and seasons, whether diet has an influence on body condition and breeding success of the females, and whether diet diversity is related to the amount of suitable forest habitat near the nests.</p> <p>We collected faecal samples from 51 different flying squirrel individuals from two different study areas near the cities of Vaasa and Pietarsaari in June of 2020. Another set of samples from 8 individuals was collected in November 2020 in Vaasa. The collected samples were sent to a laboratory in Turku, where the DNA barcoding was conducted. I then made further statistical analyses from the laboratory results using general linear models to test my study questions.</p> <p>Although the sample size was too small to obtain statistically significant results for all the research questions, my results indicated that the diet of the Siberian flying squirrel differs between males and females just like its other living habits. Male flying squirrels have more diverse diet than female flying squirrels which have more specific and narrow diet, as they also have smaller home ranges during the breeding season and are more linked to their nesting forest patch compared to males. The aspect that female flying squirrels are more specialists during breeding time is crucial for the species conservation planning. DNA barcoding studies with bigger sample sizes should be done to further investigate the relationship between diet diversity and individual's body condition and to ascertain the statistical significance to the results of this study.</p>		
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# 1 Introduction

Biodiversity is declining rapidly worldwide because of anthropogenic drivers. To slow down this ongoing biodiversity loss, it is particularly important to fill in many remaining gaps of knowledge in species life-history and ecology (Pereira et al. 2012).

## 1.1 The importance of fundamental ecology

Fundamental ecological research has an essential role in understanding biotic nature and its prerequisites in complex and changing environments (Courchamp et al. 2015). Fundamental ecology has an intrinsic value in gaining knowledge and understanding, as ecological changes often need to be predicted before they are observed. Fundamental ecological research is driven by scientific curiosity rather than a single defined goal. Unlike applied ecology, it does not have a specified objective or purpose in finding a resolution for a certain problem. The funding of fundamental ecology has been reduced continually even though its continuous improvement is essential in the constantly changing world (Courchamp et al. 2015). Nevertheless, results from fundamental ecology are often necessary for future applied ecology studies.

One of the major fundamental ecological questions is the composition of a species diet (Sutherland et al. 2004). The diet of a species is crucially linked to finding out its environmental requirements, and information about the possible changes in the diet is needed when studying the impact of environmental changes such as climate change on species (Post & Forchhammer, 2007). The amount and the quality of available food affects the individual's condition and its reproductive success (DeGabriel et al. 2009). Individuals who get less and lower quality food produce fewer offspring than those that get more and high-quality food. The reproductive success of species can be negatively affected by trophic mismatch as the climate changes, and the peak of resource availability is not synchronised with the breeding time (Post & Forchhammer, 2007).

The availability and access to different plant species is particularly important for herbivores, as they need to get all the required nutrients from them (Westoby et al. 1978), and on the other hand must avoid ingesting excessive amounts of plant toxins (Dearing et al. 2000).

The Siberian flying squirrel (*Pteromys volans*) constitutes a flagship species for biodiversity conservation efforts in Finland, both as a long-time target of conservation efforts and as a bone of contention for ecological and cost-efficient policy. It has become something of a totem animal for conservation controversies in popular press. The case of the Siberian flying squirrel shows that ecological knowledge is needed to make conservation planning effective as well as economical. Detailed ecological knowledge on the species allows identification of evidence-based conservation options, which helps to balance diversity aspects and the interests of landowners and developers (Selonen & Mäkeläinen, 2017).

## **1.2 About the study species, *Pteromys volans***

Siberian flying squirrel (*Pteromys volans*), classified as endangered in Finland, is a species living in coniferous and mixed forests. It is a nocturnal, arboreal rodent that nests in tree cavities usually made by woodpeckers, man-made nest-boxes and twig nests in boreal forests (Selonen & Mäkeläinen, 2017).

Its summer diet is based on leaves of deciduous trees and it eats mainly buds and catkins from alder (*Alnus ssp.*) and birch (*Betula ssp.*) in the wintertime (Hanski 2016:27). The abundance of alder blooms has been detected to affect the size of summer litters (Selonen & Wistbacka, 2016). Although the Siberian flying squirrel (hereafter the flying squirrel) has been much studied in Finland, the information of its dietary preferences is based on observing living individuals in their natural habitats and microscope examinations of their faeces (Mäkelä, 1981; Sulkava, P., & Sulkava, R. 1993; Mäkelä, 1996; Hanski, 2016). These methods can now be complemented with modern DNA barcoding methods that provide fast and accurate results from larger sample sizes and can greatly contribute to advance the knowledge of this species' diet (Vesterinen et al. 2016 and Rytönen et al. 2018).

Arboreal squirrels including the flying squirrel are typically inhabitants of old forests and therefore threatened by intensive forest management and logging techniques. Flying squirrels are relatively slow breeders with low mortality and metabolic rate, which means that damages to populations are not as quickly visible and hard to reverse (Selonen & Mäkeläinen, 2017).

The Siberian flying squirrel lives in northern boreal forests. Its westward spread reaches Finland and the Baltic countries, where it is relatively rare. In Finland, the flying squirrel is distributed roughly from south-western to north-eastern central Finland. In Europe, the flying squirrel prefers mature mostly spruce forests mixed with deciduous trees, such as birch (*Betula spp.*), alder (*Alnus spp.*) and aspen (*Populus spp.*). In Finland, species occurrence increases with the average forest age, the volume of spruce trees (*Picea spp.*) and the occurrence of deciduous trees within the spruce forest (Santangeli et al. 2013b).

The area of mature forests near nesting sites is not crucial for successful breeding (Hoset et al. 2017). The preference for old growth forests may be more marked in northern border at the range. (Hurme et al. 2008a). The species can also occupy urbanized areas, such as small forest patches or parks in residential areas (Mäkeläinen et al. 2015).

For flying squirrels, deciduous trees, in particular birch, alder and aspen are important food sources. Conifers, pine (*Pinus spp.*) and spruce, are also foraged. The winter and spring diet is mainly catkins from alder and birch, which forms the main winter food (Selonen & Wistbacka, 2016). Mäkelä (1996) found from an analysis of fecal samples that it forms 80% of the diet in winter. Other studies suggest that alder catkins are preferred over birch when available (Sulkava P. & Sulkava R. 1993; Mäkelä 1996; Selonen & Wistbacka, 2016).

The flying squirrel collects stores of alder catkins in cavities, nest-boxes and on tree branches (Selonen and Wistbacka, 2016). Catkins develop in summer, and the flying squirrel starts to forage them in autumn. Catkins remain on the trees during winter, and individuals continue eating catkins during the following winter and early spring, when the catkins flower. When trees grow leaves in the beginning of May, they form the main diet for the flying squirrel during the spring and summer months (Mäkelä,

1996). The flying squirrel starts mating in the middle of March and first litters are born in the end of April. During pregnancy and breeding, females continue eating catkins and buds. Catkin harvests vary yearly. Reproduction success depends on the availability of birch and alder mast before breeding. In contrast, flying squirrel breeding success is less dependent on autumn harvest (Selonen & Wistbacka, 2016).

In addition to food, flying squirrel habitats must have suitable nest cavities. This affects the dispersal of the species and the spacing of adults in the habitats. The dependence on deciduous trees for cavities and food sources may help explain the observations that the flying squirrels prefer forest edges. The species is slightly female biased in size. The female is somewhat territorial, they are polygynous and promiscuous. They have 1-2 litters per year with avg. 2.4 young per litter (Selonen & Mäkeläinen, 2017)

Diet and habitat requirements are obviously interconnected. Diet analysis gives information about habitat requirements, and conversely, information of preferred habitats is an indication of what a species looks for nutrition. Optimally, nutrient use and availability should be studied simultaneously.

