A multifaceted approach to the study of plant-eating in feline carnivores

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Chapter 1

General Introduction

1.1 Importance of dietary traits in animal studies

Animals need food to maintain their metabolism, provide energy for various activities, and support reproduction, including feeding their offspring (Pyke, 2010). The behavior, morphology, and physiology of animals are strongly linked with their dietary traits, since efficient feeding leads to increased fitness (Boag & Grant, 1981; Clauss et al., 2008; Janson & Boinski, 1992; Pyke et al., 1977; Stevens & Hume, 1998). Darwin's finches are a classic example of adaptations to different food resources in the form of diversification in beak sizes and shapes, and the resultant feeding habits (Grant & Grant, 2002). Appreciating trophic interactions is essential for assessing the ecological niche of a target species and further for understanding the structure and dynamics of ecosystems (Machovsky-Capuska et al., 2016; Thompson et al., 2012). Investigating diet has long been and continues to be one of the initial steps in studying a species' basic ecology.

1.2. Dietary adaptations in mammals

Contemporary mammals display extraordinary diversity, having adapted to occupy nearly every available ecological niche (K. E. Jones & Safi, 2011; Pineda-Munoz & Alroy, 2014). Additionally, they play a crucial role in the dynamics of the ecosystems in which they reside (Sinclair, 2003). Since mammalian biomass is large relative to their populations, they have significant impacts on the physical structure of habitats, rates of ecosystem processes, and the diversity of communities (Sinclair, 2003). Many researchers have attempted to analyze the morphology, ecology and behavior of mammals based on their diet. Mammals are roughly categorized into three groups according to their diet: carnivores, omnivores, and herbivores.

Plants are the primary food source of herbivores and are a relatively abundant resource in the environment. However, the composition of plant tissue is quite different from that of animals. Contrary to animal cell, which mainly consist of proteins and lipids, plant cell walls are rich in structural carbohydrates, especially cellulose, which is difficult for animals to digest (Karasov & Douglas, 2013; Tomme et al., 1995; Watanabe & Tokuda, 2001). In addition, many plants contain a variety of chemical compounds as an anti-predatory defense strategy (Dearing et al., 2005; Mithöfer & Boland, 2012). Phenolics are one of the ubiquitous defense compounds and known as deterrents for mammals (Levin, 1971; Mithöfer & Boland, 2012). Another feature of plant-based diet is that the nutritional quality of plant species varies with phenology. For example, in alpine environments, high-quality, sprouting graminoids generally deteriorates their nutritional quality quickly during summer, while forbs often retain their high quality for a longer period until autumn (R. Shrestha et al., 2005).

Herbivores must adapt to these features of plants to meet their nutritional needs from a plant-based diet. Thus, herbivores have developed special digestive systems (Hofmann, 1989; Vallentine, 2001). Their dentition is adapted to cut and/or grind structural polymers of plant cell walls (Reilly et al., 2001). Since vertebrates lack the capability for intrinsic cellulase production, they rely on fermentative symbioses with microbes and fungi for cellulose degradation (Karasov & Douglas, 2013). Some herbivores have evolved specific gastrointestinal structures to support a microbiota and slow the flow of digesta through the tract, allowing adequate time for the cell wall, which is slow to be digested, to break down sufficiently (Karasov & Douglas, 2013). They have developed detoxification mechanisms as well. Stomachs of ruminating mammals are alkaline and degrade a wide variety of secondary plant compounds (Freeland & Janzen, 1974). Their rumens can function on a diet of up to 50% plants containing high concentration of terpenoids and phenols (Freeland & Janzen, 1974). In response to the seasonal fluctuation of plant nutritional properties, herbivores adjust diet selection to optimize nutrient intake (Kohl et al., 2015; Westoby, 1978). In the Tianshan Mountains of China, forbs offer more protein and energy than graminoids during the warm season, and are preferred by the Siberian ibex (*Capra sibirica*). Conversely, during the cold season, graminoids constitute a significant portion of the ibex's diet (Han et al., 2020).

In contrast, carnivores have predatory and scavenging feeding strategies. Although cost to obtain them is relatively high, feeding on other animals is nutritionally more efficient than eating plants, since the chemical composition of the food item is quite similar to that of the consumer (Hayami, 1967). Additionally, the nutrient content of prey is considered to be relatively more consistent compared to plant food (Kohl et al., 2015). Carnivory has evolved repeatedly in a number of mammalian clades (Van Valkenburgh, 1991). For example, saber-toothed predators evolved in the families Felidae, Nimravidae, Thylacosmilidae, and Hyaeodontidae (Van Valkenburgh, 1991). Van Valkenburgh (1999) defined carnivores as species whose diet comprises at least 50 percent vertebrates, potentially ranging up to 100 percent. Carnivores have morphological and physiological traits characteristic of this diet. For instance, their dentition is better suited to slicing (Hamper et al., 2012; Van Valkenburgh, 1991), and their digestive tracts are shorter than those of herbivores (Stevens & Hume, 2004) owing to a decreased requirement for fermentation when digesting animal tissue as opposed to plant tissue. Additionally, taste receptor function is altered in many carnivores; there is a loss of sensitivity to sugar in fruit, heightened sensitivity to amino acid, and lower tolerance to bitter compounds (Bosch et al., 2015; P. Jiang et al., 2012; Kim et al., 2016; D. Li & Zhang, 2014; McGrane et al., 2023).

1.3. Introduction of felids

The family Felidae (felids), which includes 41 extant species (Kitchener et al., 2017), is a unique mammalian group whose members are all obligate carnivores, whose diets consist almost entirely of animal flesh and requires nutrients only found in flesh (Bradshaw, 2006; Legrand-Defretin, 1994; McGrane et al., 2023; Morris, 2002; Van Valkenburgh, 1989, 1991). Animals possessing feline traits emerged around 30 million years ago (MYA), but ancestors of extant cat species did not become apparent until the mid-Miocene period, approximately 15-20 MYA (Morris, 2002). Dental and other characteristics indicate that these animals evolved into obligate carnivores at least 15 MYA (Morris, 2002). A genome-wide phylogenetic study has indicated that each felid lineage originated in and dispersed out of Asia in the late Miocene (G. Li et al., 2016). According to Li et al. (2016), the ancestors of the Puma and Lynx lineages likely dispersed simultaneously to the Americas around 5.9 MYA, facilitated by the reopening of the Bering Land Bridge (G. Li et al., 2016), as evidenced by the subsequent first occurrence of fossil Felinae in North America (Qiu, 2003). Divergence times older than 10 MYA for the progenitors of the Caracal and Ocelot lineages suggest that the ancestors of these two lineages might have dispersed out of Asia into Africa and the Americas, respectively, via land bridges that were established earlier in the Miocene (Haq et al., 1987; Koufos et al., 2005). Throughout history, felid species have come to inhabit every continent except Australasia and Antarctica, and they are present on numerous islands (Johnson et al., 2006; Macdonald et al., 2010). There is 41 extant species (Kitchener et al., 2017), and they inhabit a diverse range of habitats, including boreal and tropical forests, savannahs, steppes, and deserts. However, many, especially the smaller tropical species, specialize in forest environments, with 32 species occurring in closed forest and woodland habitats (Johnson et al., 2006; Macdonald et al., 2010). As predators, felids often play regulatory role in the ecosystems they inhabit (Loveridge et al., 2010; Ripple et al., 2014). Felids, especially large species, provide economic and ecosystem services through direct and indirect pathways that help maintain the abundance or richness of mammals, birds, invertebrates, and herpetofauna (Ripple et al., 2014). Moreover, they influence other ecosystem processes and conditions, including disease dynamics, carbon storage, stream morphology, and crop production (Ripple et al., 2014).

The morphology of felids is highly adapted for carnivory, encompassing the traits of carnivores introduced previously. In detailing the characteristics of the felids, it is notable that they are metabolically adapted for a faster metabolism of proteins and a lower utilization of carbohydrates, as expected from their carnivorous diet (Kim et al., 2016; Zoran, 2002). For example, while adult domestic cats (*Felis catus*) require two to three times more protein in their diet than adults of omnivorous species (Morris, 2002), they lack salivary amylase, the enzyme responsible for initiating carbohydrate digestion (McGeachin & Akin, 1979). In addition, cats also have low activity of intestinal and pancreatic amylase and reduced activities of intestinal disaccharidases that break down carbohydrates in the small intestines (McGeachin & Akin, 1979; Zoran, 2002). Comparative genomics supports low level of amylase is common among the Felidae family (Kim et al., 2016). Although cats can use simple sugars, high amounts of carbohydrates in diets causes decrease protein digestibility (Zoran, 2002). Empirical studies showed that cellulose, a structural component of plant cell walls, reduced the digestibility of dry matter [(dried weight of intake – dried weight of feces)/dried weight of intake] in Amur leopard cat (*Prionailurus bengalensis euptilura*) and Turkmenistan caracal (*Caracal caracal michaelis*) (Edwards et al., 2001) and energy in domestic cat (Prola et al., 2010). Felids are likely more vulnerable to plant toxins as well. Phenolic compounds are among the most common defense chemicals in plants (Mithöfer & Boland, 2012). Pseudogenization of the UGT1A6 gene, the main enzyme for deactivating phenolic compounds, has been observed in 18 felid species (B. Shrestha et al., 2011). This pseudogenization is considered to reflect the loss of selection pressure for detoxifying various defensive chemicals found in plants, as ancestral felid species transitioned from a generalized diet (including both plant and animal sources) to a more specialized diet (exclusively animal-based) (B. Shrestha et al., 2011).

1.4. Plant-eating in felids

Interestingly, feces of these obligate carnivores have been reported to contain plant materials. Domestic cats are often observed eating plants with no apparent nutritional advantage. Edible plants for domestic cats (often grasses from the Poaceae family) are commonly sold as "cat grass." Not only the domestic species, felids in the wild are known to eat plants. In north-east Nepal, it has been reported that Tamaricaceae species were found in 9.6% of leopard (*Panthera pardus*) feces and 14.2% of snow leopard (*P. uncia*) feces during cold months, and in 6.8% of leopard feces and 12.0% of snow leopard feces during warm months (Lovari et al., 2013). In southern Brazil, plants were found from 20 to 67% of the feces from four felid species; ocelot (*Leopardus pardalis*), northern tigrina (*L. tigrinus*), puma (*Puma concolor*) and jaguarundi (*Herpailurus yagouaroundi*) (Rocha-

Mendes et al., 2010). Not only these indirect evidences, camera traps in Costa Rica captured plant consumption by wild jaguar (*P. onca*), puma and ocelot (*L. pardalis*) (Montalvo et al., 2020). In Kyrgyzstan, a video was captured of a wild snow leopard eating branches from a bush (Figure 1-1; Kinoshita et al., unpublished data). As aforementioned, morphology and physiology of felids do not suit to derive nutrition from plants, rather plant intake can have untoward effect on them. Therefore, the behavior of felids eating plants is indeed puzzling. It is hypothesized that they derive some nutrition from plants, particularly from fruit (Xiong et al., 2016); that the chemical compounds in plants serve as medicine for parasites or diseases (B. L. Hart, 2008); or that ingested plants help in the evacuation of hairballs and undigested materials (Herbst & Mills, 2010; Shultz, 2019). However, these hypotheses have not been tested.

Few studies have focused on this enigmatic plant-eating behavior, and as a result, basic information has not been accumulated. Investigating the plant-eating behavior of felid carnivores leads to a deeper comprehension of the evolution of dietary traits, especially the reasons behind their consumption of what appears to be unsuitable food. Additionally, this understanding prompts a re-assessment of the role of plants in the lives of carnivores as well as the behavioral ecology of the target species.

In this series of studies, I aimed to lay the groundwork for researching plant-eating behavior in felids. I employed a range of approaches, literature reviews to molecular-based analysis to gain insights into the relationship between feline carnivores and plants. In Chapter 2, I compiled existing information on plant-eating across all extant felid species from published sources. Then, I evaluated variations in the frequency of plant occurrence in feces and stomach contents and examined its relationship with various factors. In Chapter 3, I explored hair evacuation hypothesis, one of the primary hypotheses for the adaptive significance of plant-eating in felids. The relationship between plant intake and hair evacuation in captive snow leopards was investigated using behavioral observation and fecal analysis. In Chapter 4, I identified the dietary plant repertoire of wild snow leopards using a DNA metabarcoding approach and found out the characteristic of plant consumption of snow leopards in comparison with other sympatric mammals. I also studied the relationship between prey vertebrates and plants in the fecal samples. Additionally, I evaluated the sex differences of dietary plant composition in snow leopards. These multifaceted studies will open numerous research avenues into the plant-eating behavior of feline carnivores.

Figure 1.1. A snow leopard eating plants in Kyrgyzstan. Original video was from Kinoshita et al. unpublished data.

Chapter 2

Plant-eating carnivores: multi-species analysis on factors influencing the frequency of plant occurrence in obligate carnivores

2.1 Introduction

As mentioned in Chapter 1, felids are known to consume plants despite their predominately carnivorous dietary traits. Yet, researchers' interpretations of the presence of plant tissues in fecal samples or stomach contents are varied, possibly owing to the difficulties associated with observing this plant-eating behavior and because the amount of plant content present in these samples is often small. Some researchers believe that the presence of plant content is caused by unintentional intake (Avenant & Nel, 2002; de Villa Meza et al., 2002; Krofel et al., 2011), while others argue that there might be some advantages of plant eating (Hoppe-Dominik, 1988; Sueda et al., 2008; Tatara & Doi, 1994; Xiong et al., 2016). Indeed, observational studies indicate that felids eat plants voluntarily (Montalvo et al., 2020; Yoshimura et al., 2020) both in captivity and in the wild, which indicates that this behavior is relatively common and natural among felids. However, experimental studies suggest that cellulose intake can negatively affect energy absorption from food (Edwards et al., 2001; Prola et al., 2010). In addition, because of pseudogenization of the gene encoding a specific detoxification enzyme, felids are unable to detoxify phenolic compounds found in plants (B. Shrestha et al., 2011). Therefore, there may be some advantage for the existence of plant-eating behavior in felids. Currently, three major hypotheses have been proposed to explain the adaptive significance of plant-eating in carnivores as briefly mentioned in Chapter 1. First is the self-medication hypothesis (B. L. Hart, 2008). Many animals are known to use plants to counter parasites or diseases (B. L. Hart & Hart, 2018; Huffman, 2003; Huffman & Canon, 2000). Hart et al. (2021) reported in a questionnaire survey of owners of domestic cats that younger cats ate plants more frequently, and the authors suggested that plant consumption may be a way for individuals with low immunity to fight parasites. Second is the hair evacuation hypothesis (Shultz, 2019; Yoshimura et al., 2020). Functional carnivores often ingest their own hair while grooming, as well as the hair of their prey. Ingested plants are considered to aid in excreting hairballs (Herbst & Mills, 2010). Third is the food source hypothesis. DNA extracted from leopard cat feces included *Solanum* and *Rosoideae* species that produce berry fruits rich in sugar and nutrients (Xiong et al., 2016). Although the replacement of animal food by fruit may be subject to physiological constraints (Larivière et al., 2001), fruit may help obligate carnivores endure starvation or periods when prey animals are scarce.

Currently, knowledge about the plant-eating behavior of felids is scarce, and no comprehensive multi-species analyses have been performed. In this study, we attempted to explore and investigate factors that drive plant-eating behavior of felids in order to understand the common features of this unique behavior among felid species. To clarify whether plant eating is conserved through the evolution of Felidae, we need to evaluate the relationship of this behavior with phylogenic history. Environmental factors also need to be considered since Felids are widely distributed throughout diverse habitats (Johnson et al., 2006; Kitchener et al., 2017). In addition, given that the body mass of animals affects their diet (Carbone et al., 1999; Kleiber, 1947), its effect should be examined as well. Therefore, in this study, we focused on the aforementioned factors to elucidate their relationship with the frequency of plant consumption in extant felid species.

2.2 Materials and Methods

2.2.1 Literature search

A literature search using Web of Science (www.webofknowledge.com) was conducted on 15 September 2020 with the following keywords: "[common name of each species]" OR "[scientific name of each species]" AND "diet" OR "food". Target species were all 41 extant felid species. Common names and scientific names were obtained from the

International Union for Conservation of Nature (IUCN)/Species Survival Commission (SSC) cat specialist group (Kitchener et al., 2017). This search returned 4,100 research articles. The final output was based on the following exclusion criteria: review articles, captive studies (including domestic cats), studies that were not based on feces or gut contents (e.g., an isotope study using body hair), and non-comprehensive studies (i.e., covered only specific food items). To assess the extent of variation in the frequency of plant occurrence in the diet of carnivores, we additionally sorted these studies according to the following exclusion criteria: sample size of less than 10, and species for which no studies calculated the frequency of plant occurrence. We separated data on fruits and other plants because fruits are different from other plants in terms of energy contents and other nutrients. We only analyzed the data of non-fruit plants because the data of fruits was too scarce to be analyzed by itself. In all, 316 records from 213 studies of 24 felids (some references included records of several species) were used in the analyses.

2.2.2 Environmental factors

We included six environmental attributes: island, mean monthly precipitation, mean maximum daily temperature, mean minimum daily temperature, mean monthly normalized difference vegetation index (NDVI), and season (spring, summer, autumn, winter, dry, wet). In addition, we added sample type (feces or the digestive tract) because the remains present in the digestive tracts may be greater or lesser than those present in single feces and may not be directly comparable. Precipitation, and temperature represent climate parameters of the habitat of subject animals. Since obligate carnivores live in diverse habitats, we added these factors to know whether frequency of plant occurrence relates to specific habitats. Animals on islands often show unique traits due to limited habitat and resources (Foster, 1964), therefore, we added "Island" as a binary variable, which reflects whether the sampling site was an island or mainland including a large island with area over 10,000 km^2. We attempted to determine the effect of the abundance of vegetation on the frequency of plant occurrence in carnivores' feces and stomach contents through NDVI. Season is mainly characterized by precipitation and temperature; thus, we used the mean values of the studied season for monthly precipitation and daily temperature to consider the seasonal difference. In case there is seasonal difference independent of precipitation or temperature, we added seasons as binary variables. Climate data were obtained from the MeteoBlue database (Cano-Cruz & López-Orozco, 2015). NDVI data from the Moderate Resolution Imaging Spectroradiometer (MODIS) onboard the Terra satellite were obtained using AppEEARS (AppEEARS Team, 2019). MODIS satellite was launched in 2000; therefore, we used the data from the oldest year available for the 89 records that started sampling before 2000. The variable mean monthly precipitation was normalized (scaled into a range of 0–1) to help the convergence of Markov chain Monte Carlo (MCMC) sampling. For further details about the collection of environmental data, see the Appendix 2.3.

2.2.3 Phylogenetic factors and body mass

Phylogeny of felids was based on Li et al. (2016). To test the phylogenetic signals in the mean frequency of plant occurrence in each species, phylogenetic eigenvector regression (PVR) was conducted (Diniz-Filho et al., 1998). After extraction of pairwise phylogenetic distances from the branch duration information, the distance matrix was subjected to a principal coordinates (PCo) analysis. Following a broken-stick model (Diniz-Filho et al., 1998; Sakamoto et al., 2010), the first to fifth PCo axis (phylogenetic eigenvector 1-5, PV1-5) was retained. These five axes cumulatively explained 86% of the total variance and were included in the analysis as predictor variables for measuring phylogenetic similarity. Additionally, log-transformed body mass values were included as species-specific factors. Body mass data of all species were according to Sakamoto et al. (Sakamoto et al., 2010). Since data concerning the body mass of the African wildcat (*F. lybica*) were absent, we used the same value as that for the European wild cat (*F. silvestris*), according to International Society for Endangered Cats Canada (International Society for Endangered Cats (ISEC) Canada, 2020).

2.2.4 Statistical analysis

All analyses were performed in R v.3.6.1 (R Development Core Team 3.0.1, 2019). To explain the number of samples that contained plant materials in each study, we constructed two-part binomial (TPB) models. Since the frequency of plant occurrence has not always been reported in dietary studies on carnivores, several records in our dataset lacked values for frequency of plant occurrence. If we ignore records with missing values and apply ordinary regression models, it is likely to lead to imprecise estimation of parameters (Minami et al., 2007; Minami & Lennert-Cody, 2013). Two-part models are considered to be effective when dealing with data with many zero values or data generated from a combination of different mechanisms (Barry & Welsh, 2002; Matsuura, 2016; Minami et al., 2007; Minami & Lennert-Cody, 2013; Welsh et al., 1996). We assumed that the absence of reported plant material did not necessarily indicate that no plant material was found in the samples, as some reports mentioned that they ignored plant materials in feces or stomach samples (e.g., Móleon and Sánchez (Moleón & Gil-Sánchez, 2003), Silva-Pereira et al. (Silva-Pereira et al., 2011), Abreu et al. (Abreu et al., 2008)).

Specifically, our models assumed that the frequency of plant occurrence has not always been reported irrespective of whether the samples included plant materials, and that the probability of reporting the frequency of plant occurrence follows a Bernoulli distribution with a parameter ψ. Thus,

$$\mathrm{TPB}(y_i, N_i, \psi) = \mathrm{Bernoulli}(0|\psi) \text{ if } y_i = \mathrm{NA},$$

$$\mathrm{TPB}(y_i, N_i, \psi) = \mathrm{Bernoulli}(1|\psi) * \mathrm{Binomial}(y_i|N_i, p_i) \text{ if } y_i \neq \mathrm{NA},$$

where y_i is the number of samples that contained plant materials, N_i is the sample size, and p_i is the frequency of plant occurrence in record *i*.

2.2.4.1 Model 1: Variation in the frequency of plant occurrence in obligate carnivores

In this model, we assumed that the extent of intraspecies variation in the frequency of plant occurrence differs between species. Thus,

$$y_i \sim \mathrm{TPB}(\psi, N_i, p_i),$$

$$logit(p_i) = \alpha_j + \tau_i,$$

$$\tau_i \sim \mathrm{Normal}(0, {\theta_j}^2),$$

where α_j represents the mean frequency of plant occurrence in species j, τ_i represents the random effect which explains the overdispersion between records, and θ_j is a hyperparameter vector with a length of the number of species (Appendix 2.2).

2.2.4.2 Model 2: Environmental and non-environmental factors affecting variation in the frequency of plant consumption in obligate carnivores

In this model, we explored the factors that affect the frequency of plant occurrence observed in each study. We assessed the effect of each variable using an approach similar to the hierarchical Bayesian models:

$$y_i \sim \mathrm{TPB}(\psi, N_i, p_i),$$

$$\mathrm{logit}(p_i) = \alpha_j + \sum_{k=1}^{s} \beta_k * X\_env[i,k] + \tau_i,$$

$$\alpha_j = I + \sum_{l=1}^{t} \varepsilon_k * X\_sp[j,l] + \varphi_j,$$

$$\varphi_j \sim \mathrm{Normal}(0, \omega^2),$$

$$\tau_i \sim \mathrm{Normal}(0, {\theta_j}^2),$$

where α_j represents the species-specific intercept of species j, β are coefficients of environmental factors *X_env*, *I* is the species-independent intercept, ε are coefficients of non-environmental factors *X_sp* (i.e., body mass and phylogenetic eigenvectors), φ explains the overdispersion between species with hyperparameter ω, and τ explains the overdispersion between records with hyperparameter θ (Appendix 2.2). The number of environmental and non-environmental factors is expressed as *s* and *t*, respectively. When considering the overdispersion between records, the standard deviation of τ was assumed to differ between species since different species had different distribution areas, number of references, etc. Thus, hyperparameter θ is a vector with a length corresponding to the number of species. To consider the effect of collinearity in Model 2, we examined the correlation between environmental factors and between non-environmental factors using Pearson's product moment correlation (r), but |r| < 0.80 (Elith et al., 2006; Matsuura, 2016) in all pairs.

2.2.5 Data imputation

We estimated parameters in the models mentioned above using the original dataset (Model 1_1 and Model 2_1). In these models, missing values in the frequency of plant occurrence are treated as the same NA. However, the presence of plant material in samples has been reported in some studies even if the frequency of plant occurrence has not been reported. These descriptions are informative since they mean that missing values were at least above zero. Therefore, we attempted to impute the missing data concerning the frequency of plant occurrence so that there was no waste of information. First, we sorted the literature without information regarding the frequency of plant occurrence into two groups: literature reporting the presence of plant materials in samples and those in which the presence of plant materials has not been reported. We then imputed and replaced the 23 records from 14 references in the first group using two different methods.

2.2.5.1 Model 1_2 and Model 2_2: Data imputation with random values

First, random values were sampled from a sequence of 0.01 to 1 in increments of 0.01 to impute the frequency of plant occurrence. Then, the number of samples containing plant materials (y) was calculated as a product of random values and sample size N for each record that required imputation.

2.2.5.2 Model 1_3 and Model 2_3: Data imputation from posterior distribution of models without data imputation

First, posterior distributions of parameter p in models without data imputation (Model 1_1 and Model 2_1) were transformed into frequency distributions. The minimum unit of bins was set as 0.005 in Model 1_3 and 0.01 in Model 2_3, respectively, to avoid the inclusion of all posterior distributions into the zero bins. Frequency distributions were then transformed into ratios to decide the sampling probability of each bin. Afterwards, non-zero values were sampled according to this probability. Finally, the number of samples containing plant materials (y) was calculated as a product of p and sample size N of each dataset that required imputation. Since ψ represents the probability of the frequency of plant occurrence to be reported, estimation of ψ with the imputed dataset was considered to be inappropriate. Therefore, the parameter ψ was sampled from the posterior distribution of models without data imputation (Model 1_1 and Model 2_1).

2.2.6 Parameter estimation

We sampled all parameters using the No-U-Turn Sampler (Hoffman & Gelman, 2014) within an MCMC. We ran four parallel chains and calculated the potential scale reduction factor (Rhat; (Gelman et al., 2013; Kruschke & Liddell, 2018) to check convergence. The number of iterations was set as 5,000 with 2,000 warm-ups in the models without data imputation (Model 1_1 and Model 2_1). In models with data imputation (Model 1_2, Model 1_3, Model 2_2 and Model 2_3), MCMC sampling was repeated 10 times to reduce the potential effect of specific random value set. Thus, the number of each iteration was set as 2,000 with 1,500 warm-ups to reduce computational load for these models, and posterior distributions from each trial were cumulated. This rate was 1/2, meaning that one of every two consecutive values of posteriors was taken to reduce autocorrelation. If Rhat was 1.0 or less, the model was considered successfully converged. In addition, we conducted graphical posterior predictive checks to determine whether our models were a good fit. Models coded in Stan were compiled into C++ and run using the "rstan" package (Carpenter et al., 2017). Weakly informative priors were used according to prior recommendations from the Stan development team (Gelman, 2020) and "rstanarm" development team (Gabry & Goodrich, 2020). Specifically, intercepts (α_j and I) and coefficients (β and ε) follow Student's *t*-distribution with three degrees of freedom [Student's *t* (3,0,5)] and hyperparameters followed an exponential distribution [exp(1)].

We used a mode of posterior distribution (maximum a posteriori, MAP) with an 89% highest density interval (HDI) (Makowski et al., 2019) and a mean of posterior distribution (expected a posteriori, EAP) with a 95% Bayesian credible interval (CI) as the summary statistic. The MAP estimate is less susceptible to long tail of the posterior distribution. In contrast, the EAP estimate can indicate the tips of asymmetric posterior distribution. Thus, we reported both summary statistics. We used the HDI + ROPE (region of practical equivalence) decision rule as the basis for accepting or rejecting null values of fixed effects (Kruschke, 2018; Makowski et al., 2019). The “bayestestR” package (Makowski et al., 2019) was used to calculate MAP, HDI, and ROPE. According to Makowski et al. (Makowski et al., 2019), an 89% HDI is deemed to be more stable for an effective sample size less than 10,000. Estimated values were considered significant when the entire HDI fell outside the ROPE (i.e., the null hypothesis was rejected; (Kruschke, 2018; Makowski et al., 2019). The limits of the ROPE were set to the effect size at half of Cohen’s conventional definition of a small effect (Cohen, 1977), that is, [-0.1, 0.1], proposed by Makowski et al. (Makowski et al., 2019) and Kruschke et al. (Kruschke, 2018; Kruschke & Liddell, 2018). The “rope” function was used to calculate the overlap of HDI and ROPE. Additionally, estimated values were considered significant when the 95% CI did not include zero (Kubo, 2018).

2.3 Results

Within the 316 records that passed the exclusion criteria, the number of records dedicated to each species varied from 1 [African wildcat, Jungle cat (*F. chaus*), Canada lynx (*Lynx canadensis*)] to 55 (feral cat).

Within the 316 records, the number of records that reported the frequency of plant occurrence was 118 (37%). As for the 198 records that did not calculate the frequency of plant occurrence, 23 mentioned plant materials and 175 did not mention plants at all.

We imputed missing data with description about the presence of plants using two methods when estimating parameters. The methods used to estimate parameters when imputing missing data concerning the frequency of plant occurrence did not affect the conclusion of the analysis. Therefore, we mainly used the results obtained from data-imputed models (Model 1_3 and Model 2_3).

The frequency of plant occurrence varied substantially, from 0.005 (Pampas cat (*L. colocola*)) and 0.749 (southern tigrina (*L. guttulus*) (Table 2.1, Figure 2.1).

Within the 18 variables considered in Model 2, log-transformed body mass (MAP = -0.814 [-1.452, -0.302], EAP = -0.881 [-1.586, -0.164]) had a significant effect on the frequency of plant occurrence based on the HDI + ROPE rule (Figure 2.2-2.4, Table 2.2). In addition, "PV1" (MAP = -0.222 [-0.393, -0.036], EAP = -0.216 [-0.435, -0.0002]) was also considered significant, since the 95% CI did not include zero (Figure 2.3, Table 2.2).

Regarding the PVR (Diniz-Filho et al., 1998; Sakamoto et al., 2010), PV1 tended to have a significant positive effect on the frequency of plant occurrence in the *Panthera* and *Caracal* genera and a negative effect in other felid lineages. This effect was greater in *Panthera* than in *Caracal* (Figure 2.5). Greater body mass was associated with a reduction in the frequency of plant occurrence with a probability of 95% when estimated in a one-variable model, although the 95% CI included zero. However, PV1 showed a positive correlation with the frequency of plant occurrence with a probability of only 40%.

2.4 Discussion

2.4.1 Limitations

Our data relied on the frequency of occurrence data from previous studies. Therefore, we should acknowledge the biases and limitations of the frequency of occurrence method (reviewed in Klare et al., 2011). The frequency of occurrence method tends to overestimate the importance of small food items as it weighs the presence of small and large food items in the feces equally (Klare et al., 2011; Weaver, 1993). Although the frequency of occurrence is not always equivalent to the composition of the diet, Klare *et al.*(2011) stated that the frequency of occurrence per feces can contribute useful information about rare food items and help us understand a carnivore's ecology.. In the present study, we did not evaluate the importance of plants relative to other items for felids nor did we seek to argue that plants make up most of their diet. Rather, we attempted to estimate the frequency of plant consumption by felids and identify the factors that could affect it. Although the frequency of plant occurrence per feces/gut sample can provide valuable information on how often wild cats consume plants, it cannot evaluate the amount of plant in their feces. Further accumulation of knowledge using plant biomass calculation helps achieve a more precise assessment (Klare et al., 2011) of the importance of plant consumption in carnivores.

The present study investigated the effect of environmental attributes, which represents the traits of research areas. We could not find a clear relationship between environmental factors and the frequency of plant occurrence. However, it should be noted that it is likely that more detailed factors, such as abundance of specific plant taxa or risk of parasite infection, that were not analyzed in the present study have correlations with the frequency of plant occurrence in felids. As the plant occurrence data were based on indirect evidence (feces, remains of the digestive content), it was difficult to obtain fine-scale spatial and temporal environment data from the habitats of subject animals. This might have masked the effect of environmental factors. For example, we used the mean of NDVI during the sampling period, but it was possible that a drastic vegetation change occurred during the sampling period or during the period we did not have NDVI data for. Although seasonal difference was considered in our models, several studies have reported the frequency of plant occurrence as data throughout the year, which possibly masked the actual seasonal patterns.

Hoppe-Dominik (Hoppe-Dominik, 1988) suggested that leopards may eat plants to relieve hunger during periods of starvation. It is possible that physiological condition could confound with environmental factors. Further individual-based studies are required to test the effect of physiological conditions.

2.4.2 Phylogenetic factors

The results showed that the frequency of plant occurrence was observed to be higher in *Panthera* and *Caracal*, the two earliest diverging lineages of Felidae (Kitchener et al., 2017; G. Li et al., 2016) than other felids. This might indicate that plant-eating behavior in felids is a trace of omnivorous ancestral traits (Bradshaw, 2006; Tseng & Flynn, 2015b, 2015a). However, this effect was not significant in the HDI+ROPE rule. Besides, *Panthera* consists entirely of big cats, thus the positive effect of PV1 on the frequency of plant occurrence in *Panthera* species conflicted with the negative effect that body mass was found to have on this variable. Hence, we confirmed the effect of both variables through one-variable models and found that PV1 itself was not correlated with the frequency of plant occurrence. The significant effect of PV1 in the *Panthera* lineage may have been caused by the high frequency of plant occurrence relative to the body mass of these big cats. Although the result did not exclude the possibility that phylogeny shows a relationship with the frequency of plant occurrence in felids, it was likely to have little effect.

2.4.3 Body mass

We found that body mass showed a significant negative correlation with the frequency of plant occurrence, meaning that smaller carnivore species engaged in plant-eating behavior more frequently than larger species. The correlation was significant in Model 2_1 and Model 2_3 according to both the HDI+ROPE rule and 95% CI, but not in the model with random data imputation (Model 2_2). However, the percentage of posteriors in the ROPE was only 3.9%, and 90% CI did not include zero in Model 2_2 (Figure 2.3). In this model, the frequency of plant occurrence was imputed completely at random; therefore, unrealistic values such as 1 might have been applied and affected the posterior distribution. Hence, judging from the overall results, we concluded that body mass has a significant negative correlation with the frequency of occurrence.

One possible explanation for this correlation relates to self-medication. Kleiber’s law states that relative energy consumption is higher in smaller species (Kleiber, 1947). Maintenance metabolism (i.e., the energy required to maintain homeostasis) scales fractionally with body size; as such, smaller animals require more metabolic energy per unit of body mass (Demment & Soest, 1985). Therefore, energy loss caused by parasites has higher consequences for smaller carnivores. Moreover, Gregory et al. (Gregory et al.,

1996) suggested that host species with higher metabolic rates for their body size may show a greater number of parasite species due to increased food intake. A multi-species study of mammals in Mexico revealed that the order Carnivora showed the greatest occurrence of parasitic helminths, and that the host body mass has significant negative correlation with parasite richness (Villalobos-Segura et al., 2020). These studies support that the cost of parasites is higher in smaller felids than larger species. However, the association between parasite species richness and body weight varies depending on the subject species (Dáttilo et al., 2020), hence further quantitative study is required to confirm the relationship between host body mass and parasite richness in felids. Several animal species are known to utilize plant physical or chemical aspects against parasites or pathogens (Bosch et al., 2015; de Roode et al., 2013; B. L. Hart & Hart, 2018; Huffman, 2003). Consumption of grasses is considered to work as scouring agent against intestinal parasites such as roundworms and tapeworms in canids (Bosch et al., 2015). Small carnivores might eat plants for parasite control, since the energetic costs of parasite load are relatively high. Leopard cat (*Prionailurus bengalensis*) feces has been reported to contain parasites on *Arundinella hirta* plant (Lee et al., 2014). Nonetheless, to our knowledge, this is the only study reporting the presence of plant and parasite in the same feces of felids.

Evacuation of hair or undigested materials can be another explanation. Plant-eating behavior in felids is hypothesized to have an effect on hairball evacuation (Herbst & Mills, 2010; Shultz, 2019). Similar to the aforementioned endoparasites, a greater frequency of plant occurrence in small felids may relate to the high energy cost of an ectoparasite load. Fleas are the main ectoparasite that affect cats, and self-grooming using cornified papillae on the tongue is one of the removal strategies (B. L. Hart & Hart, 2018). As the cost of ectoparasite load increases, the intensity of grooming increases, which is likely to result in increased ingestion of its own hair by the animal.

Carnivores weighing less than 21.5 kg generally consume animals consisting of 45% or less of their own mass, while those weighing more than 21.5 kg prey mostly on animals larger than themselves (Carbone & Gittleman, 2002). Small prey consumption often includes the ingestion of indigestible parts such as fur, skin, bone, and connective tissue, besides muscle and organs, while large carnivores can selectively eat digestible parts (Clauss et al., 2010; Stirling & McEwan, 1975). In humans, dietary fiber intake is known to promote digestion and bowel movements by stimulating peristalsis and mucus secretion in the digestive tract (Chutkan et al., 2012; El-Salhy et al., 2017). Plant consumption might promote digestion or excretion of indigestible food items, which are consumed by small carnivores at a high frequency. Sugar cane-derived fibers reduced the size of hairballs in the feces of domestic cats and facilitated hair evacuation (Loureiro et al., 2014). However, cellulose, one of the main insoluble fibers, did not have such an effect (Loureiro et al., 2014), and plant intake had little effect on hair evacuation in captive snow leopards (Yoshimura et al., 2020). Owing to the aforementioned attributes of prey items, smaller carnivores are considered to be more tolerant to indigestible food items (Jethva & Jhala, 2004; Rüme et al., 2008). Indeed, Vester *et al.* demonstrated that small felids have higher digestion ability of dietary fiber (Vester et al., 2008), and Kerr *et al.* showed that tract dry matter, organic matter, fat, and energy digestibility coefficients decreased linearly with body weight in four medium-to-large cats [jaguar, cheetah (*Acinonyx jubatus*), Malayan tiger (*P. tigris corbetti*), and Siberian tiger (*P. tigris altaica*)] fed cellulose and beet pulp diets (Kerr et al., 2013). Although cellulose intake reduces dry matter and energy digestibility both in large (Kerr et al., 2013) and small felids (Edwards et al., 2001; Prola et al., 2010), smaller animals may be less affected, which could explain their increased tolerance to more frequent plant consumption. Nevertheless, this can be true whether or not plant intake has some adaptive significance for obligate carnivores, thus this does not negate the self-medication hypothesis or the hair evacuation hypothesis.

2.4.4 Conclusion

To date, little attention has been paid to the presence of plants in dietary studies of carnivores. Lack of plant data in a record does not necessarily mean that plant occurrence in samples was absent in that study. Therefore, if we had only used the data of studies that report the frequency of plant occurrence values, the analyses would have been biased. To avoid this, our methods made the best use of all information available using two-part models and Bayesian framework. We demonstrated the negative relationship of the frequency of plant occurrence with body mass. As the present study is exploratory, we cannot completely deny the alternatives. Nonetheless, our findings indicate that plant eating may have some functional significance as functional behaviors have a greater importance for smaller species that need to increase the efficiency of nutrient intake. Increased efficiency is achieved by not only increasing nutrient intake but also preventing the decrease of nutrient intake (e.g., parasites). Smaller species did not always present a higher frequency of plant occurrence than that did larger species; this may be owing to the various reasons for plant consumption and the fact that the frequency of intake varied with the primary role of the plant material. Further research is required to understand the evolution and adaptive significance of plant eating in carnivores. In particular, studies identifying plant species and their frequency of occurrence in wild carnivore samples using recently developed molecular biological methods (Monterroso et al., 2019) are important to infer the role of plant intake. Hypothesis-centered studies will provide direct evidence about the adaptive significance of plant-eating as well. By unravelling the relationship between carnivores and plants, we will be able to understand not only their behavioral ecology but also their interactions within ecosystems.

Table 2.1. Estimated frequency of plant occurrence in carnivores in Model 1_3. The numbers in parentheses represent the number of records that calculated the frequency of plant occurrence values. Estimated frequency is shown as maximum a posteriori (MAP) estimate (the mode of posterior distribution) with 89% highest density interval and expected a posteriori (EAP) estimate (the mean of posterior distribution) with 95% credible interval.

Lineage	Common name	Academic name	Number of records	MAP estimate [lower HDI, upper HDI]	EAP estimate [lower CI, upper CI]
Domestic cat	feral cat	*Felis catus*	55 (34)	0.122 [0.077, 0.167]	0.124 [0.073, 0.187]
	Jungle cat	*Felis chaus*	1 (1)	0.162 [0, 0.443]	0.237 [0.018, 0.793]
	African wildcat	*Felis lybica*	1 (1)	0.396 [0.087, 0.756]	0.436 [0.057, 0.903]
	European wildcat	*Felis silvestris*	10 (3)	0.172 [0.063, 0.248]	0.167 [0.053, 0.317]
Leopard cat	Pallas's cat	*Otocolobus manul*	2 (2)	0.238 [0.084, 0.389]	0.247 [0.071, 0.534]
	Leopard cat	*Prionailurus bengalensis*	14 (9)	0.298 [0.119, 0.507]	0.327 [0.12, 0.608]
Puma	Cheetah	*Acinonyx jubatus*	8 (5)	0.056 [0.004, 0.213]	0.11 [0.014, 0.366]
	Jaguarundi	*Herpailurus yagouaroundi*	6 (4)	0.078 [0.003, 0.494]	0.248 [0.022, 0.725]
	Puma	*Puma concolor*	43 (9)	0.027 [0.004, 0.073]	0.042 [0.007, 0.114]
Lynx	Canada lynx	*Lynx canadensis*	1 (1)	0.029 [0, 0.264]	0.13 [0.006, 0.644]
	Eurasian lynx	*Lynx lynx*	11 (3)	0.022 [0, 0.147]	0.073 [0.003, 0.361]
	Bobcat	*Lynx rufus*	21 (2)	0.062 [0.017, 0.128]	0.093 [0.025, 0.456]
Ocelot	Pampas cat	*Leopardus colocola*	2 (1)	0.005 [0, 0.212]	0.089 [0.001, 0.438]
	Geoffroy's cat	*Leopardus geoffroyi*	10 (5)	0.033 [0, 0.227]	0.112 [0.008, 0.404]
	Southern tigrina	*Leopardus guttulus*	2 (2)	0.749 [0.444, 0.94]	0.673 [0.23, 0.924]
	Ocelot	*Leopardus pardalis*	11 (6)	0.074 [0.02, 0.177]	0.105 [0.025, 0.26]
	Northern tigrina	*Leopardus tigrinus*	2 (1)	0.403 [0.226, 0.684]	0.44 [0.174, 0.81]
	Margay	*Leopardus wiedii*	4 (2)	0.416 [0.01, 0.807]	0.456 [0.03, 0.926]
Caracal	Caracal	*Caracal caracal*	10 (6)	0.127 [0.031, 0.396]	0.213 [0.038, 0.559]
	Serval	*Leptailurus serval*	2 (1)	0.024 [0, 0.129]	0.079 [0.007, 0.612]
Panthera	Jaguar	*Panthera onca*	21 (3)	0.144 [0.008, 0.328]	0.195 [0.026, 0.537]
	Leopard	*Panthera pardus*	37 (4)	0.051 [0.001, 0.23]	0.115 [0.012, 0.391]
	Tiger	*Panthera tigris*	25 (2)	0.132 [0.091, 0.207]	0.147 [0.079, 0.234]
	Snow leopard	*Panthera uncia*	17 (11)	0.259 [0.15, 0.392]	0.274 [0.141, 0.449]

Table 2.2. Estimated coefficients of fixed effects in Model 2_3. Estimated frequency is shown as maximum a posteriori (MAP) estimate (the mode of posterior distribution) with 89% highest density interval (HDI) and expected a posteriori (EAP) estimate (the mean of posterior distribution) with 95% and 90% credible intervals (CI). Bold characters represent significant fixed effects. Estimated parameters were considered as significant if the 89% HDI falls outside the region of practical equivalence (ROPE) [-0.1, 0.1] or 95% CI did not include zero.

Fixed effects	MAP estimate [lower HDI, upper HDI]	%HDI inside the ROPE	EAP estimate [lower 95%CI, upper 95%CI]	EAP estimate [lower 90%CI, upper 90%CI]
Island	0.364 [-0.448, 1.203]	0.13	0.356 [-0.651, 1.382]	0.356 [-0.495, 1.204]
Monthly precip.	1.991 [-0.119, 3.938]	0.024	1.919 [-0.553, 4.355]	1.919 [-0.195, 3.982]
Mean daily max temp.	0.003 [-0.037, 0.045]	1	0.004 [-0.048, 0.053]	0.004 [-0.039, 0.045]
Mean daily minimum temp.	-0.001 [-0.031, 0.032]	1	0.002 [-0.039, 0.038]	0.002 [-0.032, 0.033]
NDVI	-0.573 [-2.024, 0.439]	0.069	-0.785 [-2.273, 0.744]	-0.785 [-2.068, 0.48]
Spring	0.161 [-0.595, 1.343]	0.115	0.369 [-0.838, 1.588]	0.369 [-0.629, 1.374]
Summer	-0.066 [-1.063, 0.766]	0.141	-0.099 [-1.228, 1.025]	-0.099 [-1.046, 0.845]
Autumn	-0.195 [-0.919, 0.9]	0.156	-0.034 [-1.155, 1.08]	-0.034 [-0.975, 0.9]
Winter	0.029 [-0.927, 0.86]	0.152	-0.047 [-1.215, 0.987]	-0.047 [-0.999, 0.842]
Dry	0.337 [-11.104, 12.13]	0.016	-0.031 [-16.56, 16.47]	-0.031 [-12.282, 12.067]
Wet	1.202 [-1.72, 3.855]	0.038	1.224 [-2.306, 4.603]	1.224 [-1.712, 4.036]
Sample type	-0.176 [-0.762, 0.708]	0.179	-0.07 [-0.951, 0.894]	-0.07 [-0.807, 0.715]
Log body mass	**-0.814 [-1.452, -0.302]**	**0**	**-0.881 [-1.586, -0.164]**	**-0.881 [-1.469, -0.278]**
PV1	-0.222 [-0.393, -0.036]	0.135	**-0.216 [-0.435, -0.0002]**	**-0.216 [-0.399, -0.031]**
PV2	-0.042 [-0.162, 0.061]	0.771	-0.052 [-0.19, 0.09]	-0.052 [-0.165, 0.065]
PV3	0.086 [-0.075, 0.214]	0.611	0.073 [-0.111, 0.251]	0.073 [-0.081, 0.221]
PV4	0.013 [-0.157, 0.193]	0.669	0.017 [-0.194, 0.235]	0.017 [-0.162, 0.199]
PV5	-0.127 [-0.313, 0.092]	0.436	-0.107 [-0.355, 0.154]	-0.107 [-0.313, 0.106]

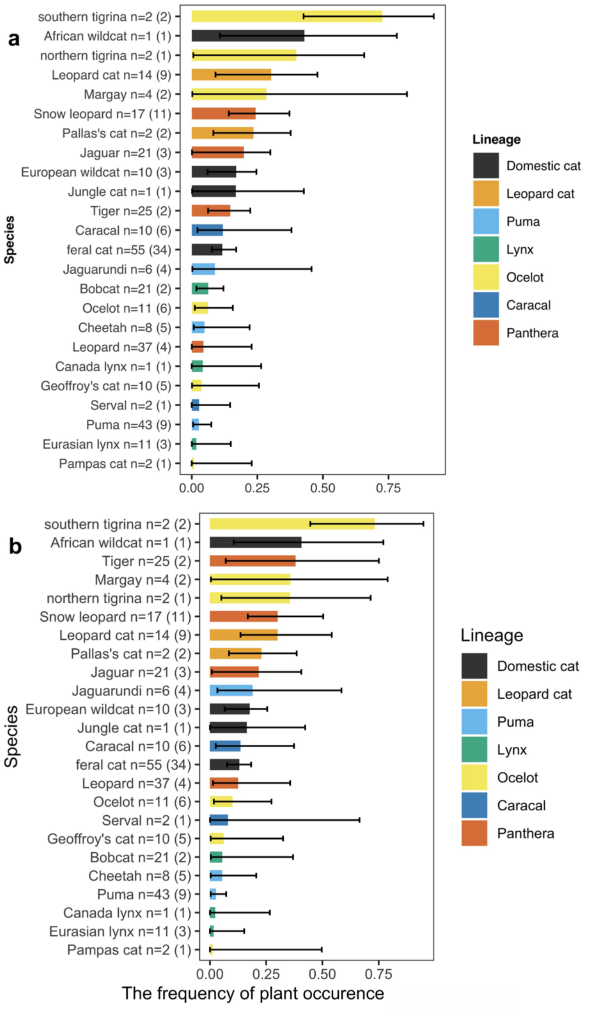


Figure 2.1. Estimated frequency of plant occurrence (a: maximum a posteriori [MAP] estimate with the 89% highest density interval [HDI], b: expected a posteriori [EAP] estimate with the 95% credible interval [CI]) of each species using Model 1_3. The numbers next to the common names of species represent the numbers of records and the numbers in the parentheses are the numbers of records showing the frequency of plant occurrence values.