Santangeli et al. (2013b) made a nation-wide study of the habitats of the Siberian flying squirrel in Finland. Their models indicated that nation-wide, the species is currently most commonly found in central-western Finland occupying fragmented cultural areas where mature spruce forests are mixed with agricultural fields. While this does not prove that such habitats are optimal, it shows that the flying squirrel is able to adapt to modified landscapes. In accordance with Desrochers et al. (2003), flying squirrel occurrence seems to relate to forest edges. The highest relative densities were found in western and south-western Finland. The species appears less common in the eastern parts of the country. The combination of variables *mature spruce dominated mixed forest*, *suitable habitat patch density*, and *agricultural field* mostly affect flying squirrel density (Santangeli et al. 2013b).

Desrochers et al. (2003) found by radio tracking that flying squirrels preferably move in borderline strips between forest and field. Agricultural fields are ploughed in productive soil. The forest edges are light which favours flowering and fruiting plants. The edges between fields and forests favour deciduous trees such as aspen,

birch and alder, which are the flying squirrel's main food species according to Hanski et al. (2000b). The preference for spruce may be explained by better shelter and higher food resources provided by old spruce-dominated forests. Compared to the 1970's the species has decreased in Eastern Finland, having been relatively common in some areas. This may be related to changes in land use (Santangeli 2013b).

Hanski & Selonen (2009) studied the natal dispersal of flying squirrels by radio tracking in southern Finland during 1998-2004. They found out that the dispersal was sex-biased, and females dispersed further away than males. Females also left the nest 2 weeks earlier than males and thus were lighter in mass than males at the dispersal time. They suggested that young females leave the nest because they are subordinate to their mothers and need to find their own breeding site. They found no indication of competition among males or between males and females.

### **1.3 Aims of this study**

The aim of my thesis is to investigate the diet composition and diet diversity of adult flying squirrels using DNA barcoding of their excrement pellets, since this has not been done in a systematic way.

Specifically, I study:

- 1) Whether the diet varies between individuals, sexes and seasons?
- 2) Whether diet has an influence on body condition and breeding success of the females?
- 3) Whether diet diversity of flying squirrel is related to the amount of suitable forest habitat?

The materials and methods part of this masters' thesis reports on collecting data on the diet of identified adult flying squirrel individuals by means of DNA barcoding of pellets, a technique that provides highly accurate information quickly and effectively from large sample size. Together with other data collected in the field, it allows studying how the diet varies between individuals, males and females and seasons,

environmental factors, such as habitat availability, and the individuals' body condition and breeding success.

#### **1.4 Research environment**

The research was done in collaboration with my supervisors, Sanna Mäkeläinen (PhD, Postdoctoral researcher in The Helsinki Lab of Ornithology) and Andrea Santangeli (PhD, Postdoctoral researcher, Research Centre for Ecological Change) at the Finnish Museum of Natural History and University of Helsinki. The terrain work pertaining to collecting the pellets and measuring the individuals was done in collaboration with Ralf Wistbacka (MSc, University of Oulu) who is running longitudinal follow-up research on the species in Ostrobothnia. DNA barcoding was done in collaboration with Eero Vesterinen (PhD, docent of molecular biology, Sveriges lantbruksuniversitet SLU, Uppsala, Sweden), who has also developed methods to study animal diet from excrements of other species (Vesterinen et al. 2018). The materials and results of the research to which this report contributes will be presented in a scientific paper to be published in an international peer-reviewed journal.

#### **1.5 Background and previous work**

Flying squirrel diet has not been studied since the early 1990's, and there are next to no international scientific publications on the topic, although the species' diet choice is referred to in many scientific and normative publications. More precise information about the dietary habits of the species is needed to support conservation, especially as the flying squirrel has been classified as endangered in the most recent Finnish red-list assessment (Hyvärinen et al. 2019).

## 1.6 eDNA and metabarcoding

Environmental DNA (eDNA) is genetic material collected from environmental samples or from remains of organisms. eDNA is found among other things in faeces, skin, air, and in animal remains (Stewart 2019). eDNA has become an important analytic tool after the invention of high-throughput DNA sequencing methods. It is used in taxonomic research and in ecology to measure biodiversity and to analyse the interaction of organisms with their environments, such as diet and other ecological requirements (Thomsen & Willerslev 2015).

DNA barcoding is a method where species can be identified by DNA sequencing short (400–800 base pairs) species-specific sections from specific genes. The sequenced DNA code is compared to similar codes in a library of sequences collected from a large number of species (Savolainen et al. 2005).

Different gene sequences are used for different groups of organisms. For most animals the most common sequence is a part of the cytochrome c oxidase I gene (COI in short) in mitochondrial DNA (Valentini et al. 2009). Other sequences are used for fungi (ITS), plants (RuBisCO), and micro-organisms. When barcoding is used to identify many organisms from one sample, the method is known as metabarcoding.

After its introduction in the early 2000's, DNA barcoding has become an important tool for ecological research. It is used in conservation to trace the presence of endangered or indicator species, or to determine the presence or absence of nutrients or stressors (Valentini et al. 2009).

DNA barcoding is useful in diet analysis from excrements and other excretions, since it is non-invasive, fast, and precise. Metabarcoding from excrements may be the only practical method when the predator's or grazer's foraging habits are difficult to observe, and the different nutrient or prey species are no longer distinguishable in the faeces (Valentini et al. 2009).

DNA barcoding can help to find new species, but for ecologists it is useful for known species identification when combined with large DNA databases of already identified species. Such databases have been constructed during this millennium (Savolainen et al. 2005).

By 2015, DNA libraries had been built for 2000 endangered species and an additional 2 891 971 specimens, all in all almost 200 000 species (The Barcode Library, Barcode of Life Data Systems). The price for identifying species from samples is no longer a major limiting factor for research. Species databases have grown quickly since then. The Global Biodiversity Information Facility (GBIF) has 420 million records and 1.45 million species names (Global Biodiversity Information Facility; [www.gbif.org](http://www.gbif.org)). The plant database Tropicos ([www.tropicos.org](http://www.tropicos.org)) has 4.4 million plant records (Pimm et al. 2015).

An early dietary study using DNA barcoding was Kowalczyk et al. (2011) which studied the diet of the European Bison in Białowieża Primeval Forest. As this research indicated, diet is closely connected with the presence of favoured nutrients, but the relation between the variety of the observed diet with food availability and species requirements is complex.

Finnish researchers (Soininen 2009 and Vesterinen 2016 and 2018) have long been in the forefront of DNA barcoding research. Kaunisto et al. (2017) applied meta-barcoding to compare the diets of three odonate species of different sizes in the same environment. The availability of prey turned to be the decisive factor rather than prey size. Rytkönen et al. (2019) study on 4 bird species applied meta-barcoding to compare the distribution of prey species in predator faeces to the availability of the prey species in the environment.

## 2 Materials and Methods

In this chapter I describe the fieldwork and the data collection, the laboratory work done by Bioname OY in Turku on the DNA metabarcoding, and my statistical analyses combining both sources of data.

### 2.1 Fieldwork

The fieldwork to collect the data was conducted in Northern Ostrobothnia, Finland in collaboration by Sanna Mäkeläinen, Andrea Santangeli, Ralf Wistbacka and myself. We collected faecal samples from 51 different adult individuals from two different study areas near the cities of Vaasa and Pietarsaari during 9-16 of June 2020.