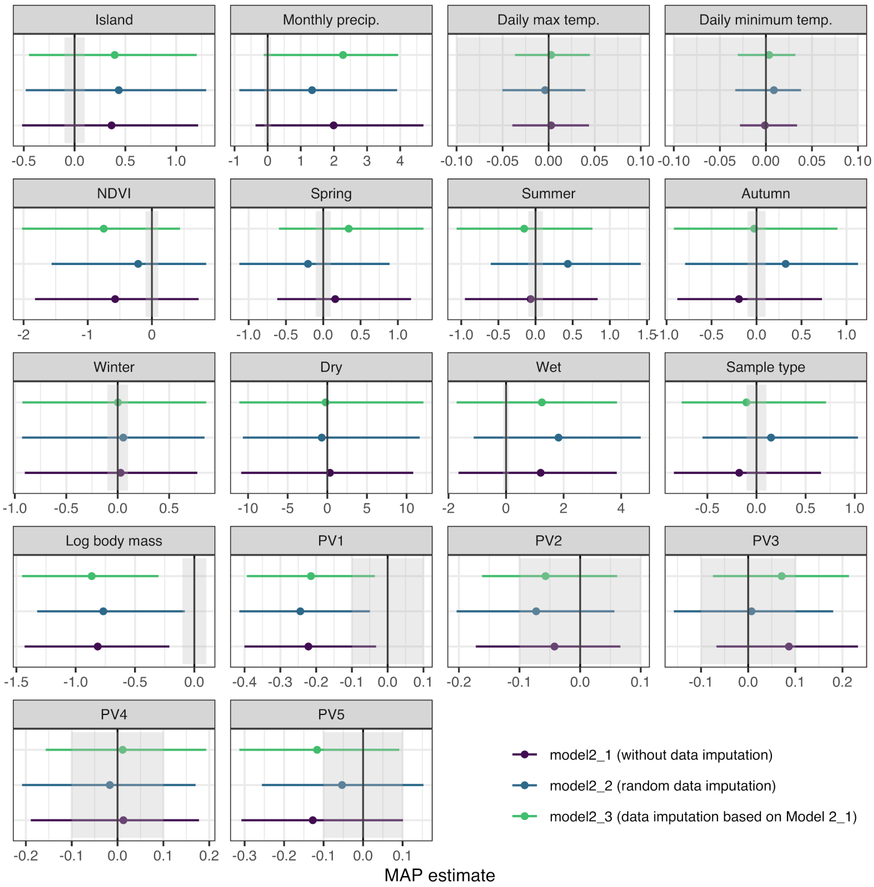


Figure 2.2. Maximum a posteriori (MAP; the mode of the posterior distribution) estimates of coefficients of fixed effects. The error bars represent 89% highest density interval (HDI) and the gray area represents the region of practical equivalence (ROPE). The black line indicates zero. Estimated parameters were considered as significant if the 89% HDI falls off from the ROPE.

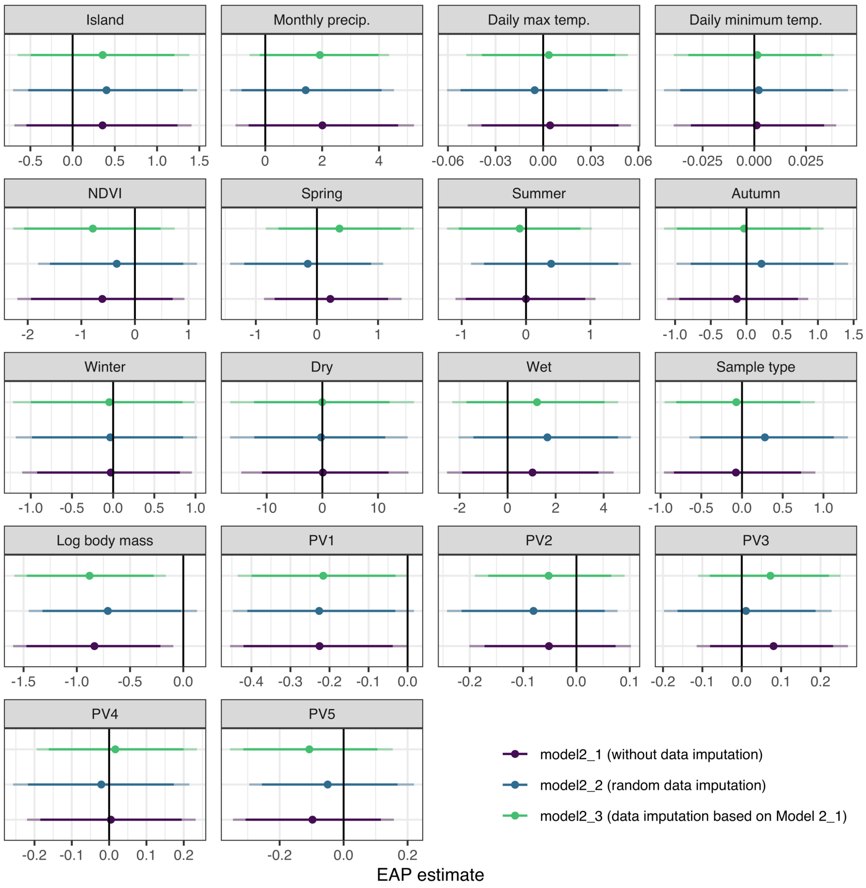


Figure 2.3. Expected a posteriori (EAP; the mean of the posterior distribution) estimates of coefficients of fixed effects. The light and thick error bars represent 95% and 90% credible interval (CI), respectively. The black line indicates zero. Estimated parameters were considered as significant if the 95% CI did not include zero.

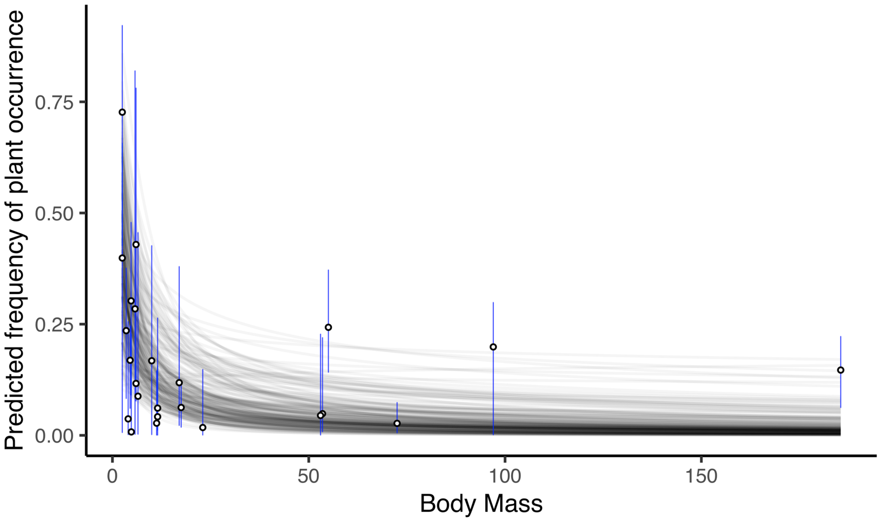


Figure 2.4. Response curve of the frequency of plant occurrence to body mass in Model 2_3. Dots are maximum a posteriori (MAP) estimates of the frequency of plant occurrence and error bars represent the 89% highest density intervals (HDI). Intercepts and slopes were randomly selected from posterior distributions.

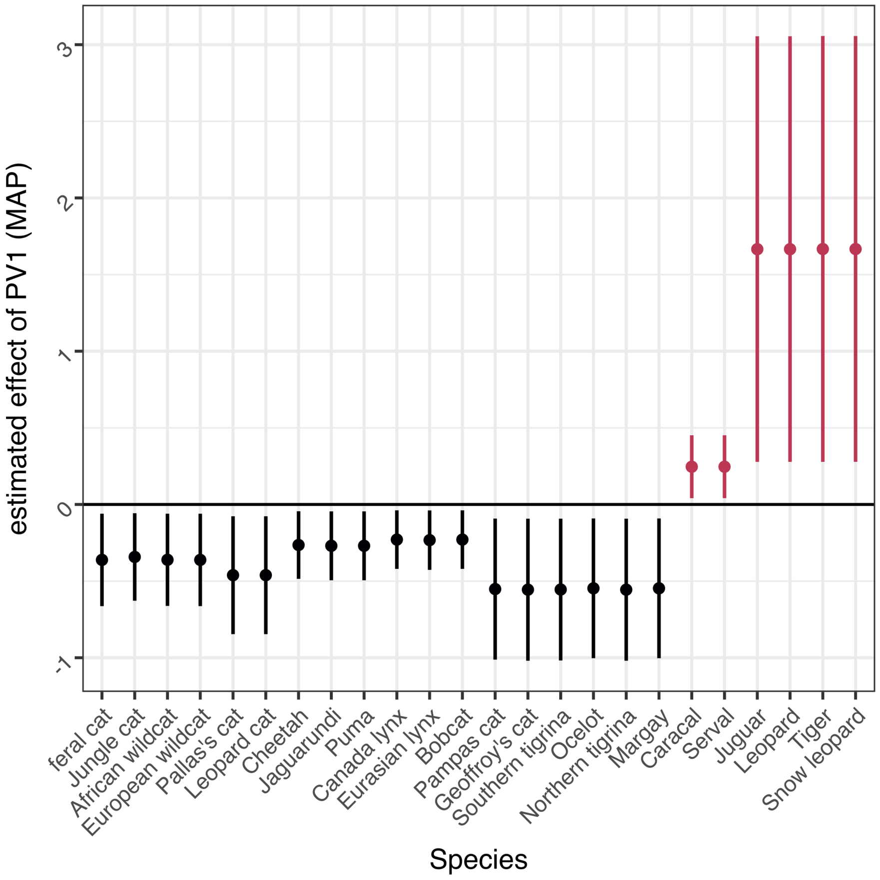


Figure 2.5. The products of PV1 and maximum a posteriori (MAP) estimates for each species in Model 2_3. Error bars reflect the 89% highest density interval (HDI) of each coefficient. Positive values are represented in red, and negative values in black.

Appendix 2.1

Here, we describe the characteristics of plant eating in each lineage. According to Li *et al.* (G. Li et al., 2016), there are eight extant felid lineages. However, only seven lineages are described here because we did not have the data from Bay cat lineage.

Appendix 2.1.1 Domestic cat lineage

This represents the most recent lineage and consists of smaller species (G. Li et al., 2016). Among the 67 records found for this group, 55 described feral cats. There were 6 studies reporting fruit detection (Biró et al., 2005; Carvalho & Gomes, 2004; Ferreira et al., 2014; Lanszki et al., 2016; Meckstroth et al., 2007; Spencer et al., 2014), with fruit possibly having been consumed as food. However, fruit was detected more frequently in domesticated cats than in feral cats living on a Croatian island (Lanszki et al., 2016), suggesting that the detection of fruit content might be associated with proximity to human activity [e.g., food provisioning or scavenging garbage (Yamane et al., 1994)]. Additionally, there were several studies showing the presence of non-fruit-bearing plants, which may have other benefits, such as parasite control (B. L. Hart, 2008; B. L. Hart & Hart, 2018; Sueda et al., 2008).

Appendix 2.1.2 Leopard cat lineage

This group consists mainly of small species inhabiting Central to South-East Asia. The two species used in this analysis had a relatively high frequency of plant occurrence. Parasites, together with *A. hirta*, were detected in leopard cat feces in Korea (Lee et al., 2014), implying that plants likely contributed to anti-parasite measures or promoted gastrointestinal tract movement (Tatara & Doi, 1994). Although no cases of fruit detection have been reported in the leopard cat, a DNA-based study of its fecal contents in China showed fruit-bearing species, suggesting its use as food (Xiong et al., 2016).

Appendix 2.1.3 Puma lineage

This group consisted of three species, each belonging to a different genus and differing in both body size and distribution range. Overall, the frequency of plant occurrence in this group was low, although one study showed a high frequency of plant occurrence in the feces of the smallest species, the jaguarundi (*Herpailurus yagouaroundi*) (Kasper et al., 2016). This high degree of intraspecies variation is reflected as a wide range of HDI [0.003, 0.494] and CIs [0.022, 0.725] (Table 1). With the exception of one study on cheetahs (*Acinonyx jubatus*) in Iran (Zamani et al., 2017), no fruit was detected. Samples of Jaguarundi and pumas have been reported to contain 28% and 20% of Cyperaceae plants, respectively (Rocha-Mendes et al., 2010). The higher presence of this family may be because of the distinctive surface of most of these grasses, which makes them easier to identify by texture, and because they tend to contain fewer toxic compounds (Hoppe-Dominik, 1988).

Appendix 2.1.4 Lynx lineage

This lineage included the relatively large Eurasian lynx (*L. lynx*) and three medium-sized species that are widely distributed in the Northern Hemisphere. The frequency of plant occurrence was relatively low in this group. Nevertheless, 7 studies reported the presence of fruit, and McKinney *et al*. reported that bobcats (*L. rufus*) in the Sonoran Desert fed more frequently on fruits and seeds during winter and spring droughts than on reptiles (Mckinney & Smith, 2007). Therefore, in this lineage, plants may serve mainly as a supplementary food source.

Appendix 2.1.5 Ocelot lineage

This group consisted of small species from Central to South America; six of the eight extant species (Kitchener et al., 2017) were used in this analysis. Fruit consumption has not been reported. The frequency of plant occurrence was high for three species and low for the others. Although these species have broad habitat selectivity, southern tigrina (*L. guttulus*), northern tigrina (*L. tigrinus*), margay (*L. wiedii*), and ocelot (*L. pardalis*) are more likely to inhabit less dry habitats (IUCN/SSC Cat Specialist Group, 2018). Indeed, the mean monthly precipitation was higher in the habitats of these four species (105, 106, 79, and 111 mm, respectively) than in the habitats of other species (Geoffroy's cat (*L. geoffroyi*): 38 mm; Pampas cat (*L. colocola*): 34 mm). Hence, the high frequency of plant occurrence in the three species may reflect hot and humid habitats where the risk of parasite and pathogen infection is relatively high (Froeschke et al., 2010; Kołodziej-Sobocińska & Ko, 2019). The ocelot's larger body mass might have caused its relatively low frequency of plant occurrence compared to that for smaller species. Additionally, ocelots, pumas, and jaguars have been observed eating wild rice containing high levels of cyclooxygenase inhibitors (Montalvo et al., 2020), which works as an anti-inflammatory agent in dogs and cats (C. J. Jones & Budsberg, 2000). However, it should be noted that studies on this topic are scarce and there is a high degree of uncertainty in the estimates.

Appendix 2.1.6 Caracal lineage

This lineage consists of medium-sized species that live mainly in Africa. Caracals (*C.*

caracal) had a higher frequency of plant consumption than servals (*Leptailurus serval*). The presence of non-fruiting plants has often been reported, and there was a study of caracals feeding on tsama melons (Melville et al., 2004). Melville *et al.* also found Kalahari sour grass (*Schmidtia kalihariensis*) in 38.8% of caracal feces (Melville et al., 2004). This is the dominant species in the Kalahari Desert, which only grows for a short period after sufficient rainfall (Dippenaar-Schoeman et al., 2018), has a distinctive odor, and has glands that secrete acidic substances (Dippenaar-Schoeman et al., 2018). Caracals may eat this plant to ingest these compounds possibly for self-medication (B. L. Hart & Hart, 2018; Huffman, 2003) or for pH control in the digestive tract (Kerr et al., 2013), although it is unclear whether these compounds have a beneficial effect. This finding further suggests that these animals might use plant odor as one of the selecting factors for consumption.

Appendix 2.1.7 Panthera lineage

These so called "big cats" constitute one of the basal lineages of extant felid species (G. Li et al., 2016). Fruit consumption has not been reported for them; however, the presence of grasses and shrubs has been detected in numerous cases [e.g., (Jumabay-Uulu et al., 2013; Ott et al., 2007; Tkachenko, 2012)]. Hoppe-Dominik stated that leopards may eat grasses to keep their digestive tract moving during starvation (Hoppe-Dominik, 1988). However, captive snow leopards also ate plants regularly even though they were fed daily (Yoshimura et al., 2020), suggesting that starvation is not always the trigger for plant-eating. Furthermore, it has been suggested that grasses are selectively eaten because they are free of secondary plant compounds, unlike those in other plant groups (Hoppe-Dominik, 1988). Indeed, undigested Poaceae and Cyperaceae plants were detected in 40%–50% of the feces of leopards (J. A. Hart et al., 1996) and tigers (Tkachenko, 2012), similar to that in the feces of puma and jaguarundi (Rocha-Mendes et al., 2010). Therefore, these plant species may be consumed not for medicinal secondary compounds but for physical traits such as hairs on their surface (Hoppe-Dominik, 1988).

Snow leopards and leopards have been reported to eat *Myricaria* shrubs in addition to grasses (Jumabay-Uulu et al., 2013; Lovari et al., 2013; Wegge et al., 2012). Tamaricaceae plants (the family that includes *Myricaria*) have been detected in 4.1%–16.9% of feces and constituted the bulk of hairballs (Lovari et al., 2013), although it is uncertain that hairballs were caused by plant intake. These *Myricaria* plants have anti-inflammatory properties and have been used as traditional medicines (Chernonosov et al., 2017; Y. Liu et al., 2009). Cold and dry climates restrict the transmission and growth of parasites (Morris, 2002), whereas low temperature increases the probability of infection in the alpine hare (Schai-Braun et al., 2019). As such, snow leopards, which had the highest frequency of plant occurrence among *Panthera* species, may utilize medicinal compounds derived from plants against parasites. Further, the relatively high frequency of plant occurrence reported in snow leopards that live in alpine environments where plants are scarce, together with no correlation with NDVI, support the possibility that plant consumption has some advantage for carnivores.

Appendix 2.2

Details of parameters in the models.

parameters	category	size	description
N_all	integer	1	the total number of records
N_sp	integer	1	the total number of species
y	vector	N_all	the number of samples contained plant of each record
ψ	numeric	1	the probability of reporting the frequency of plant occurrence
N	vector	N_all	sample size of each record
p	vector	N_all	the frequency of plant occurrence of each record
α	vector	N_sp	species-specific intercept
τ	vector	N_all	random effect of records
θ	vector	N_sp	hyperparameter for τ
β	vector	s	coefficient of environmental factors
X_env	matrix	s × N_all	environmental factor
s	integer	1	the number of environmental factors
I	numeric	1	species-independent intercept
ε	vector	N_sp	coefficient of non-environmental factors
X_sp	matrix	t × N_sp	non-environmental factor
t	integer	1	the number of non-environmental factors
φ	vector	N_sp	random effect of species
ω	vector	1	hyperparameter for φ

Appendix 2.3

Collection of Environmental factors

Appendix 2.3.1 Island

The size of the island was recorded according to the original description of references. Carrion and Valle., (Carrión & Valle, 2018) did not report the size of San Cristobal Island; thus, we used the data from Galapagos Conservancy (https://www.galapagos.org/about_galapagos/about-galapagos/the-islands/san-cristobal/). If the sampling site was on the mainland, the size was recorded as 10,000 km^2. Islands larger than 10,000 km^2 were treated in the same way as mainland (Murphy et al., 2019).

Appendix 2.3.2 Precipitation and temperature

We used the mean of monthly precipitation/temperature during the sampling period of each reference (Eckardt et al., 2019). If the reference had the information, we used it. Otherwise, the data were obtained from MeteoBlue database (Cano-Cruz & López-Orozco, 2015). The MeteoBlue climate diagrams are based on 30 years (from 1985) of hourly weather model simulations and are available for every place on Earth. We searched for the climate data of each record by the name of sampling site or by the latitude and longitude. We essentially used the midpoint of latitude and longitude of the sample site on the reference articles. If location data were not available, we searched for the filed site of each reference using Google Maps and obtained the data.

Appendix 2.3.3 NDVI

We used the monthly NDVI data for 1 km square grid ("MOD13A3_006__1_km_monthly_NDVI"). We obtained the NDVI data based on the latitude and longitude of each sampling site via AppEEARs (AppEEARS Team, 2019). First, we collected all the relevant data, from oldest to latest. Then, we calculated the mean of sampling year (e.g. if the sampling was conducted between 2003 and 2005, we calculated the mean of NDVI from 2003 to 2005 and used it as the NDVI value for the study.) Since MODIS satellite was launched in 2000, we used the data from the oldest year for the NDVI for the 89 studies that started sampling before 2000.

Appendix 2.3.4. Season

We followed the original description of each reference. If there was no description, we categorized the season based on the sampling month. In the northern hemisphere, we set spring as March to May, summer as June to August, autumn as September to November, and winter as December to February. In the southern hemisphere, we set spring as September to November, summer as December to February, autumn as March to May, and winter as June to August. The dry and wet seasons were recorded based only on the reference descriptions.

If the data were described by season in the original reference, we treated the data of each season as different record.

As “rstan” allows only numeric or integer data, we converted each season as binary variables. For example, if the study was conducted during spring and summer, the variable “spring” and “summer” is assigned a value of 1, while other seasons (“autumn”, “winter”, “dry”, “wet”) are assigned a value of 0. If the study was conducted throughout the year, “spring,” “summer,” “autumn,” and “winter” are assigned a value of 1.

Appendix 2.3.5 Sample type

We followed the original description of each reference. The data from feces and data from the remains of the digestive tract were separately recorded. As the remains present in the digestive tracts may be greater or lesser than those present in single feces and may not be directly comparable, we added sample type as a binary variable.

Chapter 3

The relationship between plant-eating and hair evacuation in snow leopards (*Panthera uncia*)

3.1 Introduction

Felids are not adapted to digest plants, and cellulose intake can hinder digestion and nutrient absorption (Edwards et al., 2001; Prola et al., 2010) as explained in Chapter 1. Given that plant ingestion is biologically costly, there should be an adaptive significance to plant-eating behavior that compensates for the potential costs. However, no reports have investigated the adaptive significance of plant ingestion in felids.

As introduced in Chapter 2, three hypotheses may explain the adaptive significance of plant ingestion in felids. In this chapter, we focused on the second hypothesis, which suggests that plant intake is related to hair evacuation (Shultz, 2019). If hair balls fill the digestive tract, they prevent digestion (Cannon, 2013). Felids often ingest their own hair while grooming, as well as the hair of their prey items while eating. Felids evacuate hair through vomiting or expelling as feces. Although it is thought that ingested plants aid in excreting hairballs (Herbst & Mills, 2010), there is still no empirical evidence of this. Therefore, the hair evacuation hypothesis has yet to be tested conclusively.

Snow leopards is an endangered cat species that live in high altitude regions (1,220 to > 5,000 m) of Central and South Asia (T. McCarthy et al., 2016), designated as VU (vulnerable) in the IUCN Red List of Endangered Species (T. McCarthy et al., 2017). Bharal (*Pseudois nayaur*) and Siberian ibex are primary prey species of snow leopards (Fox & Chundawat, 2016); their ranges almost entirely overlap with that of snow leopards (Mallon et al., 2016). Large portions of snow leopards' natural habitat are devoid of tree cover, given the predominance of alpine and desertic climate conditions in their natural range. The vegetation in their range varies from scrubland and desert to forest-alpine ecotones (Fox & Chundawat, 2016). The presence of plant material in snow leopard feces has been reported in several research areas, despite the relatively low abundance of vegetation across their habitat (Anwar Bilal et al., 2011; Chetri et al., 2017; Devkota et al., 2013; Jumabay-Uulu et al., 2013; Oli et al., 1993; Shehzad et al., 2012). For example, it was reported that plant materials occurred in 62% of fecal samples collected in Phu valley, Nepal. In some cases, fecal content consisted almost entirely of plants (Wegge et al., 2012). Snow leopards also have longer and denser hair than other felids as an adaptation to life at high altitude (Kitchener et al., 2010), which indicates a relatively high frequency of hair ingestion through grooming and thus, a correspondingly frequent evacuation. Based on the above characteristics, we chose snow leopards as a suitable felid species for which to investigate the effects of plant ingestion and hair evacuation.

In this study, we tested the hair evacuation hypothesis in captive snow leopards, through behavioral observations and analysis. Behavioral observations examined the frequency of plant ingestion and vomiting to identify the potential effect of plant intake on vomiting. We collected fecal samples and measured the amount of plants and hair and examined their statistical relationship. Together, these analyses provide quantitative evidence to test the hair evacuation hypothesis.

3.2 Methods

Management of the captive snow leopards in this study followed the Code of Ethics of the Japanese Association of Zoos and Aquariums. Sampling procedures were noninvasive and approved by each zoo and Animal Experimentation Committee of Wildlife Research Center of Kyoto University. This study complied with applicable national laws.

3.2.1 Animals

The subject animals were 13 snow leopards (7 females, 6 males) kept in zoos in Japan. All individuals were housed separately when inside. Basically, six snow leopards (Female 1–3, Male 1－3) at Tama Zoological Park used two outdoor enclosures one by one, although Female 3 and Male 3 (mother and cub) used the enclosure at the same time in

2018 only. Female 4 and Male 4 at Kobe Oji Zoo used the outdoor enclosure at the same time. Female 5 and Male 5 at Sapporo Maruyama Zoo, Female 7 and Male 6 at Nagoya Higashiyama Zoo and Botanical Gardens also used the same enclosures one at a time. Information about the animals is presented in Table 3.1. Depending on the zoo, all snow leopards were fed mainly horseflesh, chicken breast meat, and/or chicken bone. In general, their food was provided every day except on weekly fasting days. At Tama Zoological Park, snow leopards were fed whole rabbits once a week, and a bundle of straw for play was given to them on three days during the observation period in 2019. Plants were not provided as food at any zoo, however, all individuals except Female 7 and Male 6 were able to access plants in outside enclosures for at least one hour every day.

3.2.2 Behavioral observation and fecal analysis

The behavioral observation was conducted on 11 individuals. Focal animals were observed directly and/or on video while they were in the outside enclosure and their behavior was continuously recorded. Behavior (move, rest, search, marking, plant eat, play, other) was recorded based on a previous study of captive snow leopard (Freeman, 1974). Plant-eating behavior was recorded only when it was certain that they had plants that were growing in the enclosures in their mouths and bite wooden structures (e.g., benches). Since their behavior appears to be unusual during scorching weather or heavy rain, the data from approximately 19 hours (4.5% of total observation) was excluded from the analysis. Behavior bouts were defined as the same bout if a behavior was resumed in 30 seconds and no other behavior (except for move, stand, and rest) was observed. The frequency of plant-eating of each individual was calculated as bouts/hour during each observation period.

The collection of fecal samples was conducted for 10 individuals (Male 1, 2, and 3 were excluded). Whole feces were basically collected every day during the sampling period and stored in airtight plastic bags at -20 Degree Celsius until analysis. Feces excreted at one time was treated as one sample. Each sampling period was 10 to 14 days in a row. The behavioral observation was conducted simultaneously, when possible, to distinguish fecal samples and determine the order if several individuals used the same enclosure in one day or if they defecate more than once in the outside enclosure.

When it was not apparent which individual feces were from at Kobe Oji Zoo or Nagoya Higashiyama Zoo and Botanical Gardens, we used fecal DNA to identify the sex because individuals using the same enclosure were different sexes. DNA analysis used primers from Sugimoto *et al.* 2006 (Sugimoto et al., 2006). The same procedure was repeated three times, and the sex was determined only when the result was consistent.

The fecal samples were freeze-dried overnight (FDU-1200, EYLA, Tokyo), then weighed, and 0.10–0.50 g of powdery parts from each dried fecal sample were removed for other analysis if possible. The rest of the sample was then washed in tap water with 1 mm mesh to pick out undigested matters, hair, plants including pieces of wooden benches and other material (e.g., gravel). The contents from each sample were packed in airtight plastic bags, then freeze-dried overnight and weighed. Fecal samples collected in Tama Zoological Park that included rabbit hair, bone or straw were excluded from the analysis to minimize the differences between captive conditions. When deciding the order of fecal samples, samples lighter than 5 g in dried weight were not counted because at times the same individual defecated several times within a few hours and the small samples were considered as a portion of a larger fecal sample.

3.2.3 Data analysis

Data were analyzed using Microsoft Excel (Microsoft, Tokyo), and R software (version 3.6.1., R foundation for Statistical Computing 2019) (R Development Core Team 3.0.1, 2019).

To test the quantitative relationship among fecal sample contents, the amount of plant matter in a fecal sample, the amount of plant matter contained in the fecal sample evacuated before hair was excreted, and the amount of plant matter contained in the fecal sample evacuated after hair was excreted were set as fixed effect (“s-plant”, “b-plant”, “a-plant”) and the amount of hair in the fecal sample was set as the objective variable (“hair”). The amounts of plant matter contained in fecal samples evacuated before or after hair was excreted were added as variables to consider the possibility that the transition rate of the plant materials and hair could be different. The gamma distribution was selected because the objective variable was continuous and should not have a negative value. Thus, a generalized linear model (GLM) and a generalized linear mixed model (GLMM) with gamma distribution and identity link function was applied. In either dried weight of hairs or plants, significant difference was obtained among sampling units (i.e. individuals and/or sampling periods) (hair; $p < 0.001$, plant; $p <0.001$, respectively, Kruskal–Wallis rank sum test). Thus, we set “individual_period” as a random effect when creating the GLMM. Each of the three variables was applied one by one, resulting in three one-variable models. To use gamma distribution, when the amount of hair was zero, the value was replaced with 0.0001 based on the roundoff error 0.0005 (Arai & Ohta, 2006; Martín-Fernández et al., 2003) in six samples.

Bayesian estimation by “rstanarm” package version 2.19.2 was used to estimate the coefficients of the models. Normal distribution with a mean of zero was used prior because either a positive or negative estimate was allowed, and the sample sizes were small. We ran four independent Markov chains of each model. All iterations were set to

5,000 and the burn in samples were set to 2,500. The value of Rhat for all parameters was equal to 1.0, indicating convergence across the four chains (Kubo, 2018; Namba et al., 2018). We concluded that the estimate was significantly different from zero if the 95% CI range did not stride over zero.

3.3 Results

3.3.1 Plant-eating and vomiting

Behavioral observation was conducted for a total of 417 hours from September 2018 to October 2019, and 398 hours were used for the analysis. Plant-eating behavior was observed in 10 out of 11 individuals. This behavior was most frequent in Male 3 (1.19 bouts/hour) and least frequent in Female 6 (0.06 bouts/hour) (Table 3.2). The longest bout continued for 6 minutes 55 seconds (Male 3), while the shortest bout was only 2 seconds (Male 4). In each individual, these plant-eating behaviors were observed on several days. Vomiting was observed just once in Female 2 and Female 3 and twice in Male 3.

3.3.2 Plant-eating and hair evacuation in fecal samples

In total 192 fecal samples were collected from 8 individuals that were kept in enclosures with plants. Three samples from Tama Zoological Park were not used because they were mixture of several feces. Samples that were lighter than 5 g in dried weight were excluded (*n*=17). Dried weight data was not available for five of the fecal samples from Female 5; however, three samples were still included because total dried weight of feces contents was over 5 g. Live prey (rabbit) and straw were only provided at Tama Zoological Park, so samples that contained rabbit bones and hair or straw were also excluded (*n*=23). This left 147 samples that were included in the analysis. Of the 147 samples, 141 samples (96%) contained snow leopard hair and 95 samples (65%) contained plant matter. As shown in Figure 3.1, plants were sometimes evacuated in an undigested state. Additionally, 14 fecal samples from Female 7 and 15 samples from Male 6 were collected. These two were kept in enclosures without plants but four samples from Male 6 contained pieces of wood from the wooden bench. One sample from Female 7 that was lighter than 5 g was excluded. The results of sex identification were not consistent in three samples thus we didn’t use them in the study. Dried weight of feces, and the amounts of hair and plants included in fecal samples are presented in Table 3.3.

The scatter plot shows the relationship between the amounts of hair and plant included in fecal samples (Figure 3.2). Hairs were evacuated in fecal samples regardless of the presence or absence of plants. Following analyses were conducted on individuals, except Female 7 because there were no plants growing in the enclosures at Nagoya Higashiyama Zoo and Botanical Gardens and no plant was collected from its feces. Prior to modeling for the estimation of the quantitative relationship between amounts of plants and hairs evacuated, we removed fecal samples if the defecation order of samples was not clear. When data of the previous or next fecal sample was not available, they were also excluded. A total of 107 samples were used in this analysis. To consider the possibility that the transition rate of plant materials and hair will be different, three variables (the amount of plant contained in the same fecal sample, and in the samples evacuated before and after hair was excreted) were set as fixed effects (Figure in S2 File). The estimated coefficients of fixed effects in each of the one-variable models are shown in Table 3.4. None of the three variables were significantly different from zero (Table 3.4). In order to find out if there was a sampling unit that had a relationship between hair and plant in the fecal sample, we constructed generalized linear models for each sampling unit. However, irrespective of sampling unit, the estimated coefficients of three variables were not significantly different from zero (Appendix 3.1).

3.4 Discussion

The results of this study confirm that captive snow leopards eat plants fairly frequently and this behavior did not induce vomiting. Together with findings from reports of plant containment in snow leopard feces (Jumabay-Uulu et al., 2013; Wegge et al., 2012), our results suggest that plant-eating is a normal behavior for this species, both in the wild and in captivity. Therefore, growing plants in captive snow leopards' enclosures might be more suitable to bring out their natural behavior in captivity, contributing to the enrichment and thus improving their welfare.

In fecal samples, the amount of hair did not increase in relation to the amount of plants ingested and there was no quantitative relationship between them. Therefore, we conclude that ingested plants do not have an immediate function to evacuate hair. Our data clarified that the traditional hypothesis that ingested plants help to excrete hairballs is not necessarily credible.

In the wild, snow leopards move daily across long distances (Fox & Chundawat, 2016) (e.g., 12 km/day in Mongolia (T. M. McCarthy et al., 2005)). Their broad home range and rugged habitat make it difficult to observe their natural behavior and hinder our ability to consistently collect fecal samples from the same individual. Studies on captive individuals have enabled detailed observations of this plant-eating behavior, allowing continuous sampling to test this relationship with a time difference. This study further demonstrates the importance of studying captive individuals to understand wildlife.

We observed plant-eating behavior in all but one of the snow leopards (Table 3.2), despite daily feeding for the study duration. Hoppe-Dominik (1988) suggested that leopards intentionally eat grass during periods of prolonged starvation to keep their digestive system functioning. However, our results indicate that plant-eating is also common in well-fed captive snow leopards. Contrary to the frequent plant-eating, we rarely observed vomiting. The frequency was not consistent with that of plant-eating, thus we conclude that snow leopards did not eat plants to promote vomit hairballs through stimulation of the throat or stomach.

In this study, hairs were evacuated in feces, regardless of the presence or absence of plants in the enclosures. Also, the amount of hair and plant in fecal samples were varied among individuals and/or sampling terms, indicating individual differences and/or differences between sampling periods in the amount of hair and plants within the enclosures. The length of coat hair of snow leopards is reported to differ with the seasons (Hemmer, 1972). Additionally, depending on the season and the zoo, the abundance and composition of vegetation within the enclosures appeared to vary. This might have caused the individual differences in hair and plant matter in the fecal samples. When creating the GLMM, the difference between individual and/or sampling period was taken into consideration. Still, the results revealed that all three variables did not have significant effects on the amount of hair contained within a fecal sample. Furthermore, the amount of plant matter in fecal samples had no significant relationship with the amount of hair, regardless of time difference. Although we cannot rule out a causative relationship between the amount of plant in feces and hair evacuation, this study was the first to provide evidence that plant matter in feces had no quantitative effect on hair evacuation.

In this study, we obtained continuous data from captive snow leopards to estimate the relationship between plant ingestion and hair evacuation over a period of time. However, captive animals might ingest much less hair from their diet than in the wild because they are mainly fed meat as opposed to live prey. Therefore, we should note that the effect of plant intake may be underestimated due to the lack of prey hair ingestion. Also, plant composition was different from their wild habitat. In some habitats, it was reported that many feces of snow leopards contain *Myricaria spp.* (Jumabay-Uulu et al., 2013), but snow leopards also intake other plant species including grasses in other habitats (Schaller et al., 1988). Although in this study we let snow leopards voluntarily select when and which plant to eat, there was a chance that the effect of plant intake was not detected because the plant species they ate in zoos did not have the required traits.

As stated in introduction, several factors are expected to make strict carnivores eat plants. Our study tested one hypothesis about the adaptive significance of plant-eating in strict carnivores for the first time. We confirmed that snow leopards voluntary and frequently eat plants. However, our results did not support the hair evacuation hypothesis, therefore the advantage of plant intake for snow leopards is still unclear. Further studies are required to evaluate the effects of plant intake not only on physical aspects but also on chemical aspects such as antibiotic compounds. Information about the plant species that snow leopards use in the wild may provide a novel hypothesis to be tested. Another area of research that requires further investigation would be to identify the driving factors of carnivore plant consumption in the wild. Carnivores are known to be indifferent to sugars, as demonstrated by a study on domestic cats that revealed a lack of sweet taste receptors (Lei et al., 2015), bitter taste receptors for plant secondary metabolites (Hu & Shi, 2013). Thus olfactory clues (Bol et al., 2017), or plant texture (Hoppe-Dominik, 1988) might be influencing carnivore plant-eating behavior. To truly understand their ecology, we should pay attention not only to the prey animals but also to the plant species present in the feces of strict carnivores.

Table 3.1 Focal invidivual characteristics.

Individual ID	Name	Sex	Age at study	Location
Female 1	Asahi	Female	8	Tama Zoological Park
Female 2	Mirucha	Female	11	
Female 3	Mimi	Female	10	
Male 1	Valdemar	Male	14	
Male 2	Kovo	Male	5	
Male 3	Fuku	Male	1	
Female 4	Yukko	Female	10	Kobe Oji zoo
Male 4	Fubuki	Male	2	
Female 5	Sizim	Female	9	Sapporo Maruyama zoo
Male 5	Akbar	Male	14	
Female 6	Supica	Female	14	Omuta city zoo / Kumamoto City Zoological and Botanical Gardens
Female 7	Rian	Female	9	Nagoya Higashiyama Zoo and Botanical Gardens
Male 6	Yukichi	Male	10	

Age at study is age at the time of the latest study.

Table 3.2 Plant-eating and vomiting behavior of snow leopards observed in zoos in Japan. Mean bout length of plant-eating with standard deviation (±SD).

Location	Individual	Observation period	plant eat (bout)	vomit (bout)	observed time (h)	Frequency of plant-eating (bout/h)	Bout length of plant-eating (min)
Tama Zoological Park	Female 1	Sep 22, 2018 to Dec 2, 2018	23	0	22.9	1.00	1.0 ± 0.9
	Female 2		3	0	26.5	0.11	0.7 ± 0.7
	Female 3		14	0	38.8	0.36	0.3 ± 0.2
	Female 1	Oct 4, 2019 to Oct 16, 2019	15	0	19.8	0.76	0.7 ± 0.6
	Female 2		10	1	15.5	0.65	0.4 ± 0.6
	Female 3		10	1	26.5	0.38	0.5 ± 0.6
	Male 1	Sep 22, 2018 to Dec 2, 2018	0	0	8.8	0.00	-
	Male 2		25	0	30.4	0.82	0.9 ± 0.8
	Male 3		29	2	24.3	1.19	1.0 ± 1.5
Kobe Oji Zoo	Female 4	May 22, 2019 to Jun 4, 2019.	8	0	105.7	0.08	1.7 ± 1.6
	Male 4		9	0	105.3	0.09	0.7 ± 0.5
Sapporo Maruyama Zoo	Female 5	Sep 10, 2019 to Sep 23, 2019.	10	0	21.6	0.46	1.1 ± 1.1
	Male 5		10	0	20.8	0.48	0.9 ± 0.8
Kumamoto City Zoological and Botanical Gardens	Female 6	Aug 1, 2019 to Aug 14, 2019.	2	0	36.3	0.06	1.4 ± 0.2

Table 3.3 The mean total hair and plant amounts (g, dried weight) contained in fecal samples (mean ± SD).

Location	Individual	Sampling period	Sampling unit	Hair in feces (g)	Plant in feces (g)	Feces weight (g)
Tama Zoological Park	Female 1	Oct 4, 2019 to Oct 16, 2019.	f1 ($n=8$)	0.53 ± 0.47	0.10 ± 0.11	37.67 ± 20.11
	Female 2		f2 ($n=7$)	1.19 ± 2.49	0.03 ± 0.04	26.72 ± 19.83
	Female 3		f3 ($n=11$)	2.41 ± 1.40	0.04 ± 0.02	22.17 ± 9.69
	Male 1 Male 2 Male 3	No data		No data	No data	No data
Kobe Oji Zoo	Female 4	Aug 24, 2018 to Sep 5, 2018	f4_1 ($n=12$)	2.80 ± 2.12	0.35 ± 0.31	45.51 ± 19.12
		May 22, 2019 to Jun 4, 2019.	f4_2 ($n=18$)	2.30 ± 1.65	0.05 ± 0.12	50.17 ± 29.59
		Aug 26, 2019 to Sep 4, 2019.	f4_3 ($n=13$)	1.45 ± 1.15	0.05 ± 0.08	41.60 ± 16.95
	Male 4	May 22, 2019 to Jun 4, 2019.	m4_1 ($n=11$)	3.73 ± 3.47	0.03 ± 0.13	35.16 ± 11.51
		Aug 26, 2019 to Sep 4, 2019.	m4_2 ($n=9$)	1.20 ± 0.90	0.23 ± 0.26	34.31 ± 10.87
Sapporo Maruyama Zoo	Female 5	Sep 10, 2019 to Sep 23, 2019.	f5 ($n=16$)	1.28 ± 0.83	0.10 ± 0.12	27.05 ± 11.96*
	Male 5		m5 ($n=17$)	0.16 ± 0.14	0.14 ± 0.13	34.98 ± 18.54
Omuta city zoo	Female 6	Jul 20, 2018 to Aug 2, 2018.	f6_1 ($n=13$)	3.36 ± 1.95	0.12 ± 0.14	25.39 ± 6.59
Kumamoto City Zoological and Botanical Gardens	Female 6	Aug 1, 2019 to Aug 14, 2019.	f6_2 ($n=12$)	1.60 ± 0.75	0.07 ± 0.09	37.67 ± 14.31
Nagoya Higashiyama Zoo and Botanical Gardens	Female 7	Mar 2, 2020 to Mar 11, 2020	f7 ($n=13$)	1.85 ± 1.61	0.00 ± 0.00	40.81 ± 23.34
	Male 6		m6 ($n=15$)	1.41 ± 1.29	0.06 ± 0.14	34.76 ± 20.94

*Data was not available for three samples.

Table 3.4 The effect of plants on the amount of hair in the fecal sample, as estimated by GLMM (Bayesian estimation). Coefficients with SD.

Models	Model 1	Model 2	Model 3
Objective variable	The amount of hair in fecal sample		
Fixed effect	The amount of plant contained in the same sample	The amount of plant contained in the sample evacuated before hair was excreted	The amount of plant contained in the sample evacuated after hair was excreted
Estimate (± SD)	0.685 ± 0.974	0.852 ± 0.901	0.673 ± 1.105
95% CI range	-0.970 - 2.869	-0.639 - 2.938	-1.142 - 3.255

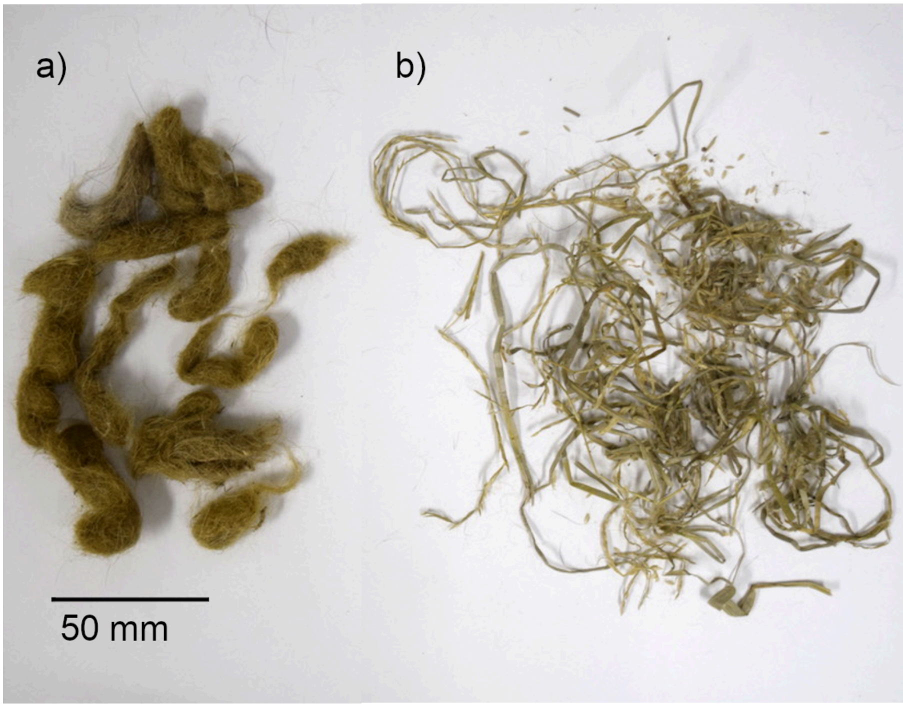


Figure 3.1 Hair and plant collected from snow leopard fecal sample. Example of a) hair and b) plants collected from one fecal sample.