Another set of samples from 8 individuals was collected during 5-6 of November 2020 in Vaasa by Sanna Mäkeläinen and Ralf Wistbacka. These study areas have been monitored for flying squirrels since 1993 (Selonen & Wistbacka 2016). The studied flying squirrels live in man-made nest boxes (Figure 1, right). In the Vaasa region (Figure 2, below), (including Mustasaari municipality) the study area consists of spruce-forest patches, clear cuts, parks in residential areas and agricultural fields. The other area is in the municipalities of Luoto and Pietarsaari (Figure 2, above), where the examined nest boxes were in spruce dominated mixed forests in the shoreline, clear-cuts and cultivated pine forests (Selonen & Wistbacka 2016).



Figure 1 Left: Measuring the skull diameter. Right: A typical nest box

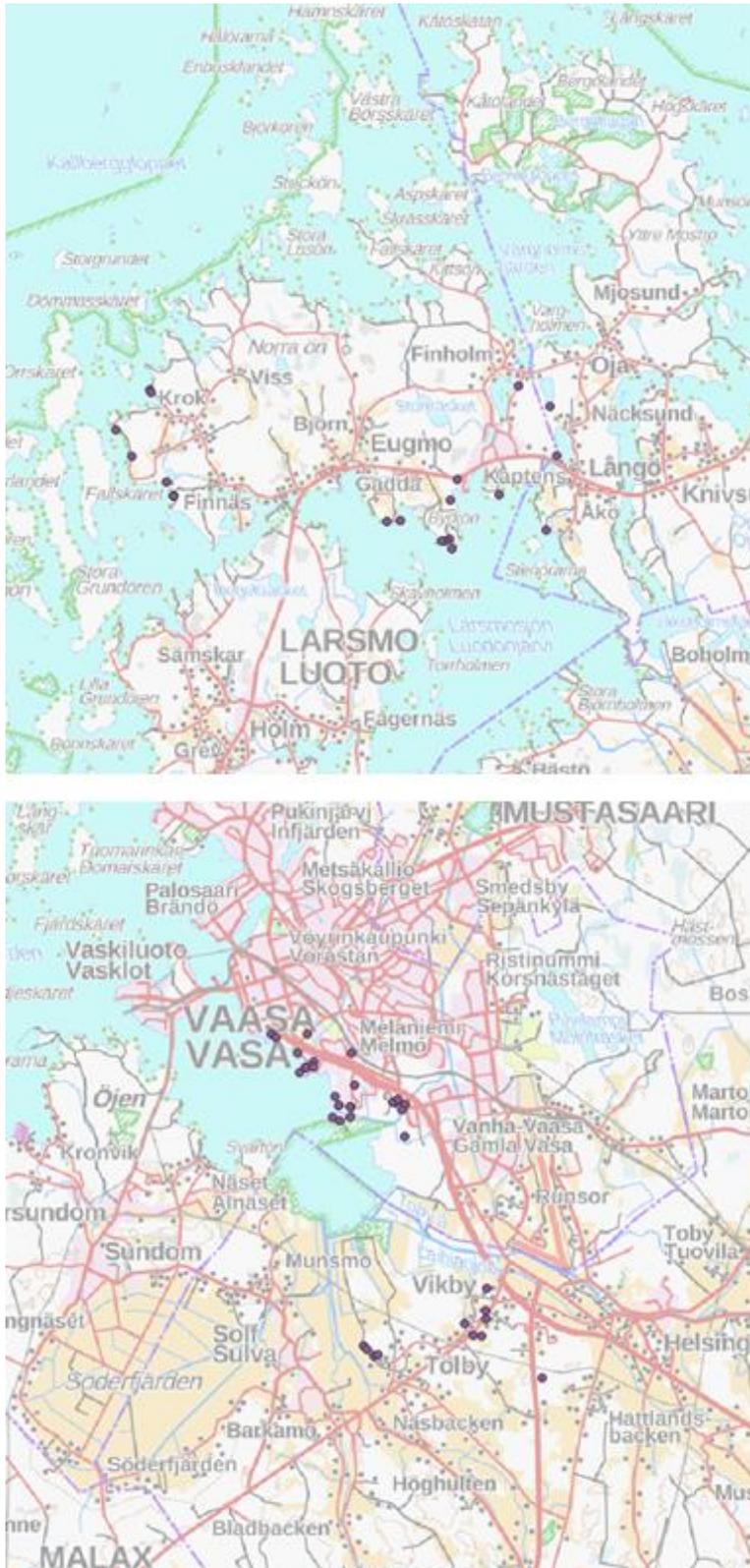


Figure 2 Maps of the field work areas in Luoto (above) and Vaasa (below) regions. Inspected nest boxes are marked with purple dots.

During the inspection of the boxes, new individuals were tagged and weighed by putting them in a bag individually and weighing the bag with a scale. The weight of the bag was then reduced from the total weight. The bag was also weighed regularly. The length of femur and the diameter of skull of individuals were measured with a vernier calliper. Also, their sex and age (adult/pup) were determined. Additionally, it was marked down how many pups every female individual had and if the females seemed to be pregnant or lactating. All the observations were written down for further analyses. In connection with the inspection, the pellets produced by the individual were stored in separate sample tubes in reagent (RNA-later). The samples were collected using rubber gloves to prevent contamination. Finally, the samples were stored in a freezer at -20 degrees Celsius until the start of the laboratory analyses in April 2021.

## **2.2 Laboratory work by Bioname OY**

Pellets (appr. 1-5 pellets per individual) were put in tubes containing reagent and sent to Bioname OY, a laboratory in Turku, where DNA barcoding and related bioinformatics were conducted during 11 weeks in the spring of 2021. The laboratory work consisted of 1) extracting the DNA using a reagent intended for faecal samples (QuiaAmp PowerFecale), 2) a polymerase chain reaction (PCR) phase to multiply the DNA for recognition, 3) preparation of a gene library (following Vesterinen et. al 2016) and DNA clean-up, and 4) sequencing the DNA, where the precise order of the nucleotides was determined. After the laboratory work, the DNA data was processed and analysed using statistical software and compared to international databases for definitive species identification.

Bioname OY processed a semi-scale next-generation sequencing analysis for the samples as we requested. Pellets had low diversity of dietary plants so sequencing depth was aimed to 10,000-50,000 reads per sample using one locus which matched the output. The average sequence depth per sample was almost 22,000 reads per sample. Bioname OY delivered final data tables ready for further statistical analyses and the raw sequence data for publications (Bioname OY report 2021).

### 2.3 Materials on suitable forest habitats

For evaluating suitable forest habitats, I obtained land cover data based on forest classification for the study areas by Wistbacka et al. (2018). The area described there as suitable habitat for breeding females is layered spruce dominated mixed forest with different sized and aged trees. The mean diameter of tree trunks is 16 cm, which means mature forest and the proportion of deciduous trees is over 5 percent.

### 2.4 Calculation of body condition and diet diversity indices

I constructed a data set containing the information collected in the field: every inspected individual's body mass, femur and skull length, the nest box location and number of pups for the females, including expert observations on the condition of the females. The fieldwork data on each individual was then combined with the DNA data received from the laboratory by Bioname OY.

I calculated a measure of the individuals' body condition using Scaled Mass Index (SMI) for each measured adult individual (Peig & Green, 2009). The Scaled Mass Index indicates the body condition of the individual as in the study by Santangeli et al. (2019). It scales the individuals body mass by the expected value as if all individuals had the same body size.

The scaled mass formula used was:

$$\hat{M}_i = M_i \left[ \frac{L_0}{L_i} \right]^{b_{SMA}}$$

where  $M_i$  is individual body mass,  $L_i$  is length and  $L_0$  sample average length. As exponent  $b_{SMA}$  I used 2. According to Peig & Green (2009) the value for vertebrates is lower than the cubic estimate (3). Value 2 coincides with body mass index (BMI) used for humans. Since the individuals' body length was not measured, I used the sum of femur and skull length as a substitute. I included SMI also for the females but removed ones that had been marked as probably pregnant or lactating in the field from tests involving body condition.