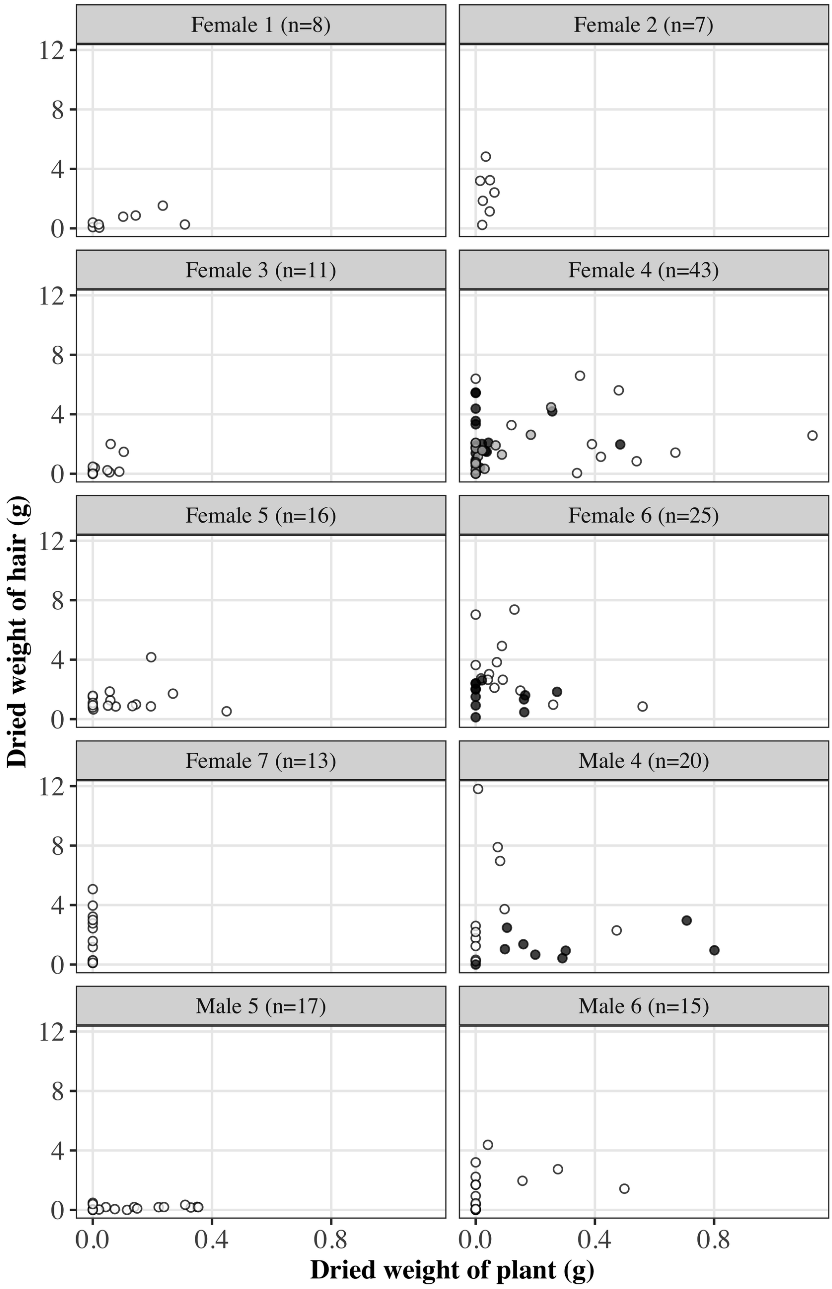


Figure 3.2 Total dried weight of plant and hair contained in snow leopard fecal samples. White, black and gray represent the first, second, and third sampling period, respectively. For the details of the individuals, see Table 3.3.

Chapter 4

Metabarcoding analysis insights into the link between prey and plant intake in the alpine large cat carnivore, snow leopard

4.1 Introduction

As shown in Chapter 2, plant material has been reported in the feces of 24 out of 41 extant felid species; notably, snow leopard feces frequently contained plant materials, despite their alpine habitat where vegetation is typically sparse (Yoshimura et al., 2021). Previous studies have made cursory mentions of grasses and bushes (in particular *Myricaria spp.*) (Fox & Chundawat, 2016) and 45% of their feces contained the shrub *Myricaria spp.* (Jumabay-Uulu et al., 2013) in prey animal surveys but have not investigated the plant species further. Therefore, it is unclear if *Myricaria spp.* is more frequently consumed against other plants and if this is a phenomenon unique to snow leopards compared to other animals. We believe that investigating the plant repertoire consumed by wild snow leopards in alpine environments will deepen our understanding of the plant-eating behavior despite limited plant resources.

The molecular approach using the next generation sequencing (NGS) is widely used in diet analysis for many animals (G. Liu et al., 2021). Deoxyribonucleic Acid

(DNA) metabarcoding analyses DNA contained in samples using high-throughput sequencing. It uses small, highly variable universal primers (barcodes) to identify animals and plants (Pompanon et al., 2012; Thuo et al., 2019). Relative to morphological identification of undigested remains in feces, DNA metabarcoding has higher sensitivity, broader taxonomic coverage and relatively cost-efficient (Pompanon et al., 2012; Thuo et al., 2019). This technique is considered suitable for identifying the potentially diverse dietary plant in carnivores. However, application of metabarcoding method for plant identification in felids is quite limited. A study of leopard cat in China is the only case at the moment (Xiong et al., 2016) and there are few studies that use this method for large cat species.

The primary objective of this study was to explore the reasons why felids eat plants. By identifying plant species in the feces of wild snow leopards, we aimed to understand the feature of plants they frequently consume. This would enable us to infer the function of plant-eating in felids. Based on Illumina sequencing data, we first revealed the frequently consumed prey and plant taxa in snow leopard feces. Simultaneous investigation of prey and plant consumption allows us to evaluate the possibility of secondary consumption through the gut content of prey. Additionally, we identified the dietary plant species consumed by other herbivorous mammals inhabiting the same alpine ecosystem. These included ibex and argali (*Ovis ammon*) that constitute the primary prey for snow leopard, wolf (*Canis lupus*) that is another apex predator species in its habitat, and red fox (*Vulpes vulpes*) that functions as a mid-level predator and omnivore. By contrasting the dietary composition of the other mammal species, we can understand the characteristics of plant eating of the snow leopard. Specifically, which plant characterizes the snow leopard plant-eating. A machine-learning based classification approach was applied to clarify the difference in dietary plant composition of snow leopard and other mammals. A study of Puma showed the sex affects the species and size of prey (Bernard et al., 2023). Additionally, the difference in reproductive roles between sexes influences their behavior and energy requirements (Oftedal & Gittleman, 1989), potentially impacting plant-eating behavior. Consequently, we tried to find out whether the dietary composition differs between sexes in snow leopards.

4.2 Materials and Methods

4.2.1 Ethical note

This research adhered to the legal requirements of the governments of Kyrgyzstan and Japan. All sampling procedures were noninvasive, granted by the State Agency on Environment Protection and Forestry (now Ministry of Natural Resources, Ecology and

Technical Supervision) of the government of Kyrgyzstan, and carried out according to the guidelines for animal studies in the wild and ethics in animal research issued by the Wildlife Research Center of Kyoto University.

4.2.2 Study area

The Sarychat-Ertash Reserve (42°02′N 78°25′E) spans 1,341 km² in the Central Tien-Shan Mountain range's Uch-Kol River basin. It is characterized by altitudes of 2,000–5,500 m and experiences a cold continental climate with mean monthly temperatures in June and January of +4.2 and −21.5 °C respectively, and annual precipitation of 295 mm. The Reserve's vegetation consists of arid grasslands, wet meadows, and tundra cushion plants (*The Sarychat-Ertash State Reserve Management Plan 2007–2015*, 2007). Snow leopard, wolf and red fox are the most common carnivores; brown bear (*Ursus arctos*), lynx, Palla's cat (*Otocolobus manul*) and stone marten (*Martes foina*) are the other carnivores found there. In addition to ibex and argali, potential snow leopard and wolf prey species include marmot (*Marmota baibacina*), hare (*Lepus tolai*), pika (*Ochotona roylei*), and birds such as snowcock (*Tetraogallus himalayensis*) and chukar partridge (*Alectoris chukar*). There are reports of four species of mustelids and four vole species in the area (*The Sarychat-Ertash State Reserve Management Plan 2007–2015*, 2007). Historically affected by human activities such as livestock grazing and illegal hunting, only a small part of the Reserve's buffer zone is used for seasonal livestock grazing.

4.2.3 Sample collection

The fecal samples were collected in November 2017, March and September 2018, May 2019, October 2022, and May 2023. High water levels in the summer and thick snow cover in the winter prevented fieldwork in these seasons during the year. Fecal samples were collected opportunistically. We collected fecal droppings of ungulates in addition to those of carnivores in the autumn of 2022. Typically, whole feces were collected into plastic bags with silica gel after photographing it in its natural setting. Geographical coordinates, altitude, and sampling time were recorded. Since refrigerating facilities were not available at the study site, fecal samples were stored in a dark place at ambient temperature until they were brought to Bishkek city. We took and preserved samples for DNA extraction in two ways, surface swabs with the lysis buffer and inner parts with *RNAlater*. Samples in the lysis buffer were utilized for species and sex identification due to the expected higher concentration of host DNA. The samples taken from the inner region of feces were used for diet analysis to reduce the environmental contamination. Surface of each feces was swabbed by sterile cotton swab and preserved in sterilized 2 ml plastic tubes with 1 ml lysis buffer [0.5% sodium dodecyl sulfate (SDS), 100 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0), 100 mM Tris–HCl (pH 8.0), and 10 mM NaCl (Longmire et al., 1997)], mixed by tapping the tube, and kept in dark boxes at ambient temperature for later processing. We cut the feces with sterile tweezers and transferred the inner parts into 2 ml sterilized plastic tubes with 1 ml RNA*later* solution (Thermo Fisher Scientific, Waltham, MA, USA). The contents in the tube were mixed by tapping the tube, and they were kept in a dark box at ambient temperature for later processing.

4.2.4 DNA extraction

All experimental procedures were performed under sterile conditions, as recommended by Hayakawa et al. (2018). DNA from each fecal sample was extracted and purified using the QIAmp DNA Fast Stool Mini Kit (Qiagen, Hilden, Germany). DNA from samples stored in the lysis buffer was extracted according to the manufacturer's protocol. Samples stored in RNA*later* were first precipitated and then washed twice with 1 ml of phosphate-buffered saline (PBS) (pH 7.4) (centrifugation speed: 20,000 ×g for 10 min). Each of the processed samples was beaten using four zirconia beads (3 mm in diameter) and 1 mg zirconia/ silica beads (0.1 mm in diameter) in a 2 ml plastic tube at 4,200 rpm for 5 min. The DNA samples were then purified using the QIAmp DNA Fast Stool Mini Kit and eluted in 100 µl of Buffer ATE with 30 min of incubation at ambient temperature. The DNA concentrations were estimated with a Qubit dsDNA HS

Assay Kit and a Qubit fluorometer (Thermo Fisher Scientific). The purified DNA samples were stored at 4 °C.

4.2.5 Host species identification

We used molecular species identification to identify the specific origin of each fecal sample. To accomplish that we designed a 16S rRNA primer pair (16SrRNA_L2513_felid: GCCTGTTTACCAAAAACATCAC; 16SrRNA_H2714_felid: CTCCATAGGGTCTTCTCGTCTT) to amplify an ~244 bp (excluding the primers) mitochondrial 16S rRNA gene sequence. The PCR conditions and programs are provided in Appendix 4.1. The PCR products were purified by using a High Pure PCR Product Purification Kit (Roche, Basel, Switzerland). Direct sequencing was performed using the Big Dye 3.1 Terminator cycle-sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The cycle sequencing products were purified by ethanol precipitation and nucleotide sequences were determined using an ABI PRISM 3130xl genetic analyzer (Applied Biosystems). Forward and reverse complement sequences were aligned using MEGA11 (Tamura et al., 2021). The resulting sequences were searched in the GenBank nucleotide (nt) database and species identity was determined based on the matches with the highest similarity scores (95–100%).

4.2.6 Sex identification

After identification of species as snow leopard, we identified sex of the individual that the sample belonged to. We used one set of four primer targeting introns of Zinc-finger in X chromosome (ZFX-PF/PR) and DEAD box polypeptide in Y chromosome (DBY7-PF/PR) (Sugimoto et al., 2006). The PCR conditions and programs are provided in Appendix 4.1. PCR products were electrophoresed and visualized on 2.0% agarose gels. The same procedure was repeated at least twice, and the sex was determined only when the results were consistent.

4.2.7 Library preparation and amplicon sequencing

Library preparation and amplicon sequencing were performed with the MiSeq system (Illumina, Inc., San Diego, CA, USA) according to the manufacturer's protocol with modifications optimized for our sample as follows. Five different marker sets were used to analyze species' diets. A universal vertebrate 12SV5 marker (Riaz et al., 2011); three universal plant markers: Uniplant (Moorhouse-Gann et al., 2018), rbcL mini-barcode (Erickson et al., 2017; Kress & Erickson, 2007), trnL-g/h (Taberlet et al., 2007); and finally one Poaceae specific marker (ITSPoa) (Baamrane et al., 2012) to increase the taxonomic resolution for grasses, which are expected to be abundant in the environment. These primers were fused with 3-6-mer Ns and specific overhang adapters 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-(forward primer)-3′ and 5′-

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-(reverse primer)-3′. PCR was performed using the KAPA HiFi HotStart ReadyMix PCR Kit (Kapa Biosystems, Inc., Wilmington, MA, USA) with 200 nM of each primer and 25 ng DNA as the template in a total volume of 25 µl. The PCR conditions and programs are in Appendix 4.1. When 25 ng DNA was unavailable due to low DNA yield, the available maximum volume of the DNA solution was used in the PCR. The resulting amplicons were visualized on agarose gels.

Each PCR product (20 µl) was purified using 36 µl Agencourt AMPure XP beads (Beckman Coulter, Inc., Carlsbad, CA, USA) with 80% ethanol washes. Each of the purified PCR products was eluted in 10 mM Tris–HCl (pH 8.5). Using the KAPA HiFi HotStart ReadyMix PCR Kit and the Illumina Nextera XT Index Kit v2, specific dual indices and sequencing adapters were attached to each amplicon by PCR conducted in a 50 µl solution containing 5 µl of each of the forward and reverse primers and 5 µl of the first purified PCR solution. The resulting amplicons were visualized on agarose gels. Each product (45 µl) was purified using Agencourt AMPure XP beads with 80% ethanol washes. Each of the purified products from the second PCR was eluted in 27.5 µl of 10 mM Tris–HCl (pH 8.5).

The ’NA concentration of each product was measured with a Qubit ds’NA HS

Assay Kit. Products were mixed in the same amount of DNA concentrations to form the pooled sequencing library. Fragment size distribution of the library was estimated with an Agilent 2200 TapeStation (Agilent Technologies, Inc., La Jolla, CA, USA). The library was diluted to 15 pM and subjected to a sequencing run mixed with other libraries unrelated to this study and 30% PhiX spike-in on an Illumina MiSeq sequencing platform using the MiSeq Reagent Kit v3 (600 cycles). Sequencing was separately operated in four different runs. The read lengths from the MiSeq run were 301 bp (forward sequences), 8 bp (forward indices), 8 bp (reverse indices), and 301 bp (reverse sequences). Although quality scores of nucleotides at the 3′-end of Illumina sequences are generally low, the amplicon sizes of this study were smaller than the number of cycles of the kit (i.e., 600). Therefore, overlapping regions of the forward and reverse reads were used to restore these low-quality sequences in the following bioinformatics procedure.

4.2.8 Bioinformatics

As suggested in Toju et al. (2016), we converted the raw MiSeq BCL data into FASTQ data by ourselves using the bcl2fastq v2.20.422 program distributed by Illumina to prevent the potential demultiplexing errors, and we then demultiplexed the FASTQ data using the program Claident v0.9.2022.04.28. In the demultiplexing and primer-trimming process with Claident, all the sequencing reads containing low quality (quality scores <30) index sequences were eliminated and no mismatch between input and output index sequences was tolerated. Adapter sequences were trimmed using Skewer (https:// sourceforge.net/projects/skewer) (H. Jiang et al., 2014) and the forward and reverse sequences were corrected with DADA2 (https://github.com/benjjneb/dada2) (Callahan et al., 2016) package on R programming interface (R Development Core Team 3.0.1, 2019). Reads containing ambiguous bases were removed and trimming lengths were adjusted based on sequence quality profiles, so that Q-scores remained above 30. Error model calculation (for R1F/R2R read pairs and then R2F/ R1R read pairs), read correction, read merging and chimera removal was performed at default settings implemented in DADA2. All the resulting amplicon sequencing variant (ASV) table were curated with LULU (Frøslev et al., 2017) package on R to remove spurious ASVs. As the aim of the present study was to detect and identify species, and not intraspecific variation, we decided to create clusters of sequences, instead of denoising and creating ASV (Antich et al., 2021; Lynggaard et al., 2022). According to the developers of LULU algorithm, incorporation of DADA2 and LULU is a safe pathway for producing reliable and accurate metabarcoding data (Frøslev et al., 2017). The LULU curation requires an external algorithm to produce the match list. Thus we used VSEARCH v2.21.1 as recommended by the developers (Frøslev et al., 2017).

The remaining operational taxonomic units (OTUs) were then subjected to molecular taxonomic identification based on the automatic database search algorithm of the query-centric auto-*k*-nearest-neighbor (Qcauto) method (Tanabe & Toju, 2013) and subsequent taxonomic assignment with the lowest common ancestor (LCA) algorithm (Huson et al., 2007) using Claident. Among the filtered databases bundled with Claident, we used the 'animals_mt_genus' and 'animals_mt_species' sub-databases for 12SV5 region; 'plants_rbcL_genus' and 'plants_rbcL_species' sub-databases for rbcL mini-barcode; 'plants_cp_genus' and 'plants_cp_species' sub-databases for trnL g-h; 'overall_genus'; 'overall_species' databases for Uniplant and ITS1Poa. The Qcauto search information was then subjected to taxonomic assignment with the LCA algorithm (LCA/genus results). As the default setting of the LCA algorithm sometimes returns conservative results, additional taxonomic assignment was conducted with a relaxed setting tolerating 5% mismatches of taxonomic information among database sequences in the LCA process (relaxed-LCA/genus) (Tanabe & Toju, 2013). The overall identification results were obtained by merging the LCA/species, LCA/genus and relaxed-LCA/ genus results in this priority order using the 'clmergeassign' command of Claident. Since the Qcauto method is conservative (Tanabe & Toju, 2013), we conducted additional megablast search for 12SV5 marker and complemented the taxonomic assignment. If an OTU was assigned to several species and we knew which candidate species inhabit the study area (Davletbakov et al., 2015), we assigned the inhabiting species to the OTU. When several local species assigned with same probability or no species was assigned with >95% match, we kept the QCauto result.

Index hopping rate of MiSeq is estimated to be 0.001 (MacConaill et al., 2018). To ensure that index hopping did not result in false positives, the reads of the OTU in the samples were removed whenever the number of reads of an OTU detected in each sample were <0.001 of the number of reads of the OTU detected in all samples (Tsukamoto et al., 2021). A recent study showed that a combination of a sample-based threshold with removal of maximum taxon contamination is an optimal method to remove artefacts (Drake et al., 2021). Following the suggested filtering process (Drake et al., 2021), read counts within a sample that are less than a proportion of the total sample read count for that sample were removed. We decided the threshold proportion to 0.01 and 0.05 for the universal markers (Ando et al., 2018; da Silva et al., 2019; Erickson et al., 2017) and ITS1Poa (de Barba et al., 2014), respectively. Threshold proportion of 12SV5 varied from 0.001 (Xiong et al., 2017) to 0.05 (Shao et al., 2021) thus we choose 0.01 as other universal markers. In addition, we removed any read counts within each OTU that lower than the highest read count within a negative control or blank cells for that OTU (Drake et al., 2021). Based on the molecular taxonomic identification results, nontarget OTUs (non-vertebrate and human in 12SV5, non-plant for the three universal plant markers, and those not in Poaceae family for ITS1Poa) were excluded. The OTUs from host carnivore species were also excluded in the 12SV5 dataset. The sequencing read set of each sample was rarefied to the minimum coverage rate among the analyzed samples (Chao & Jost, 2012) using vegan (Oksanen et al., 2022) package of R. The coverage rate of each marker was 1.00, 1.00, 0.83, 0.83, 1.00, for 12SV5, Uniplant, rbcL, trnL, ITS1Poa, respectively.

In order to overcome problems of primer specificity and bias, we integrated information from the four molecular markers used for plant identification using the python 3.0 script (da Silva et al., 2019). The script provides a single list of taxa detected per sample controlling for duplications by collapsing less resolved taxa detected by one marker with higher resolved taxa detected using a different marker (da Silva et al., 2019). The ITS1Poa marker was Poaceae specific marker to improve the resolution of grasses. Considering that Poaceae are common and fecal samples are often on the ground with grasses, Poaceae specific amplification may increase the risk of amplification of rare sequences contaminated from the environment. Therefore, data from the ITS1Poa marker was only merged to the samples in which Poaceae sequence was detected by other three universal makers.

4.2.9 Statistical analysis

Visualization and basic statistical analyses were performed using the phyloseq v1.26.1 (McMurdie & Holmes, 2013) package in R. Dietary data were summarized across samples using two occurrence-based metrics commonly used in molecular dietary data analysis: (1) frequency of occurrence and (2) weighted percent of occurrence (Deagle et al., 2019). The number of samples that contain a given food item is expressed as FOO whereas wPOO weighs each occurrence according to the number of food items in the sample (i.e. lower weights to individual food taxa in a mixed meal), which is considered to be more biologically realistic (Deagle et al., 2019). Since we merged data from multiple markers, we did not use a sequence abundance-based metric. The samples without any prey or plant OTUs were excluded from subsequent statistical analyses.

We used machine learning models using the randomForest package (Liaw & Wiener, 2002) in R to determine which plant genera best discriminated whether a sample came from snow leopard or other sympatric mammals based on the sample-plant matrix (Goldberg et al., 2020; Urban et al., 2022). RandomForest evaluates an ensemble of decision trees to perform classification (Liaw & Wiener, 2002), in this instance, it classifies snow leopards and other mammals based on the plant composition in their fecal samples. Random forest models are considered to be robust against overfitting and known to have high predictive accuracy (Breiman, 2001). We tuned the random forest models to determine the number of variables (mtry) to try at each node of the tree that resulted in the lowest out-of-bag (OOB) error rates using randomForest function. OOB error is an internal validation method, estimating the prediction error of random forest models by using bootstrap samples not included in the construction of each tree. Since the number of samples were biased toward snow leopard, classwt option with inverse of the ratio of the sample size was used to enforce penalties for errors in minority category. Random forests estimate the variable importance. Thus, we were able to identify which plant genera represents the snow leopard feces. Random forests provide two indicators for variable importance: mean decrease accuracy (MDA) and mean decrease gini (MDG). MDG is considered to be more stable than MDA (Calle & Urrea, 2011), therefore we used MDG as the indicator.

A post hoc probabilistic co-occurrence analysis was conducted to show which taxa are simultaneously present in the same fecal samples of predators using package cooccur (Griffith et al., 2016) in R. A prey-specific co-occurrence would indicate secondary predation of prey gut content (Tercel et al., 2021). In addition, we summed up all prey OI as single “prey” OTU and evaluated co-occurrence with each plant OTU that indicated accidental intake from the environment such as grasses on the ground.

The difference in the dietary composition between each sex of snow leopard were assessed by permutational analysis of variance (PERMANOVA) using the adonis2 function with 999 permutations and visualized by non-metric multidimensional scaling (NMDS) using Bray-Curtis dissimilarity, as implemented in vegan (Oksanen et al., 2022).

Constrained analysis of principal coordinates (CAP) (Anderson & Willis, 2003) was performed to evaluate dietary composition differences between male and female snow leopard (model 1:dietary animal composition, model 2: dietary plant composition) while accounting for the effects of sampling season and spatial autocorrelation. Spatial autocorrelation variables were added to consider the effect of spatial proximity on the sample. We first generated a set of Moran's eigenvectors from the coordinates of each sampling point using distance-based Moran's eigenvector maps (MEMs) (Legendre & Legendre, 2012). We then identified positive MEMs that significantly ($p < 0.05$) described spatial patterns using the function “moranNP.randtest” using the R package adespatial (Dray et al., 2021). The four CAP models were constructed by setting the Bray-Curtis dissimilarity as a response variable. The models included “Sex”, “Sampling season", "Altitude" and MEM vectors as explanatory variables. April and May were defined as spring while September and October were defined as autumn. The variables' variance inflation factors (VIF) were computed to check the collinearity. If the variable's VIF was above 20, the variable was excluded from the model, resulting in different degrees of freedom in MEMs. The significance of models and explanatory variables were tested using permutational analysis of variance with 999 permutations.

4.3 Results

4.3.1 Summary of sequence data

We collected 150 mammal fecal samples in total out of which, we could genetically identify the host species of 126 samples. These samples (90 snow leopards; 7 wolves; 9 red foxes; 3 brown bears; 9 ibexes; 7 argali; 1 marmots) were used in the dietary analysis. We obtained 16,623,180 raw sequence reads after demultiplexing (27,567 reads per sample on average). The 11,453,323 (20,862 reads per sample on average) that passed the filtering processes were used as curated OTUs in the following analysis.

The results from FOO and wPOO were qualitatively similar, thus we show wPOO-based results for subsequent dietary analysis in the main text.

4.3.2 Prey

In total 13 OTUs were found from predator samples. Wild ungulates and marmot were the frequently detected prey species in Sarychat-Ertash for the larger predators. Ibex was the main prey of snow leopards, whereas wolves and red foxes preyed more on marmots. We detected smaller mammals mainly in red fox fecal samples (Figure 4.1).

There were 51 samples from male and 27 samples from female snow leopard. We could not determine the sex in the remaining 12 samples. Figure 4.2 is the NMDS plot of prey diet composition based on Bray-Curtis dissimilarity (stress: 0.138). Remarkably, we found no OTU of argali in samples from female snow leopards (Figure 4.3). However, the result of PERMANOVA (999 permutation, p=0.141) as well as CAP model (p=0.601) did not show a significant difference between male and female for prey composition. The CAP model controlled the effect of spatial proximity and sampling season and explained 85% of total variance.

4.3.3 Plant

The Uniplant, rbcL, trnL, ITS1Poa markers detected 69, 34, 62 and 48 OTUs respectively. The composition of detected plant taxa was similar among Uniplant and rbcL, but trnL showed different compositions (Apendix 4.3-4.5). The merged OTU table contained 141 OTUs. Figure 4.4 shows the five most frequent plant genera and figure 4.5 shows the three most frequent plant families in each mammal species. Three plant families, Asteraceae, Tamaricaceae (including *Myricaria spp.*) and Poaceae were frequently present in snow leopard samples from Sarychat-Ertash nature reserve (Figure 4.4 and 4.6). While Asteraceae and Poaceae were also detected from other five mammals except marmots, Tamaricaceae was rarely detected in other species. Wolf and fox samples often contained grasses (*Poa spp.*, *Stipa spp.*), whereas ungulates typically consumed Asteraceae, Poaceae and Chenopodiaceae (*Lepidium spp.*, *Chenopodium spp.*, *Krascheninnikovia spp.*) in autumn.

We achieved a final OOB error rate of 12.61%. The model correctly identified snow leopards in 78% of the samples it labeled as snow leopards (precision), and it correctly found 83% of the actual snow leopard samples in the dataset (recall). Figure 4.6 shows the important plant taxa to classify whether each sample was from snow leopard or other sympatric mammals. The top 30 important plant genera based on MDG were shown in the figure. *Myricaria spp.* was notably important plant genera to distinguish snow leopard samples from other sympatric mammals, and wPOO of this genus was higher in the snow leopard samples.

The post-hoc co-occurrence analysis showed that *Myricaria spp.*, which was a representative plant genus in snow leopard feces, was negatively co-occurring with prey DNA (p=0.00005) while Festuca, Rosaceae and *Ephedra spp.* OTUs in snow leopard feces co-occurred with ibex OTU (*p*=0.002, 0.006, and 0.006, respectively) (Figure 4.7).

The difference of dietary plant composition between sexes are visualized in the NMDS plot (stress: 0.067) where we removed outliers to make it easy to interpret (Figure 4.8). The CAP model explained 49% of the total variances and showed that plant composition was different among sex (999 permutation test, *p*=0.009), although the result from PERMANOVA was a little above the significance threshold (*p*=0.051). The effect of the sampling season (spring or autumn) was marginally significant with *p*-value below 0.1. *Myricaria spp.* was not detected from female samples in autumn, when the presence of *Ephedra spp.*, Asteraceae, Poaceae, and Crassulaceae increased. In case of males, *Myricaria spp.* was detected during both seasons.

4.4 Discussion

4.4.1 Prey

Carnivores in Sarychat-Ertash relied on wild ungulates and marmots as reported in previous microhistological research (Jumabay-Uulu et al., 2013). Conducted between June and October in 2009 at the same study site as ours, Jumabay-Uulu (2013) reported higher occurrence of argali than ibex in the diets of snow leopard and wolves (18:3 for snow leopards, and 12:8 for wolves). In contrast, our study finds occurrence of argali to be lower than that of ibex in the carnivore diets (Jumabay-Uulu et al., 2013). This variation may be due to differences in methodology (e.g., microhistological vs. molecular, sampling season, sampling location) or ecological factors (e.g., changes in relative abundance). Many argali died due to unexpected heavy snowfall in 2022 (Zhumabai-uulu, *pers. comm.*), which might have influenced the proportion of argali and ibex in carnivores' diets. Samples from red fox contained more small mammals such as rodents. Ungulates OTUs from red fox samples are more likely to be from scavenging given their small body size. One snow leopard sample contained a small number of red fox DNA reads ([red fox] : [snow leopard]=180 : 8468), and two red fox samples contained a small number of snow leopard DNA reads ([red fox] : [snow leopard]=23892 : 1194 and 26974 : 703). Red fox sometimes scavenges from snow leopard kill thus snow leopard DNA in red fox samples was probably a byproduct of scavenging. Red fox sometimes defecate close to the scrapings of snow leopard (Janečka et al., 2011) and snow leopard is known to kill smaller predators such as red fox to avoid scavenging (Samelius et al., 2023).

We did not find a significant difference of dietary prey items between sexes in snow leopard samples. Figure 4.2 shows that two female samples showed different prey composition from that of males. One sample contained *Tetraogallus himalayensis* and another contained unidentified Artiodactyla (the order to which ungulates belong). Unidentified Artiodactyla OTU was believed to be a byproduct of DNA degradation since the sample contained *Capra sibirica* OTU as well. The limited diversity of potential prey mammals in the study area might have obscured any sex differences. Notably, only male samples contained traces of argali (Figure 4.3). Although we did not identify individuals, argali was detected from male samples collected in 2018, 2022 and 2023. Females (36-43 kg), being slightly smaller than males (only males reaching the 43-52 kg range), might prey on argali (60-185 kg) less often due to its larger size compared to ibex (30-100kg) (Fox & Chundawat, 2016; University of Michigan Museum of Zoology, 2020). It will require further detailed investigation to determine whether this difference between male and female snow leopard's consumption of argali was an artifact of the size difference between male and female snow leopards (Fox & Chundawat, 2016; University of Michigan Museum of Zoology, 2020), or the possibility that with their larger home ranges (Johansson et al., 2018), male snow leopards were more likely to venture into sub-optimal habitat such as rolling terrain that are used by argali (Mallon et al., 2016).

4.4.2 Plant

We found various plant taxa from snow leopard samples. The frequent detection of the genus *Myricaria* agreed with previous observation-based reports in the same study sites

(Jumabay-Uulu et al., 2013). However, the result showed that some plant OTUs co-occurred with prey OTUs. The *Festuca*, Rosaceae and *Ephedra* OTUs in snow leopard feces were positively detected with ibex OTU indicating the possibility of secondary consumption from ibex gut content. Although *Ephedra spp.* was not detected from ibex samples collected in this study, a previous study reported that livestock ate *Ephedra*'s young shoot in early spring in China (Mikage et al., 2008). In this study, ibex samples were collected in autumn thus seasonal food plant fluctuation might have prevented the detection of *Ephedra* OTUs. It is also reported that Siberian ibex in eastern Tianshan, China preferred eating forbes (Asteraceae, Gentianaceae, Rosaceae, Fabaceae) that has higher nutritional value than graminoids during the warm season and graminoids occupied a high proportion of their diet during the cold season (Han et al., 2020). Since our co-occurrence analysis is only exploratory, it does not necessarily confirm ecological interactions (Tercel et al., 2021). However, given that plants that positively co-occurred were reported to be frequently consumed by ungulates, their presence in snow leopard feces is more likely attributable to secondary consumption rather than voluntary intake by the snow leopards.

On the other hand, *Myricaria spp.*, the representative plant in snow leopards, tended to be detected from samples which did not contain any prey OTUs. This suggests that snow leopards intentionally consumed this bush more frequently, particularly when their digestive tracts were empty. The factors that cause snow leopards to intake *Myricaria spp.* may have some relationship to whether the individual obtained prey or not. In domestic cats, it has been hypothesized that constant availability of food (ad libitum feeding) may reduce the inclination to ingest alternative items such as plastic. (Demontigny-Bédard et al., 2016). When the digestive tracts of felids are empty, they may exhibit a tendency to bite hard objects as a means to compensate for their appetite. One species in this genus, *Myricaria bracteata*, has been used in traditional Tibetan medicine and contains anti-inflammatory compounds (J. B. Liu et al., 2015), although its medicinal effects have not been specifically tested on snow leopards. Therefore, intake of *Myricariaia spp.* and the failure to acquire prey may be related to the individual health condition of the snow leopards.

The results showed a significant difference of plant composition between male and female snow leopards. The potential effect of the sampling season was also indicated. Female samples tended to contain *Ephedra spp.* and Asteraceae as often as *Myricaria spp.*. Besides, no *Myricaria spp.* were detected from female samples collected in autumn (Figure 4.9). As previously mentioned, *Ephedra spp.* and Asteraceae were suspected to be instances of secondary consumption. Since snow leopard give birth in mainly early summer (Johansson et al., 2020), this difference could be resulting from the seasonal behavioral differences among male and female. The genus *Myricaria* is not a dominant plant in the study area and is sporadically distributed along rivers. Although there is little information about snow leopard nursing behavior in the wild, during nursing period, mother may have preferred to stay closer to the cubs than proactively look for the *Myricaria spp.* patch. A female captive snow leopard exhibited a lower frequency of plant-eating behavior in the year she shared an enclosure with her cub, as compared to the following year when the cub became independent (Yoshimura et al., 2020). While our results are indicative, it is important to consider the caveat of small sample size especially in case of female feces. The CAP model explained only half of the total variance, thus there is a possibility of other factors, not included in this study, affecting the presence of plants in snow leopard diet.

A inter-regional sampling with a specific study design is required to better understand the relationship between snow leopard and plant. This will shed light on regional differences and similarity. *Myricaria spp.* was often detected in snow leopard diet from other countries such as Nepal and India, but frequent containment of feather grass was reported in Mongolia (Fox & Chundawat, 2016). Comparison of plant repertoire in different regions will provide answers to why snow leopards selectively intake on *Myricaria spp.* in this study area and identifying commonalities will lead to understanding the adaptive significance of plant-eating. In addition, a comprehensive vegetation survey is necessary to evaluate the preference in light of availability.

4.4.3 Limitation

Due to challenging terrain that limited human access, we could not establish a clear transect for sampling, and we did not identify individual animals for each fecal sample, possibly leading to sampling bias. Seasonal constraints further limited our study; for example, high water levels and deep snow prevented sampling in summer and winter. Additionally, the number of fecal samples from species other than snow leopards was limited, restricting our ability to perform statistical comparisons between species. While metabarcoding is powerful for diet analysis, its high sensitivity can also pick up environmental contamination or accidental intake (Tercel et al., 2021). Sometimes there was amplification bias as we found in plant markers in this study (Appendix 4.3 and 4.4). Although we took steps to minimize these biases, they could not be entirely eliminated. The resolution of the markers was not enough to identify plant OTUs at the species level, and the lack of a comprehensive vegetation survey in the area could have led to inflated diversity estimates for plant OTUs.

4.4.4 Conclusion

In this study we applied a molecular-based approach to comprehensively investigate animal and plant in feces of mammals in the alpine habitat of Kyrgyzstan. Detected prey items from large carnivores agreed with previous study in the same study site. Red fox, a mesocarnivore, consumed smaller mammals as well. Although statistical significance was not detected, consumption of argali was biased toward male snow leopards indicating the possibility of prey selection according to the predator's body size.

We focused on dietary plants and highlighted the feature of plant repertoire in snow leopard feces. As mentioned in an observation-based report, the genus *Myricaria* characterized the snow leopard samples. We found the plant was negatively co-occurred with prey DNA, indicating the consumption of this bush when the digestive tracts were empty. This suggests the importance of simultaneous investigation of prey and plant in carnivore diet. Since this study was exploratory, adaptive significance of plant-eating behavior remains a mystery. However, our results lay the foundation for formulating hypotheses and provide a cue to determine the direction of further research.

Unveiling the relationship between snow leopard and plant, obligate carnivores and plants in general, improve our understanding of not only their behavior and ecology but also evolution of diet repertoire and animal-plant interaction in the ecosystems.

Table 4.1. List of primers used in the study.

Usage	Name	Primer sequence (5’ – 3’)	Reference
Species identification	16SrRNA_L2513_felid	GCCTGTTTACCAAAAACATCAC	This study
	16SrRNA_H2714_felid	CTCCATAGGGTCTTCTCGTCTT	
Sex identification	ZFX-PF	TACCGAGCGATATAGCTCCAG	Sugimoto et al. (2006)
	ZFX-PR	GTGTTCCTACGTTAAGCTATTG	
	DBY7-PF	CTCATGAAGCCCTATTTTTGGTTG	
	DBY7-PR	ACGGCGTCCGTATCTTCCA	
Diet analysis	12SV5F	TAGAACAGGCTCCTCTAG	Riaz et al. (2011)
	12SV5R	TTAGATACCCCACTATGC	
	UniplantF	TGTGAATTGCARRATYCMG	Moorhouse Gann et al. (2018)
	UniplantR	CCCGHYTGAYYTGRGGTCDC	
	rbcL-F	CTTACCAGYCTTGATCGTTACAAAGG	Erickson et al. (2017)
	rbcL-R	GTAAAATCAAGTCCACCRCG	Kress and Erickson. (2007)
	trnL-g	GGGCAATCCTGAGCCAA	Taberlet et al. (2007)
	trnL-h	CCATTGAGTCTCTGCACCTATC	
	ITS1-F	GATATCCGTTGCCGAGAGTC	Baamrane et al. (2012)
	ITS1Poa-R	CCGAAGGCGTCAAGGAACAC	

Table 4.2. The number of identified taxa for each marker. The total number of plant OTUs after merging the four plant markers is labeled as "Merged".

Marker	Order	Family	Genus	Species	OTUs
12SV5	5	7	11	11	13
Uniplant	11	17	25	5	69
rbcL	9	13	13	1	34
trnL	20	28	19	0	62
ITS1Poa	1	1	10	1	48
Merged	20	29	44	7	141

Table 4.3. Constrained analysis of principal coordinates (CAP) for factors structuring the prey and plant composition in snow leopard samples. Models included sex, altitude, sampling season and the MEM vectors (i.e. horizontal spatial structure) as explanatory variables. Model 1 included wPOO-based OTU matrix of prey and Model 2 included that of plant. Significant variables are highlighted in bold.

Model	OTU matrix	Explanatory variable	d.f.	F	p
Model 1	Prey	Sex	1	0.7	0.601
		Altitude	1	1.4	0.273
		Season	1	1.7	0.206
		MEMs	18	2.1	0.027
Model 2	Plant	**Sex**	1	2.5	0.009
		Altitude	1	0.4	0.990
		Season	1	1.6	0.090
		MEMs	21	1.4	0.004

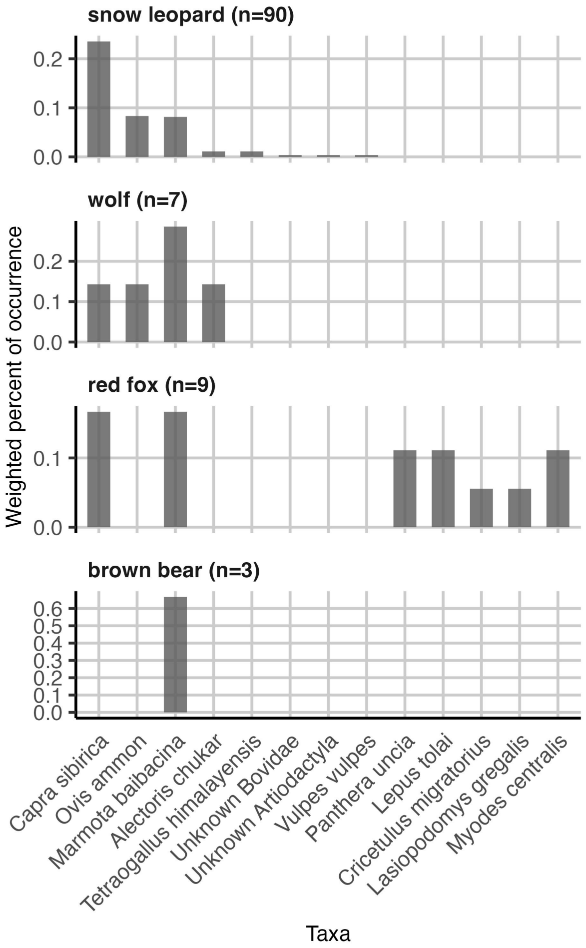


Figure 4.1. Weighted percent of occurrence of vertebrate taxa for predators. The number in the parentheses shows the number of fecal samples.

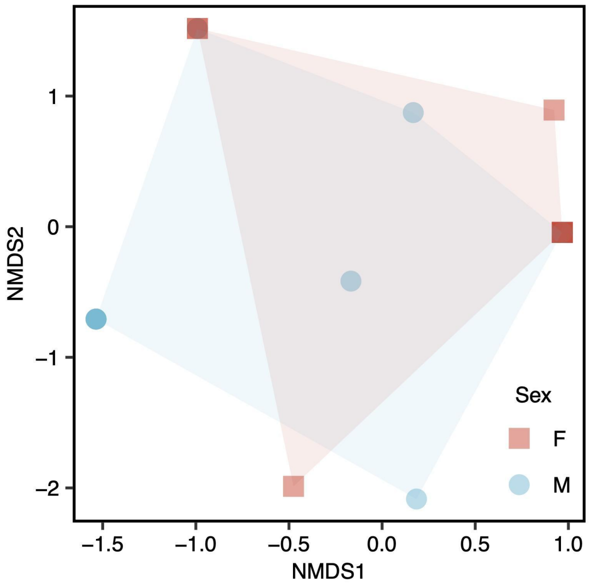


Figure 4.2. NMDS plot of wPOO-based Bray-Curtis dissimilarity of vertebrates from snow leopard samples. Color and shape correspond to different sex.

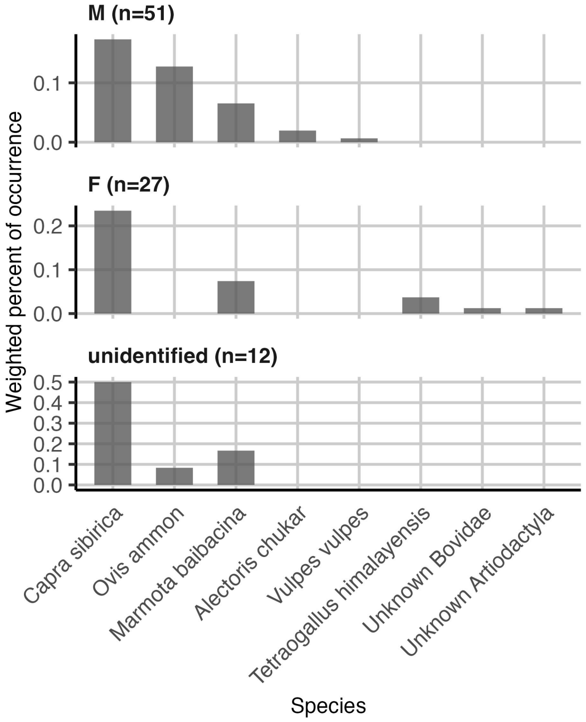


Figure 4.3. Weighted percent of occurrence of vertebrate taxa in snow leopard feces. The caption "M" represents male samples and "F" represents female samples. The number in the parentheses shows the number of fecal samples.

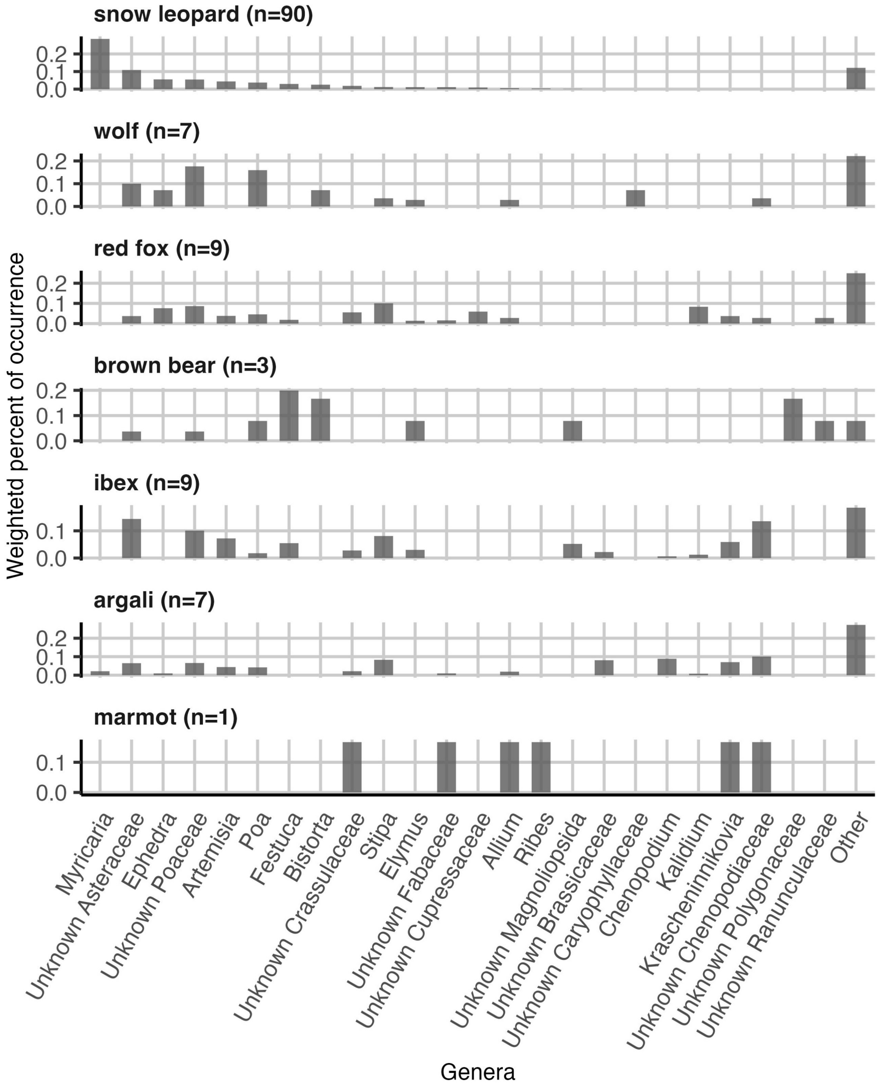


Figure 4.4. Weighted percent of occurrence of the five most frequent plant genera in feces from each mammal. Less frequent taxa were summarized as “Other”. The number in the parentheses shows the number of fecal samples.