I also calculated a diet diversity index ( $H$ ) for each individual's diet using the Shannon diversity formula (Spellberg & Fedor, 2003). Since the quantity of DNA replicates is not known to indicate quantity of the item in the diet (Vesterinen et al. 2016), I chose to use the presence/absence of a food item, measured by the proportion of specimens containing it as  $p$  in the Shannon formula:

$$H' = - \sum_{i=1}^S p_i \ln p_i$$

## 2.5 Statistical analyses

After the plant composition at the genus level in the excrement pellets was determined, I ran statistical models using the `glmmTMB` package in R version 4.1.1, following statistical procedures described in Santangeli et al. (2020). In the statistical models I have assumed throughout normal (gaussian) distributions. I used logit link function when the result values were between 1 and 0.

I obtained a correlation plot (Figure 3) of all variables using the library `psych` and the command `pairs` in R, to see how the variables correlate and how to fit them into generalized linear models.

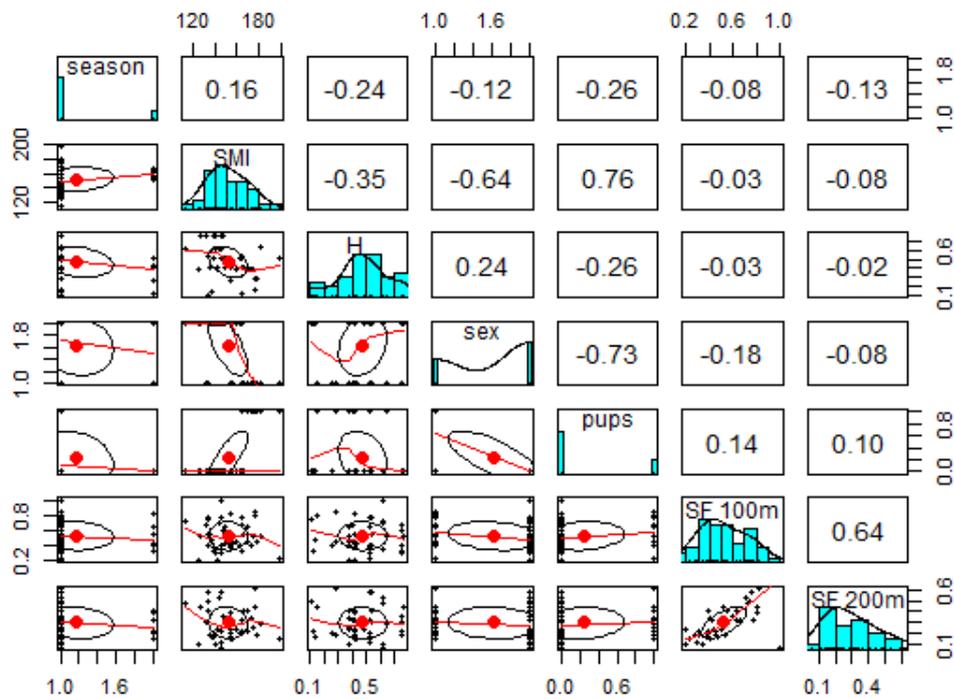


Figure 3 Correlation plot of all variables: season, body condition (SMI), diet diversity (H), sex, breeding success (pups), suitable forest habitat in within 100 meter radius (SF 100m) and suitable forest habitat within 200 meter radius (SF 200m). Sex and breeding success (pups) correlates with body condition (SMI). Suitable forest habitat within 100m radius (SF 100 m) is included in the 200m forest habitat (SF 200 m) and thus correlates strongly.

By diet composition I mean the presence and absence of different genera in the samples, determined from raw data. Diet diversity refers to the Shannon diversity index (H) calculated from the distribution, which is used as one variable in general linear models.

To examine the how diet diversity varies between sexes and seasons, I fit the variables into the generalized linear model using beta regression, because the response variable diet diversity was measured in the interval 0-1. The explanatory variables in this model were season and sex which are categorical variables (female/male and June/November). I also fitted the variable of sex into another model with only data from June since the sample size in November was so small (only 8 samples altogether).

Next, I studied whether the diversity of an individual's diet has an effect on its body condition (SMI) and on the breeding success of the females. The first part was addressed as following general linear model: body condition (SMI) ~ sex + diet diversity (H) + interaction term H\*sex, using data on individuals in June. I also tested the effect of season on SMI while controlling for sex and diet diversity, but this was not significant ( $z = 0.17$ ,  $p = 0.86$ ). The interaction term addresses the interdependence of diet diversity and sex.

To test the effect of diet diversity to the breeding success of females, I ran the following model: female breeding success (0 for no pups and 1 for pups) ~ diet diversity (females in June).

Before the analyses related to body condition, I checked for possibly pregnant females using field notes and removed them from the data (Santangeli et al. 2019). The suspected pregnant females were significantly heavier than the rest by Welch t-test. On this basis, I also excluded two of the heaviest remaining females as possible outliers.

Finally, I tested my third question, whether the diet diversity is related to the availability of suitable forest habitat in the neighbourhood of the individual within a radius of 100 and 200 metres from the nest box. I did this by fitting the following variables into two general linear models, since the two suitable forest habitat variables are nested inside each other and thus correlated strongly, using beta regression:

diet diversity (June) ~ suitable forest habitat within 100 meter radius (SF 100m) + sex + interaction term SF 100m\*sex

diet diversity (June) ~ suitable forest habitat within 200 meter radius (SF 200m) + sex + interaction term SF 200m\*sex

I excluded the November data from consideration in these tests to avoid complexity.

### 3 Results

#### 3.1 The diet composition revealed by DNA barcoding

We had in total 59 samples (equivalent to number of individuals). 51 samples were collected in June (27 from females and 24 from males) and 8 in November (4 from females and 4 from males). A total of 12 different candidate food item genera were found in the pellets of 59 individual flying squirrels by DNA barcoding. From these candidate food items I removed 4, (Sphagnum, Dyopteris, Polypodia and Mesangiospermae) since they were very rare, and it is highly likely that they are contamination from fieldwork and do not belong to flying squirrels' diet. The remaining food item genera were: Acer (Maple), Alnus (Alder), Betula (Birch), Malus (Apple), Picea (Spruce), Pinus (Pine), Populus (Aspen) and Salix (Willow).

As shown in Figure 4, the most common plant item found in the pellets of individuals was Pinus (found in 55 individuals' pellets) followed by Betula (found in 40 individuals' pellets) and the least common were Acer and Salix, found in pellets of two individuals, respectively. Alnus was found in 34 individuals' pellets and Populus in 35. Malus (apple) was found in 23 individuals' pellets. It is a new food item to be observed in the flying squirrel diet.

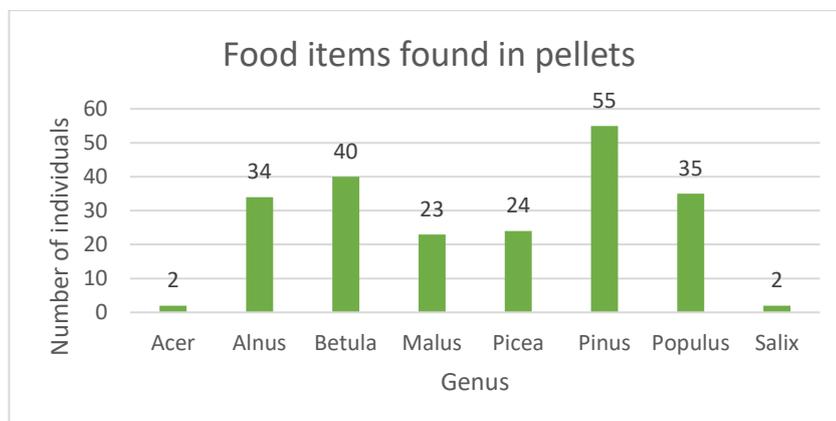


Figure 4 Food items (different tree genera) found in pellets by the number of individual flying squirrels. Figure is drawn from raw data.