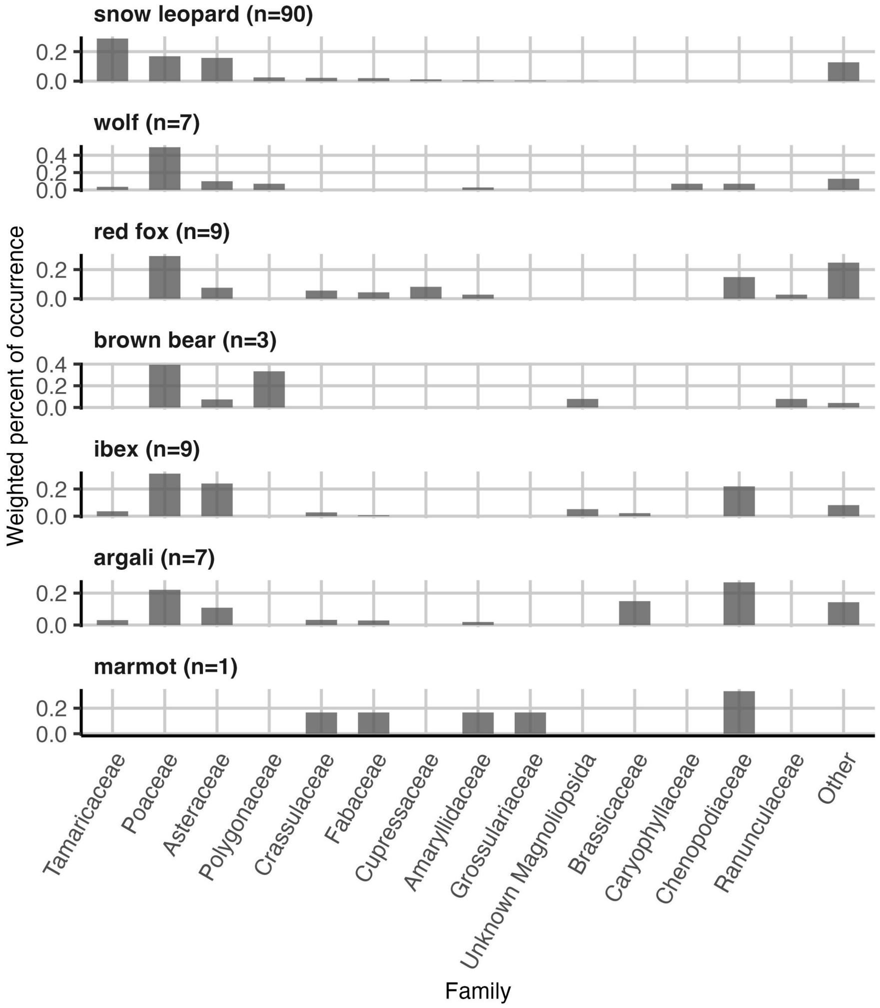


Figure 4.5. Weighted percent of occurrence of the three most frequent plant families in feces from each mammal. Less frequent taxa were summarized as “Other”. The number in the parentheses shows the number of fecal samples.

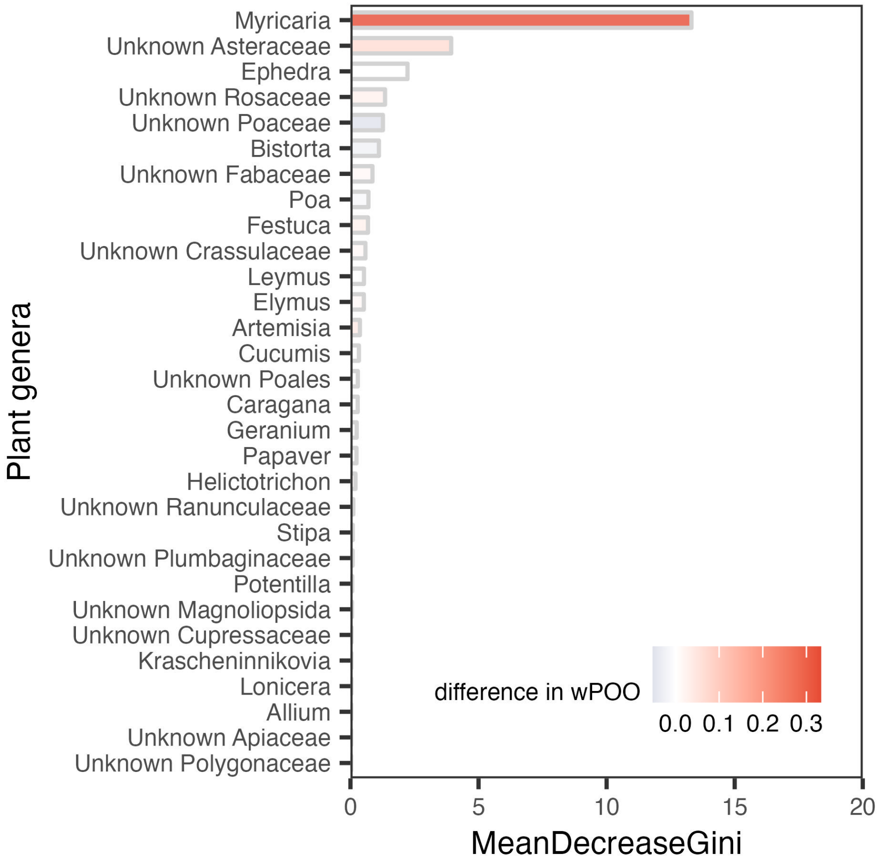


Figure 4.6. Variable importance contribution of the random forest model. Only the top 30 genera are included representing the plant genera that are most important to the model's ability to distinguish between a sample from snow leopard and a sample from other mammals.

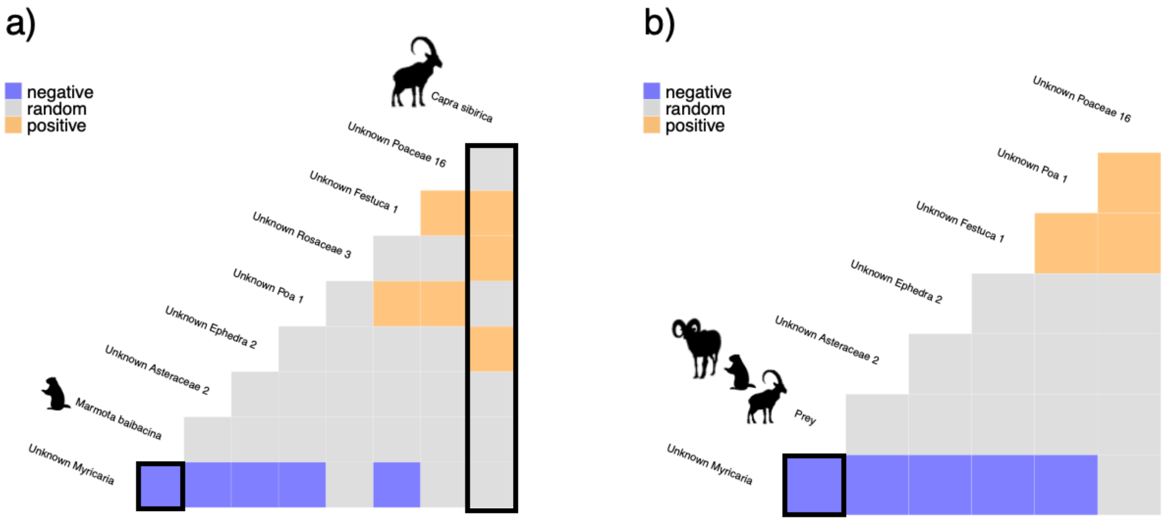


Figure 4.7. Co-occurrence matrix of plant OTUs and a) each prey OTUs, b) summarized prey OTU. Names of OTUs are positioned to indicate the columns and rows that represent their pairwise relationships with other OTUs. The color of each cell represents positive, negative, and random co-occurrence. Cells that show positive and negative co-occurrence of prey and plant were highlighted.

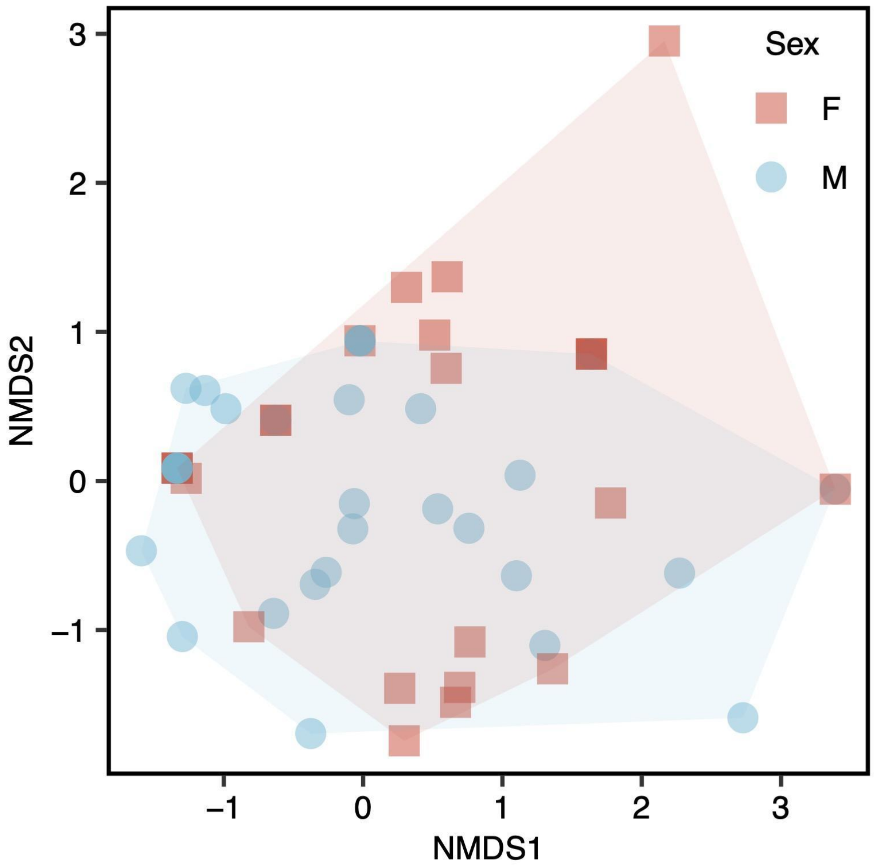


Figure 4.8. NMDS plot of wPOO-based Bray-Curtis dissimilarity of plants from snow leopard samples. Color and shape correspond to different sex.

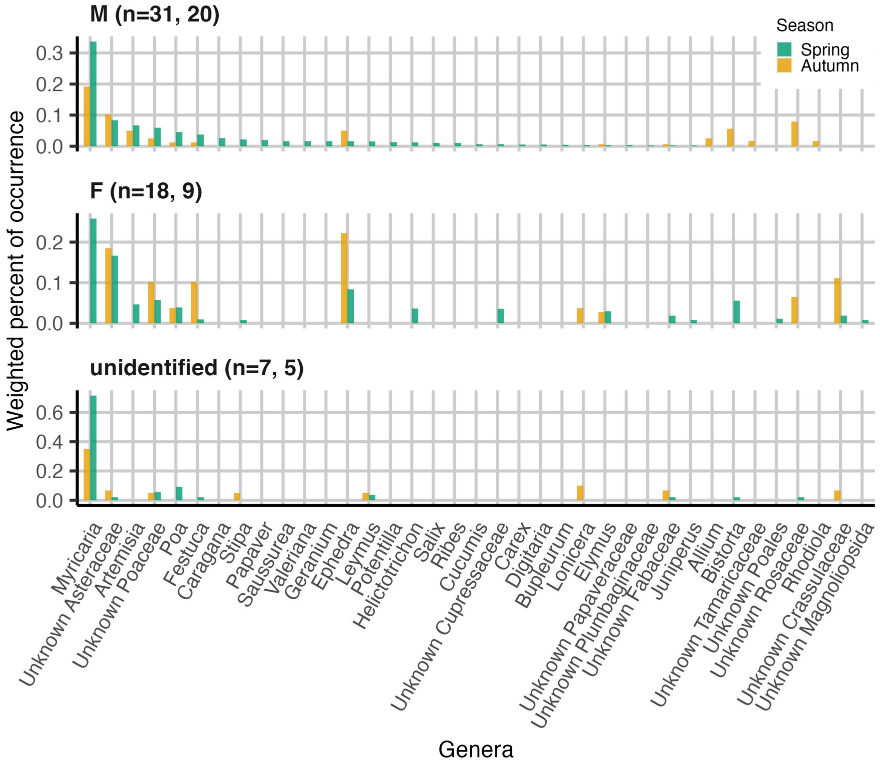


Figure 4.9. Weighted percent of occurrence of plants from snow leopard samples. The caption “M" represents males and “F” represents females. The numbers in the parentheses are the number of samples collected in spring and autumn, respectively. The color corresponds to different sampling seasons.

Appendix 4.1

Species identification PCR condition

	Volume per 15μl reaction (μl)
Primer F (20μM)	0.3
Primer R (20μM)	0.3
LA Taq HS	0.15
10X LA PCR Buffer II (Mg2+ plus)	1.5
dNTP	2.4
H2O	8.2
T4gene32protein	0.15

Thermal cycle

94°C 10 min
94°C 10 s ┐
60°C 30 s | 36 cycles
72°C 20 s ┘
72°C 10 min

Snow leopard sex identification PCR condition

	Volume per 10μl reaction (μl)
ZFX Primer F (10μM)	0.25
ZFX Primer R (10μM)	0.25
DBY7 Primer F (20μM)	0.25
DBY7 Primer R (20μM)	0.25
AmpliTaq Gold 360 Master Mix	5
H2O	2
T4gene32protein	0.1

Thermal cycle

95°C 9 min

94°C 30 s ┐

56°C 30 s | 45 cycles

72°C 30 s ┘

72°C 5 min

Snow leopard diet analysis PCR thermal cycles

12SV5 (Riaz et al., 2011)

95°C 3 min

98°C 30 s ┐ 35 cycles

60°C 30 s ┘

UniplantF - UniplantR (ITS2) (Moorhouse-Gann et al., 2018)

95°C 3 min

98°C 30 s ┐ 40 cycles

56°C 30 s ┘

rbcL mini-barcode F - rbcLaR (Erickson et al., 2017)

95°C 3 min
98°C 30 s ┐
65°C 30 s | 15 cycles
72°C 5 s ┘
98°C 30 s ┐
63°C 30 s | 25 cycles
72°C 5 s ┘
72°C 3 min

trnL-g - trnL-h (Taberlet et al., 2007)

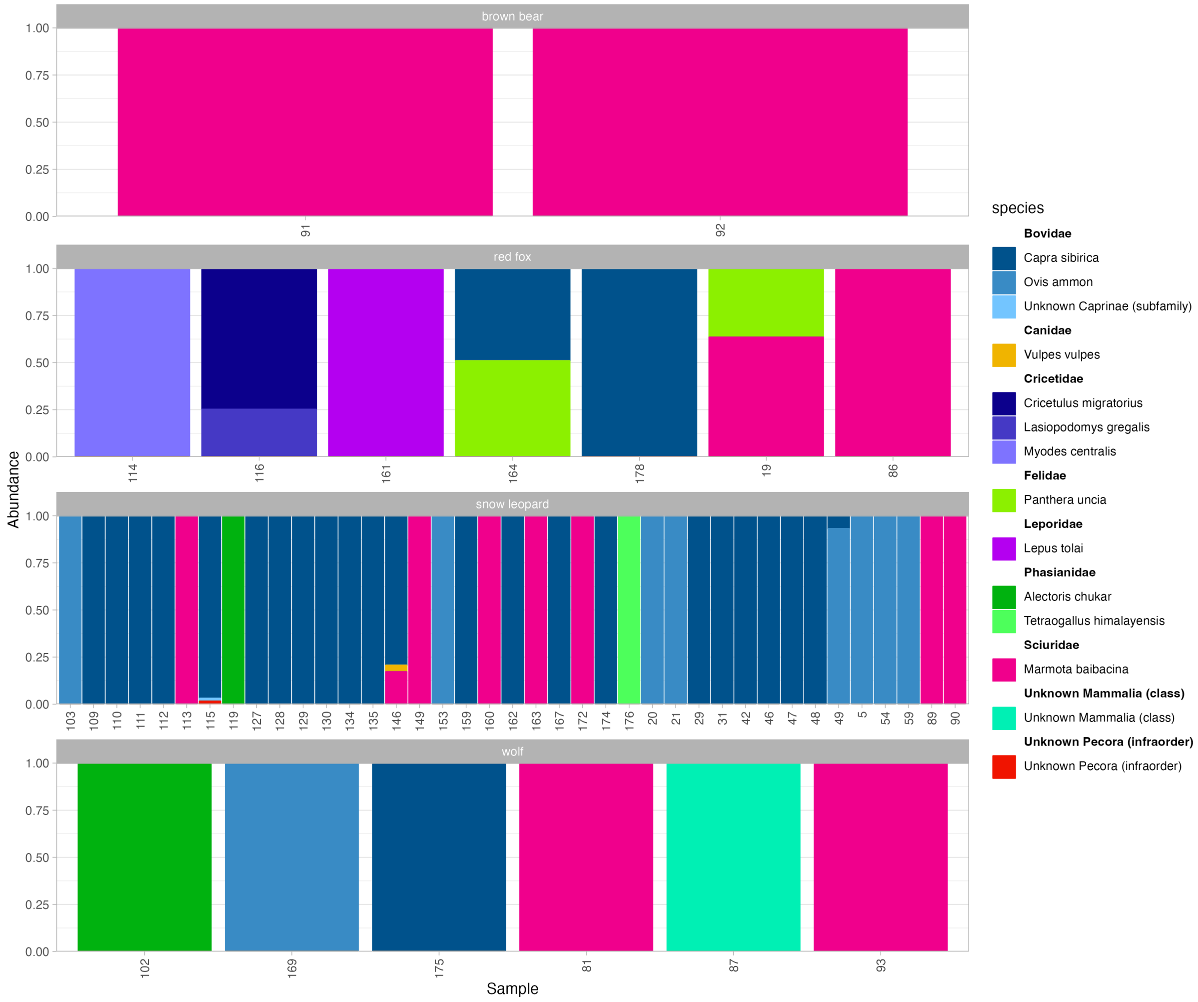
95°C 3 min
98°C 30 s ┐ 35 cycles
60°C 30 s ┘

ITS1F - ITS1PoaR (Baamrane et al., 2012)

95°C 3 min
98°C 30 s ┐ 15 cycles
65°C 30 s ┘
98°C 30 s ┐ 25 cycles
60°C 30 s ┘

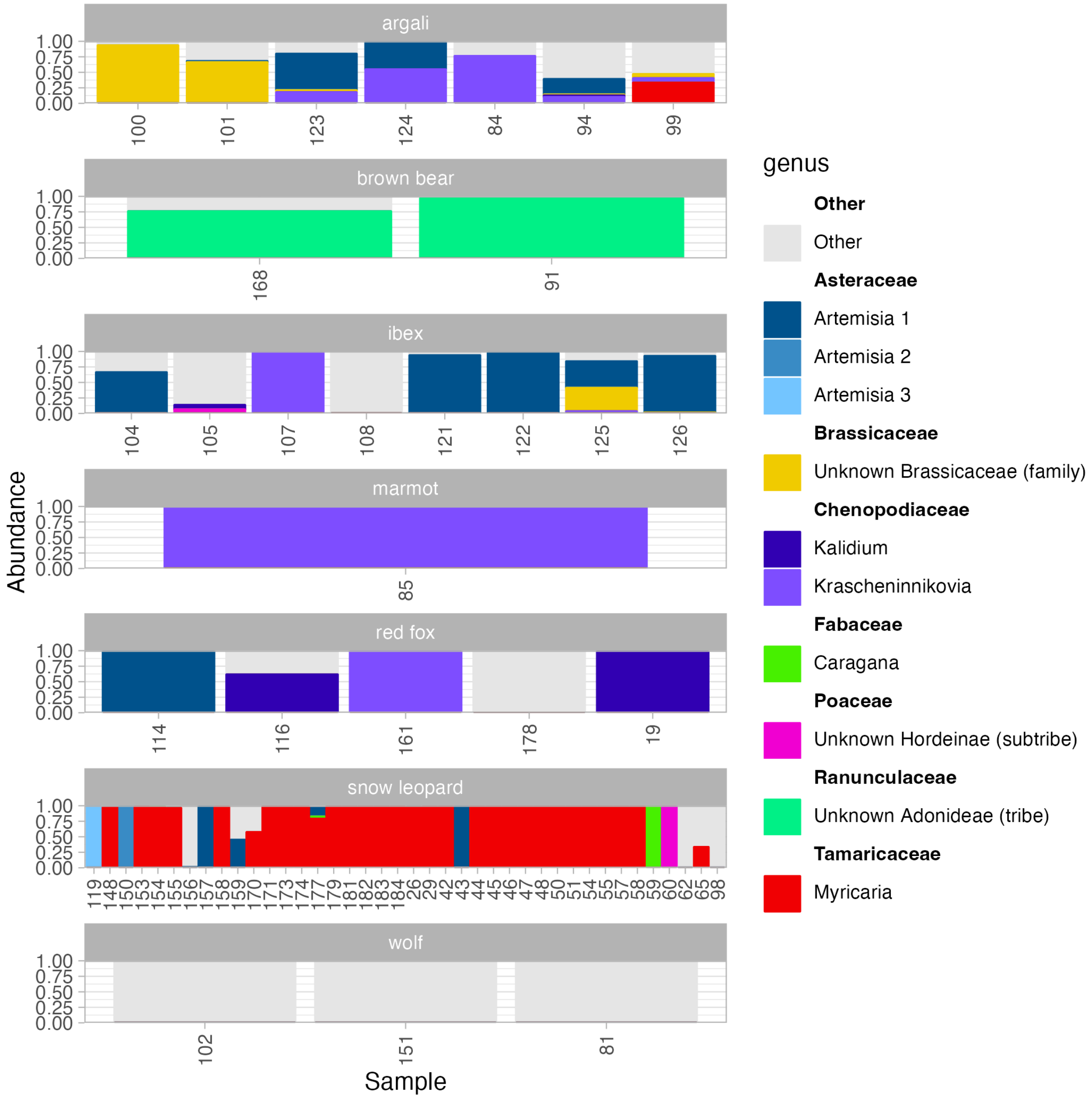
Appendix 4.2

Relative read abundance of 12SV5.



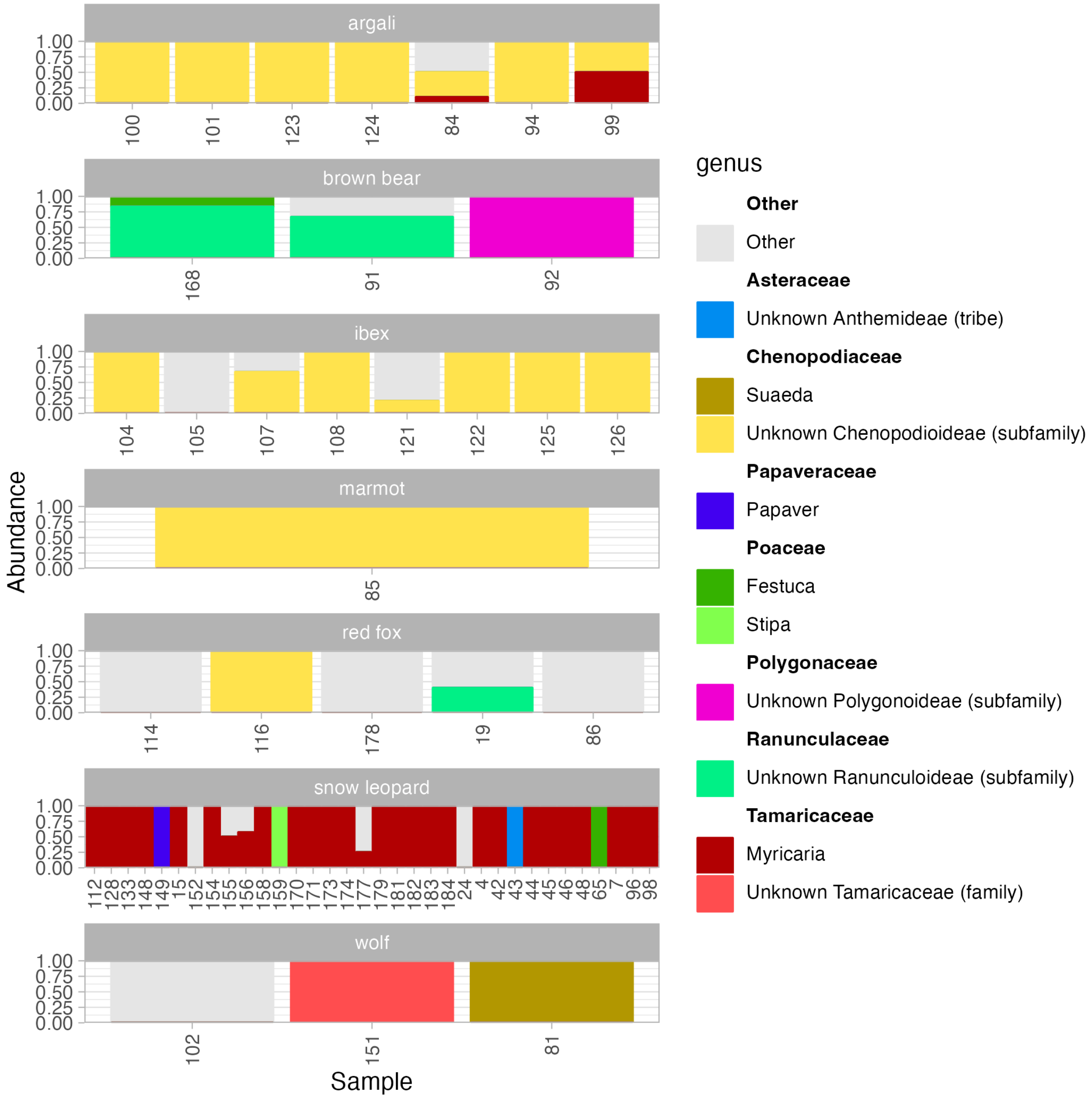
Appendix 4.3

Relative read abundance of Uniplant. The top 10 frequent plant genera were shown.