### 3.2 Does the diet composition and diet diversity vary between individuals, sexes and seasons?

Here I show the results for my first study question: whether the diet varies between individuals, sexes and seasons.

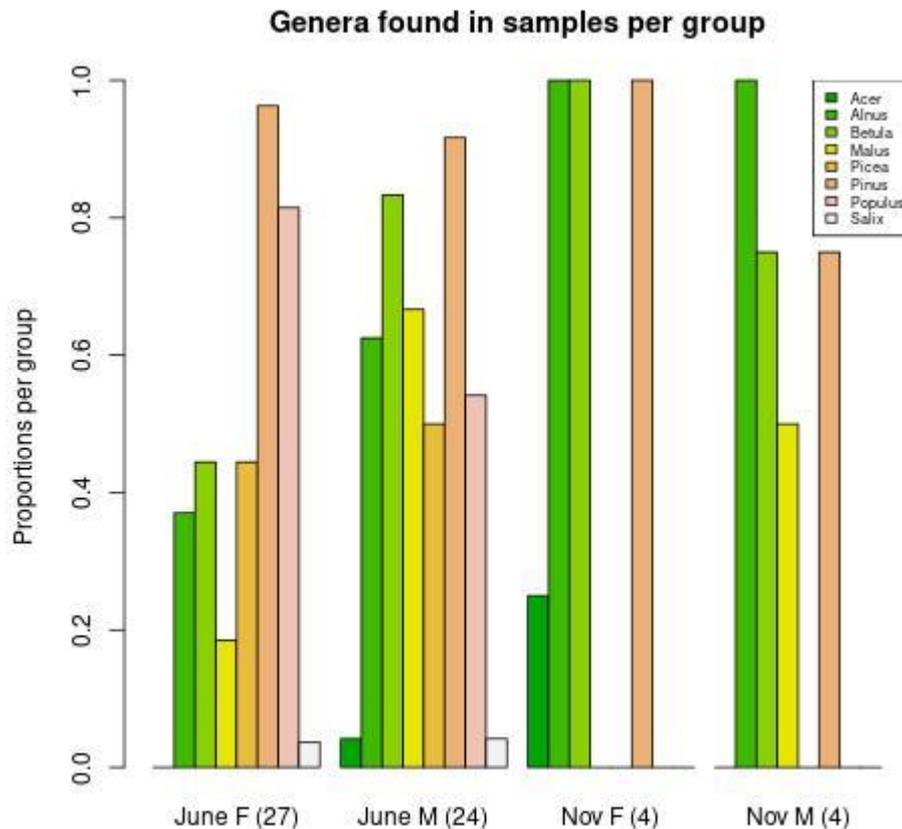


Figure 5 Food items found in pellets per group, the groups are females in June (June F), males in June (June M), females in November (Nov F) and males in November (Nov M). The bars show proportions per total of samples in each group. The values in brackets are the total number of samples in each group. Note that the fractions do not sum to 1, because the genera are not exclusive. Figure is drawn from raw data.

Figure 5 shows how the food items found in pellets varies between season and sex. Differences between males and females are marked in the summer but not so in the fall. The distribution by sex in our data is balanced (F 31/ M 29). New genus Malus seems to be more present among the pellets of males than the pellets of females (Figure 6).

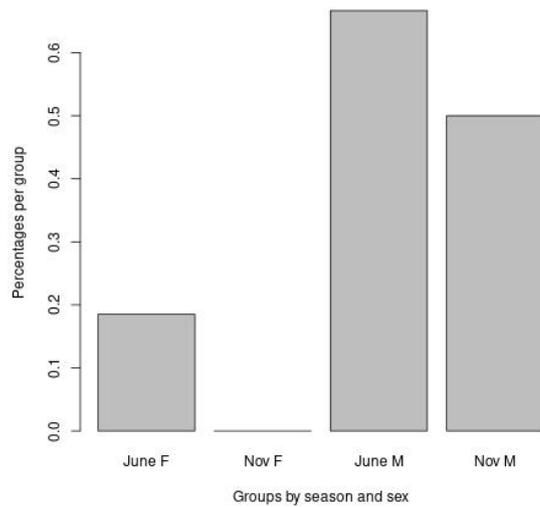


Figure 6 *Malus* (genus of apple) found in flying squirrel pellets by groups (June F = June females, Nov F = November females, June M = June males and Nov M = November males). Figure is drawn from raw data. *Malus* was found in 67 % of male samples in June, and in 50 % of male samples in November.

As seen in Figure 6, *Malus* (genus of apple) was found in 19 % (5/27) of female samples and in 67 % (16/24) of male samples collected in June. In November it was found in 50 % (2/4) of male samples and 0 % (0/4) of female samples.

Next, I tested with beta regression if the diet diversity (H) varies between sexes and seasons. As Figure 7 shows, the diet diversity was higher in June than November, but the effect of season was not significant (Table 1). The diet diversity seems to be higher in males than females (Figure 8, Table 2).

Table 1 Results of a general linear model where response variable is diet diversity of the flying squirrel and the explanatory variables are sex (F for female and M for male) and season (June and November). The model results are not statistically significant.

Variable	Estimate	SE	z	p
<b>Intercept</b>	-0.2088	0.1723	-1.212	0.225
<b>Season (Nov)</b>	-0.3732	0.2642	-1.413	0.158
<b>Sex (M)</b>	0.2587	0.2058	1.257	0.209

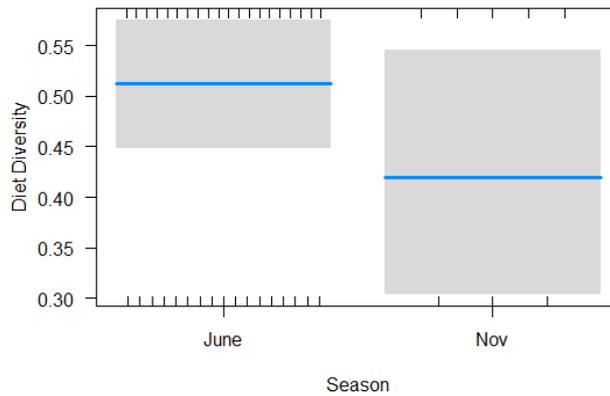


Figure 7 General linear model results from Table 1 suggest that diet diversity is higher in flying squirrel pellet samples collected in June than samples collected in November (Nov). Response variable is diet diversity, and the explanatory variables are season + sex. The grey areas show the 95% confidence intervals. Sample sizes are 51 for June and 8 for November.

Table 2 This table shows the modelled variables on the response variable diet diversity ( $H$ ) with data of flying squirrels from June. The explanatory variable is sex. The female is taken as the reference category, to which the effect of male is computed. The results are statistically significant.

Variable	Estimate	SE	z	p
<b>Intercept</b>	-0.2881	0.1245	-2.313	<b>0.027</b>
<b>Sex (M)</b>	0.3614	0.1808	1.999	<b>0.0456</b>

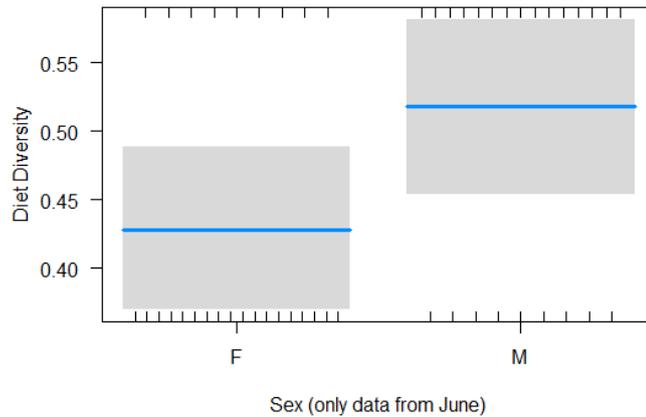


Figure 8 Figure is drawn from general linear model of diet diversity versus flying squirrel sex (F for females and M for males). Results showed in Table 2. Females have lower diet diversity than males in June. The grey areas show the 95% confidence intervals. Sample size is 27 for females and 24 for males.

### 3.3 Is diet diversity related to the body condition and breeding success of females?

In this section I study whether diet diversity (H) has an influence on the body condition (SMI) of all individuals in June and on breeding success of the females in June. As the Figure 9 and Table 3 show, diet diversity seems to be negatively correlated with the body condition of females in June but not with that of males in June.

Table 3 This table shows the effect of modelled variables on the response variable body condition (SMI) with interaction (sex\*diet diversity), using data only from June. Sex correlated significantly with body condition. The female is taken as the reference category, to which the effect of male is computed.

Variable	Estimate	SE	z	p
<b>Intercept</b>	190.879	9.943	19.197	<b>&lt;0.001</b>
<b>Sex (M)</b>	-48.467	12.617	-3.841	<b>&lt;0.001</b>
<b>Diet diversity (H)</b>	-51.638	21.506	-2.401	<b>0.016</b>
<b>Sex * Diet diversity</b>	48.881	25.688	1.903	0.058

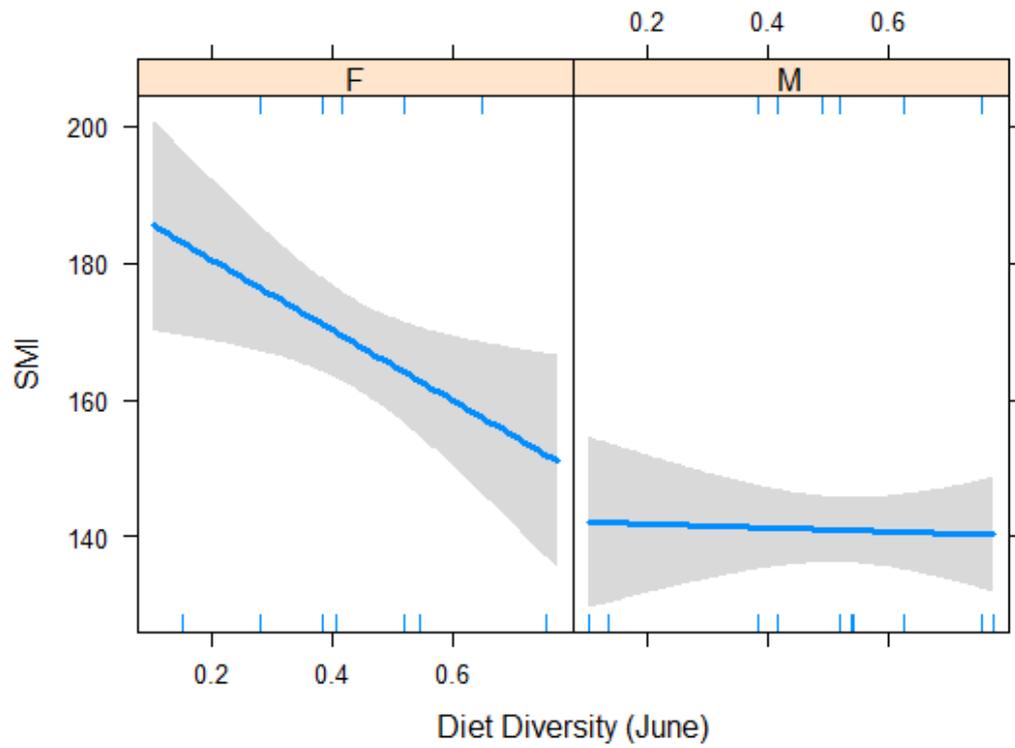
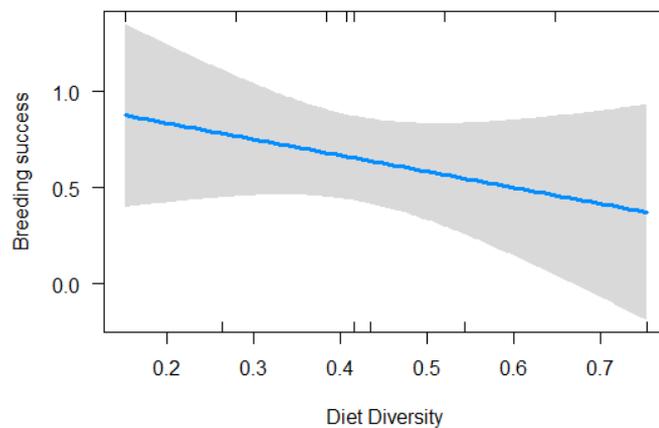


Figure 9 The body condition (SMI) is negatively affected by diet diversity in female (F) flying squirrels in June. The body condition does not affect the diet diversity in male (M) flying squirrels in June. The grey areas show the 95% confidence intervals. Figure is drawn from model results (Table 3). Sample sizes are 24 for males and 13 for females after removing the possibly pregnant ones.

Next, I tested whether diet diversity affects the breeding success of females. The breeding success is measured by 1 as having pups and 0 as not having pups.

*Table 4 The general model results where the response variable is breeding success (pups or no pups) of flying squirrel females and the explanatory variable is diet diversity (H). The sample size is 27. The model results are not significant.*

Variable	Estimate	SE	z	p
<b>Intercept</b>	1.0019	0.3554	2.819	0.00482
<b>Diet diversity (H)</b>	-0.8387	0.7971	-1.052	0.29272



*Figure 10 Breeding success of flying squirrel females is negatively related with diet diversity. Figure is drawn from general linear model results showed in Table 4. The model results were not significant. Data is shown by ticks in x-axis. The grey areas show the 95% confidence intervals. Sample size is 27 females in June.*

The model results were not significant (Table 4), but as seen in Figure 10, the breeding success (pups or no pups) of females is weakly negatively related with the diet diversity.

### 3.4 Does the proportion of suitable forest habitat affect diet diversity?

Finally, I tested my third study question: whether the proportion of suitable forest habitat affects diet diversity using data on all individuals in June. There was not a significant correlation between the diet diversity and suitable forest habitat within 100 meters (Table 5) or 200 meters radius (Table 6) when modelling with all data from June.

*Table 5 General linear model results explaining the diet diversity of flying squirrels in June with the proportion of suitable forest habitats within 100 meter radius (SF 100m). The explanatory variable is diet diversity and explanatory variables are SF 100m and sex. An interaction term (SF 100m \* Sex) was included in the model. The female is taken as the reference category, to which the effect of male is computed. Model results are not statistically significant.*

Variable	Estimate	SE	z	p
<b>Intercept</b>	0.07037	0.44797	0.157	0.875
<b>SF 100m</b>	-0.57125	0.73577	-0.776	0.438
<b>Sex (M)</b>	0.07926	0.56669	0.140	0.889
<b>SF 100m * Sex</b>	0.41937	0.97382	0.431	0.667

*Table 6 General linear model results explaining the diet diversity of flying squirrels in June with the proportion of suitable forest habitats within 200 meter radius (SF 200m). Diet diversity of female flying squirrels is negatively affected by the proportion of suitable forest habitat within 200 meter radius. The explanatory variable is diet diversity and explanatory variables are SF 200m and sex. An interaction term (SF 200m \* sex) was included in the model. The female is taken as the reference category, to which the effect of male is computed. Model results are not statistically significant*

Variable	Estimate	SE	z	p
<b>Intercept</b>	0.3405	0.3853	0.884	0.377
<b>SF 200m</b>	-1.8613	1.1283	-1.650	0.099
<b>Sex (M)</b>	-0.2802	0.4650	-0.603	0.547
<b>SF 200m * Sex</b>	1.9058	1.3621	1.399	0.162

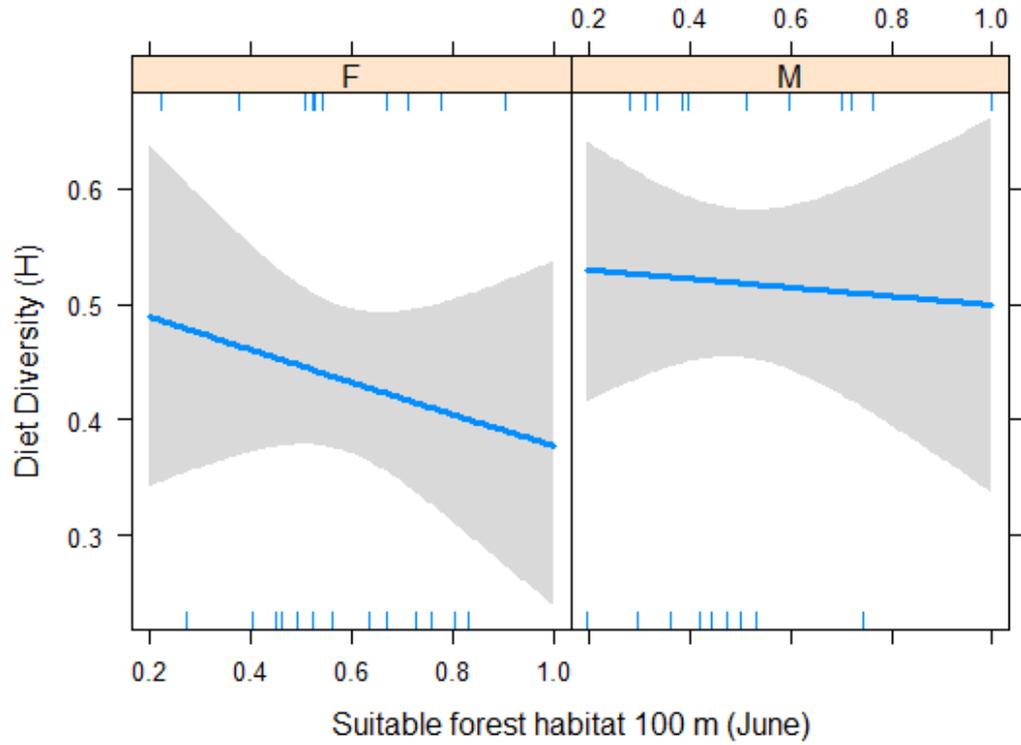


Figure 11 Diet diversity decreases with the suitable forest habitat within 100 meter radius especially in flying squirrel females (F for females and M for males). Figure is drawn from model results showed in Table 5. The grey areas show the 95% confidence intervals. The results were not significant. Sample sizes are 27 for females and 24 for males.

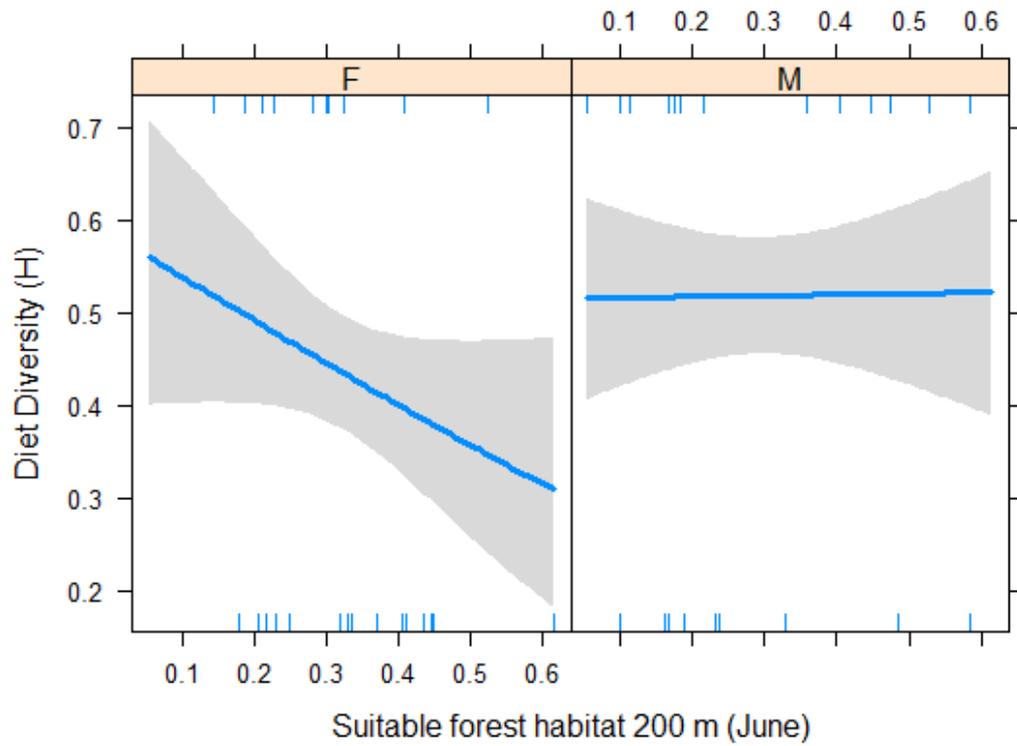


Figure 12 This figure shows how diet diversity ( $H$ ) decreases in female flying squirrels ( $F$ ) with the suitable forest habitat radius within 200 meters. For males ( $M$ ) the effect of suitable forest habitat proportion is not clear, but it seems to be rather increasing instead of decreasing. The grey areas show the 95% confidence intervals. Figure is drawn from model results showed in Table 6. Sample sizes are 27 for females and 24 for males.

## 4 Discussion

### 4.1 Interpretation of the results

In my data, the tree genera *Betula* and *Populus* were not found in the pellets of flying squirrels as frequently as expected from earlier studies on flying squirrel foraging. Instead, *Pinus* was found in almost all samples. This does not rule out that the individuals are still including all of them in their diets, because the sample size is so small. It is possible that *Betula* and *Populus* would have been present in other pellets from the same individual. One individual from November had two different pellet samples in two different tubes, one of them contained two genus (*Betula* and *Populus*) and the other three (*Betula*, *Populus* and *Pinus*). This shows that composition of food items can vary between pellets from the same individual. The reason could also be a seasonal issue if *Betula* and *Populus* are more in favour earlier in the season, or a difference in availability of tree species in the study area.

A total of 16 male individuals in June had foraged *Malus* compared to only 5 females that foraged *Malus*. There might be apple trees somewhere near the study area or the males move further from the nest box to eat them. Samples from Luoto and Pietarsaari contained relatively less *Malus* than those from Vaasa. The presence of *Malus* in the diet might be related to some individuals living in urban areas where apple trees are cultivated in gardens.

Half of the flying squirrels inspected in June had eaten *Picea* (Spruce) but no spruce DNA was found in the pellets in November. One explanation could be that the sample size in autumn was smaller than in June, only 8 samples altogether, and they were collected in only one study area in Vaasa. In the field study by Suorsa (1999) flying squirrels were observed eating pinecones in September. It was also observed that most of the summer diet consists of deciduous trees (*Betula* and *Populus*) and the proportion of coniferous trees (Pine) in their diet increases towards winter.

The number of DNA reads found in the pellets is not known to indicate the proportion of the food item in the individuals' diet (Vesterinen et al. 2016). Absence

from the small sample does not guarantee that the food item is not at all eaten by the individual. The differences in the frequency of the food items are more likely to become visible in larger sample sizes.

The results show that male flying squirrels have a more diverse diet than female flying squirrels. This goes in line with the idea that males have larger home ranges and travel further away to find females (Santangeli et al. 2013b). They have more time and opportunity to eat different kinds of trees. They are not necessarily constrained by the central place of the nest box like females. In this sense, my results support the central place foraging theory, where costs involved in foraging are related to travel to and from a central place like nest. My observations accord with the predictions of central place foraging theory that diet diversity should be inversely related to abundance of preferred food (Rockwood & Hubbell, 1987).

This study indicates more specific and narrow summer diet of the female flying squirrels, which have smaller home ranges during the breeding season and are more linked to their nesting forest patch compared to males that can roam around larger areas and are not necessarily linked to a particular forest patch (Santangeli et al. 2013b).

Naturally, in late autumn the diet is narrower than in summer for both sexes, since deciduous tree leaves have fallen.

Interestingly, body condition seemed to be decreasing with the diet diversity, especially for male flying squirrels. With this small sample size, it is difficult to tell the reason for this. Possibly, condition correlates positively with food abundance, and when food is abundant, there is less reason to vary the diet (Rockwood & Hubbell, 1987).

My results suggest that diet diversity seems to be weakly related to suitable forest habitat proportions near the nests, except for the female flying squirrels in June. A possible partial explanation to this is that the flying squirrel nest boxes are placed in known suitable habitats. The tendency for diet diversity to decrease with suitable forest habitat availability was at first surprising. An explanation for this could be that female flying squirrels might have a more diverse diet in less suitable habitats because their home range is even smaller in the breeding time, as they are in a hurry

to get back in the nest box to take care of the young. If the habitat surrounding the nest box is suitable, they have suitable foraging trees nearer and can use all the foraging time in one or two trees. If the habitat is less suitable, they need to travel further away and, on the way, might forage other trees as well. The diet diversity in male flying squirrels tended to slightly increase with the proportion of suitable habitat in 200 meters. This could be because the eating habits of males are different, and they travel further away from the nest boxes to forage (Santangeli et al. 2013a). On the way they can eat more diverse food items that they come across. Mäkeläinen et al. (2016) found out that male flying squirrels moved shorter nightly distances if their home ranges consisted of more fragmented, urban habitats. However, the relation of habitat fragmentation to diet diversity is not clear without further study.

## **4.2 Potential sources of error**

Contamination while collecting samples is possible, since the conditions in the field cannot be completely sterile, although we used rubber gloves and collected the pellets quickly and directly from the flying squirrel as they were produced. Errors could have been made during measurement of the individuals because it had to be done quickly in order not to disturb the wild animals longer than necessary. However, our team had years of experience in collecting these measurements, and the same person collected all these, minimising observer biases.

The most important potential reason for uncertainty is the small sample size of the pellets. Only 1-5 pellets were collected per individual, which is a small amount. Absence from the small sample does not guarantee that the food item is not at all eaten by the individual, as seen in the sample from November where two samples taken from one individual had different food item composition. Also, the number of individuals becomes small when divided into groups by sex and season, which might prevent significant patterns to emerge.

## **4.3 Future research**

DNA barcoding is a new method for the study of flying squirrel diet. With these new, accurate methods, it might be possible to find further new food items for the flying

squirrel. We found *Malus* (genus for apple) in 23 individuals' pellets, which is a previously unobserved food item in the flying squirrel diet. Further analysis may reveal specific seasonal preference for particular plants or trees that have been overlooked earlier and may prove relevant for conserving the species habitats.

It is not known for certain whether flying squirrels are fully herbivorous or if they eat some animal-based items as well. In some old literature of flying squirrels, observation cases of flying squirrels eating animal-based food items are mentioned (Hanski, 2016). In this study, DNA analysis was done only for plants.

In further study, it would be interesting to see whether DNA barcoding results give any indication of other than plant-based food items. For example, the red squirrel (*Sciurus vulgaris*) is known to eat animal-based food items, such as birds, eggs and insects but also fungi (Moller, 1983).

Another set of open questions concerns food choice vs. food availability. According to optimal foraging theory, an animal should optimise the nutritive benefits of each food item against the costs of finding or storing it (Vesterinen et al. 2016).

Rubino et al. (2012) wanted to find out why Eurasian red squirrels (*Sciurus vulgaris*) prefer spruce seeds over fir (*Abies*) seeds, even though fir seeds are more energy efficient. They found out that fir seeds contained more secondary metabolites which are toxic for squirrels in high amounts. It would be interesting to see if flying squirrels also seasonally prefer some tree species. This might be caused by high concentrations of secondary metabolites in some Finnish tree species.

In further studies it would be interesting to see if some individuals have to move further, i.e. have great home ranges to get particular food items; it is not obvious in the current situation that individuals get to eat what they like or would optimally prefer what they get to eat. This could be achieved by combining the diet analyses by DNA metabarcoding with GPS tracking of flying squirrel movement and space use.

According to optimal foraging theory, species are pickier when the food is abundant and less selective when it is scarce. Vesterinen et al. (2016), did not find any evidence that this would be the case with insectivorous bats. Further study is needed to know to what extent my findings for flying squirrels support the theory.

The present findings suggest that diet diversity is sex specific in the flying squirrel just like its other living habits.

Flying squirrel home range sizes and their habitat compositions have been studied by radio tracking the daily movements of individuals (Hanski et al. 2000a; Mäkeläinen et al. 2016). By estimating the availability and presence of food items within area around the nest that represents an average home range size for a male and female, it would be possible to investigate whether they prefer particular tree species. Such combined nesting and feeding maps would constitute a definite improvement on the nesting and resting places (Santangeli et al. 2013) considered in current conservation regulations.

In addition to my actual study questions, I tested how municipality (Luoto, Vaasa and Mustasaari) affected the diet diversity and it seemed to have influence. I wanted to test this because I noticed that municipality correlated with the average of suitable forest habitat. I also noticed that in maps from Natural research Institute (Luke 2021), the proportion of coniferous trees was much higher in Luoto area than in Vaasa area. My hypothesis is that Vaasa area would consist more suitable habitat for the flying squirrels considering that it has more deciduous trees. Diet diversity was higher in rural areas in Luoto and Mustasaari compared to residential areas of Vaasa. In the light of my previous speculation about diet diversity, this result might indicate that flying squirrels have adapted well to residential areas (Mäkeläinen et al. 2015). Residential areas have parks and yards which might have older trees and untouched nature compared to rural areas with commercial forests which are more affected by clear cuts.

## 5 Conclusion

In conclusion, this study indicates that the diet of the Siberian flying squirrel varies between males and females just like its other living habits. The results show that male flying squirrels have more diverse diet than females during breeding time. This goes in line with the idea that males have larger home ranges and travel further away to find females. They have more time and opportunity to eat different kinds of trees. Female flying squirrels have more specific and narrow diet, as they also have smaller home ranges during the breeding season and are more linked to their nesting forest patch compared to males. The aspect that female flying squirrels are more specialists during breeding time is vital for the species conservation planning. It is important to retain the favoured tree species for the females to be able to settle and breed successfully. More DNA barcoding studies with bigger sample sizes should be done to further investigate the relationship between diet diversity and individual's body condition and to ascertain the statistical significance to the results of this study.

## 6 Acknowledgements

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