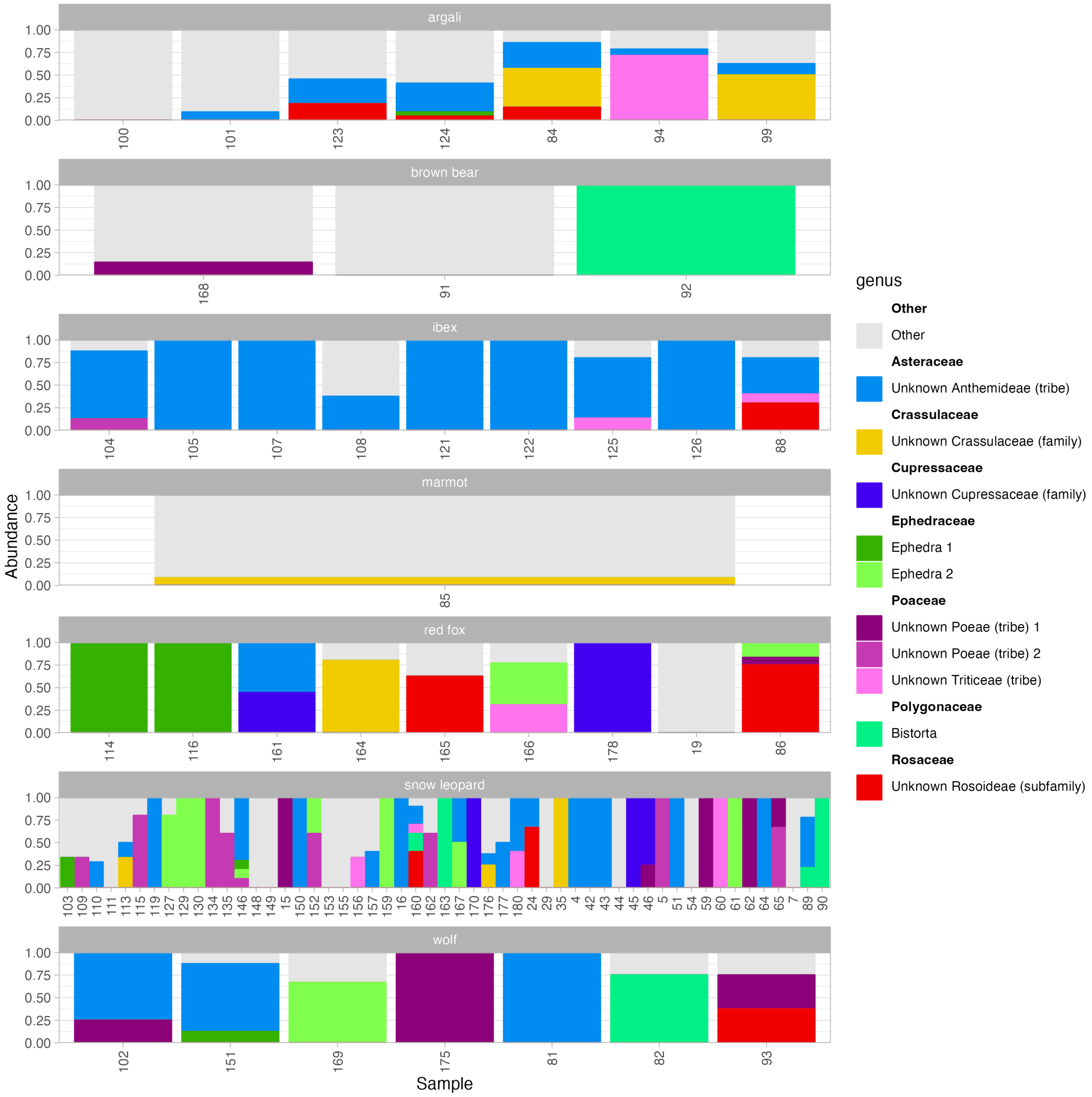
Appendix 4.4

Relative read abundance of rbcL. The top 10 frequent plant genera were shown.



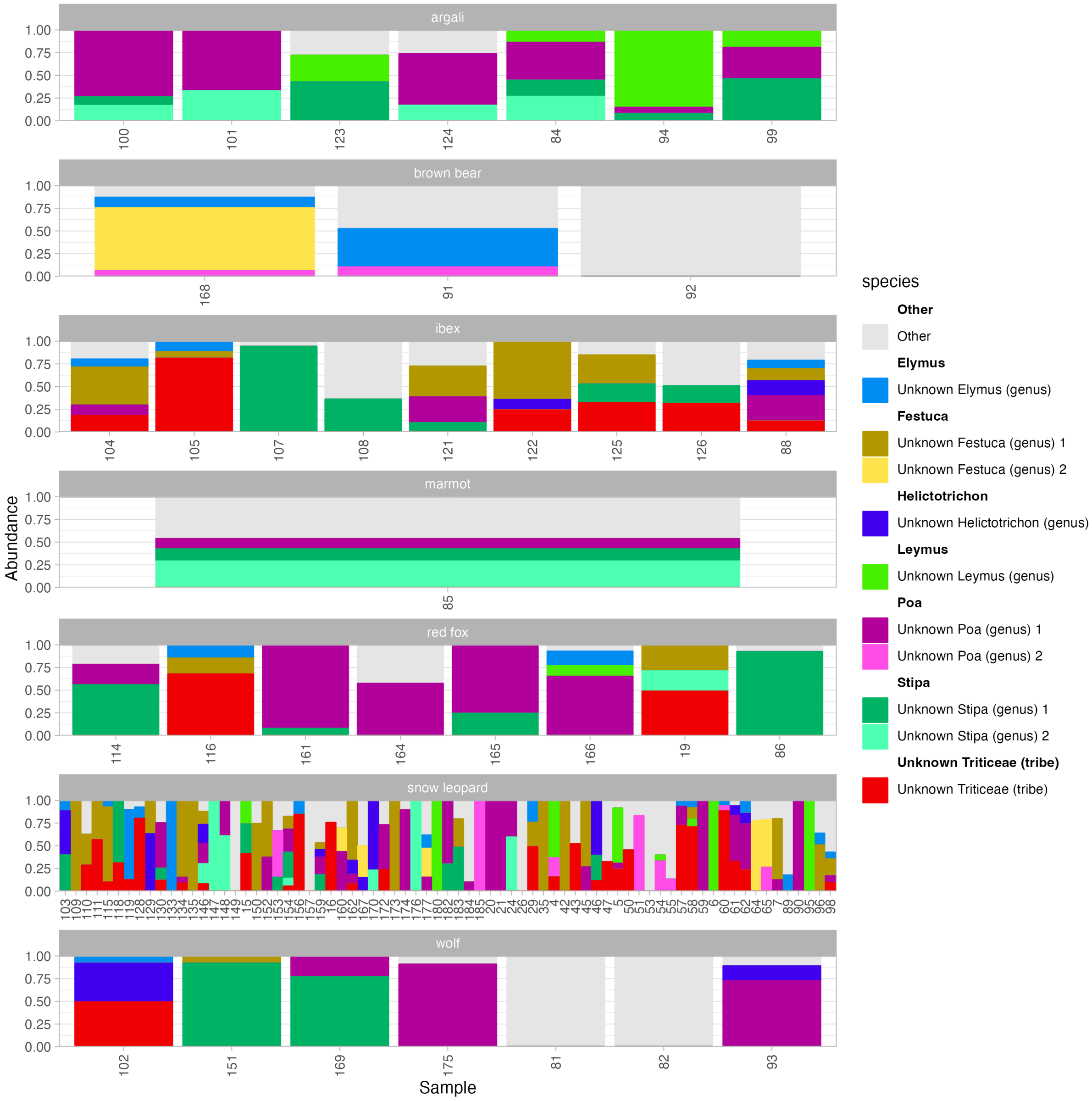
Appendix 4.5

Relative read abundance of trnL. The top 10 frequent plant genera were shown.



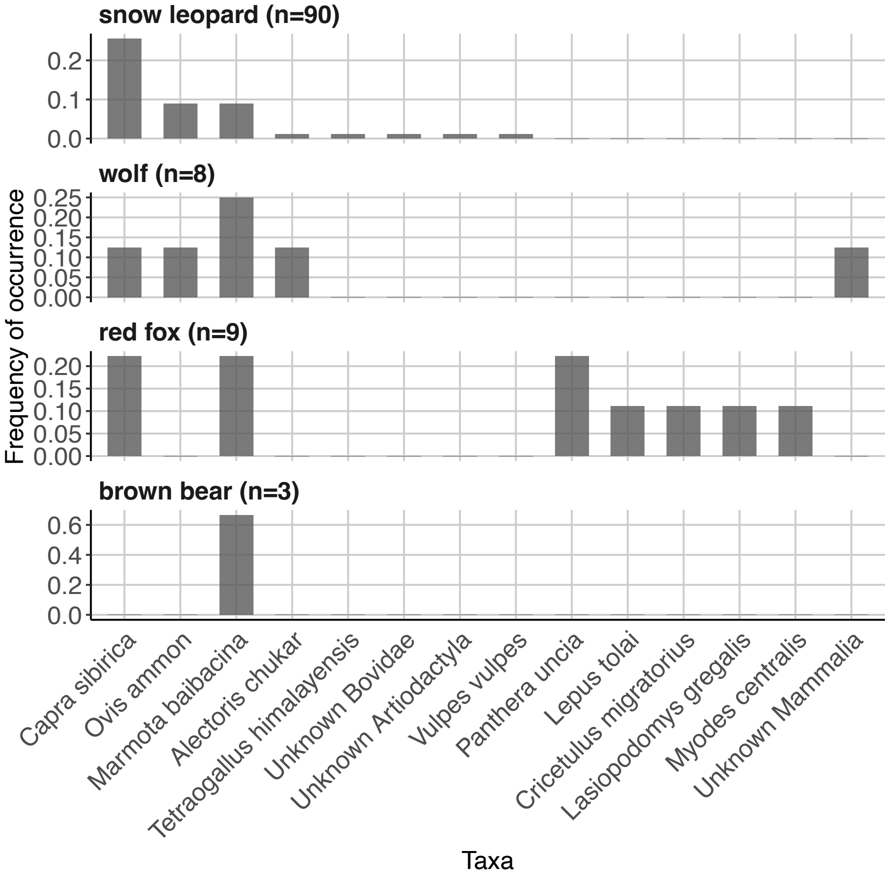
Appendix 4.6

Relative read abundance of ITS1Poa. The top 10 frequent genera were shown.



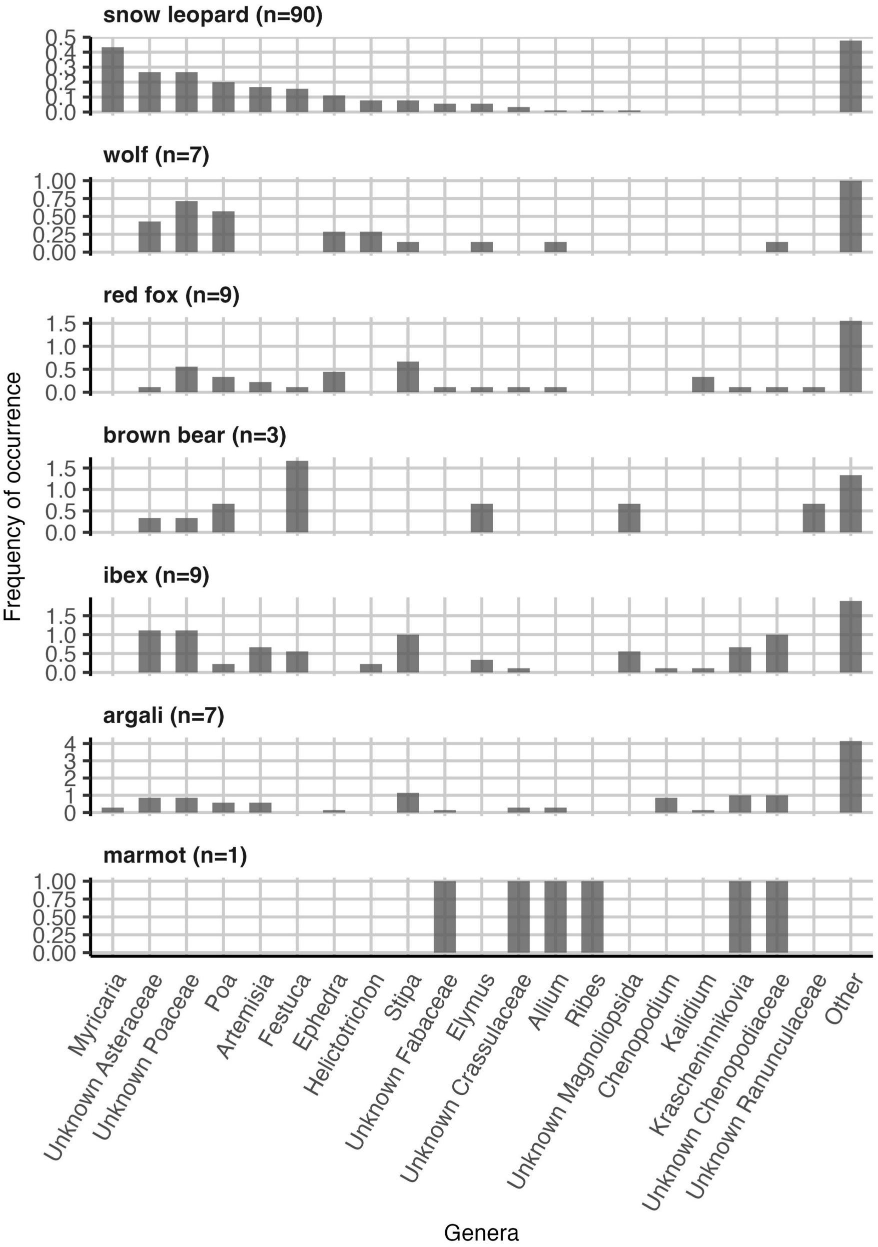
Appendix 4.7

The FOO of Vertebrate species from mammal species.



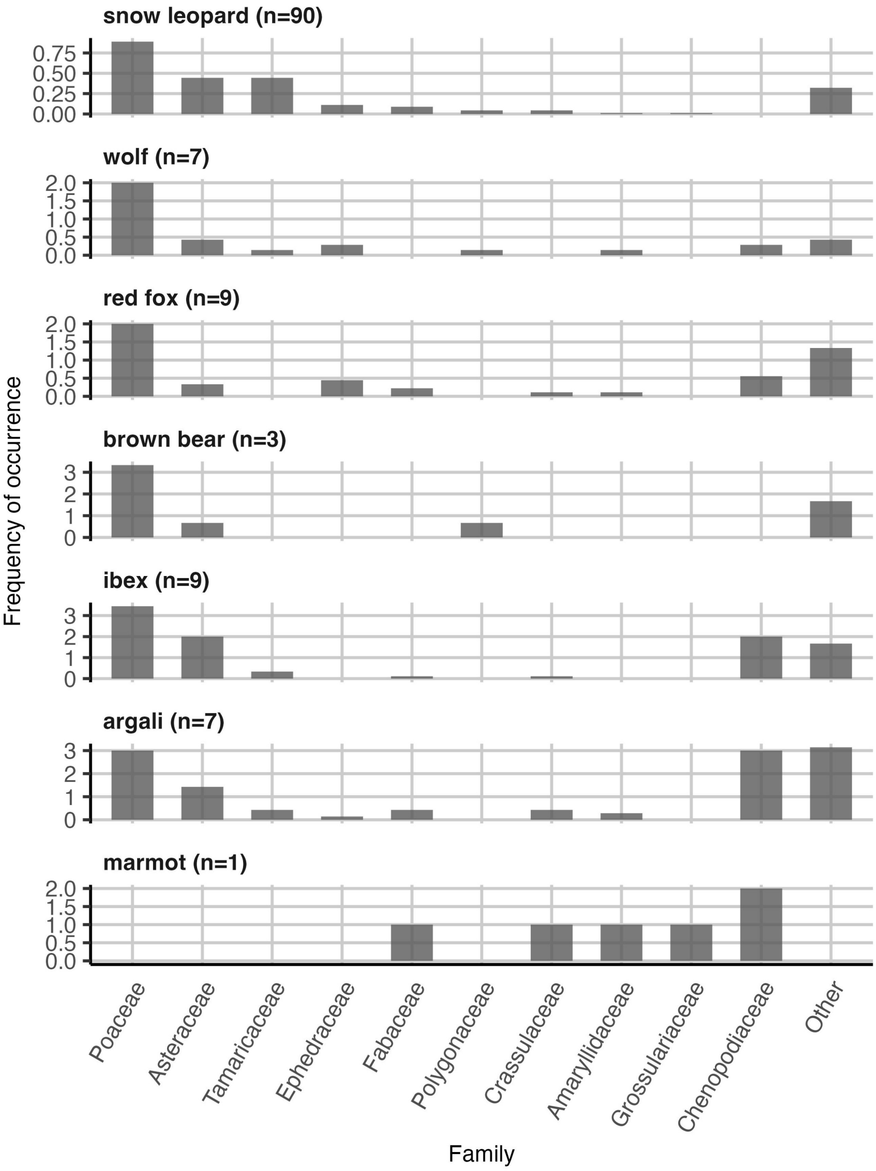
Appendix 4.8

Top 5 frequent plant genera for each mammal species.



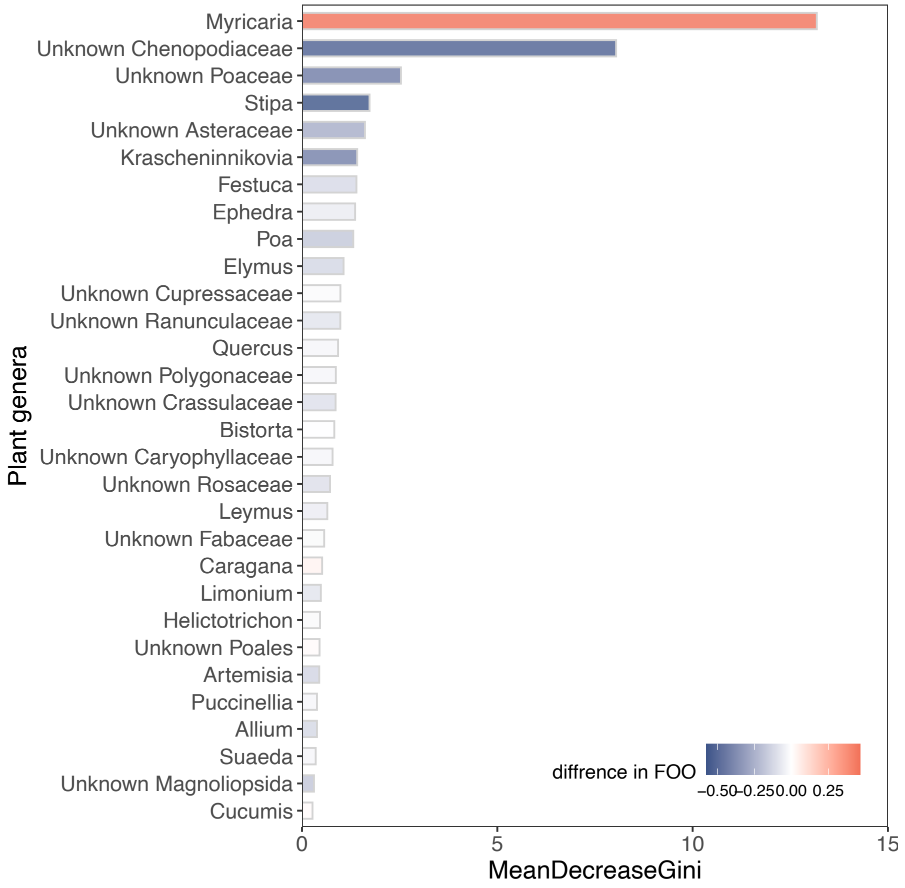
Appendix 4.9

Top 3 frequent plant families for each mammal species.



Appendix 4.10

Variable importance contribution of the random forest model based on FOO. Only the top 30 genera are included representing t’e plant genera that are most important to the model's ability to distinguish between a sample from snow leopard and a sample from other mammals.



Chapter 5

General discussion

5.1. Key findings and contributions

This series of studies is one of the pioneering studies in plant-eating behavior of felid carnivores. It encompassed multi-species comparisons, establishing a correlation between body mass and plant occurrence frequency. Furthermore, the studies tested the hair evacuation hypothesis in captive snow leopards, which are the smallest *Panthera* species. This hypothesis is one of the leading hypotheses explaining the adaptive significance of plant-eating in felids. Contrary to expectations, plant ingestion did not induce vomiting or facilitate hair evacuation in feces. The research also documented the plant diet of wild snow leopards in Kyrgyzstan, revealing a distinct aspect of their plant-eating behavior. It showed a negative co-occurrence between prey and *Myricaria spp.*, which was the representative plant in snow leopard feces. Additionally, the data implied dietary differences between male and female snow leopards, possibly due to ecological variations during reproductive seasons.

The data gathered will serve as a foundation for future studies on plant-eating behavior of felids. I observed inter-species variations in the frequency of plant occurrence in feces and stomach contents. For efficient data collection on plant consumption, it is advisable to target species that exhibit a high frequency of plant occurrence in their diet. The absence of vomiting and hair evacuation in captive snow leopard feces following plant ingestion suggests a function of plant diet other than hair evacuation and underscored the necessity of verifying established hypotheses and formulating new ones.

The research provided evidence of variation in plant-eating behavior among felids. The data show that plant-eating is widespread among extant felids, with notable variations across species, regions, and even individual animals. For instance, the study revealed that captive snow leopards are less likely to vomit after ingesting plants compared to domestic cats, 27–37% of whom do vomit (B. L. Hart et al., 2021). This underscores the need for not only data from a representative species but also data from each species in the family Felidae to fully understand the complex plant-eating behavior. Domestic cats, the most familiar felid, are the most suitable for conducting controlled studies and gathering organized data. While it is crucial to fully leverage data from such representative species, caution is necessary when extrapolating these findings to other members of the Felidae family.

5.2. Insights into the reasons behind plant-eating behavior

5.2.1. Adaptation

The negative correlation between species' body mass and plant occurrence frequency, discussed in Chapter 2, implies that smaller species, which typically consume smaller prey, may ingest more indigestible parts, and thus consume plants more frequently to facilitate the evacuation of these parts. This provides indirect support for the hair evacuation hypothesis. However, Chapter 3 presented findings that did not support this hypothesis, as plant ingestion in captive snow leopards did not promote hair evacuation. Chapter 4 further countered the hypothesis by observing a negative co-occurrence of representative plant and prey DNA in feces, which suggests that if plants aided in expelling indigestible parts, plant and prey DNA would more likely co-occur. Additionally, recent studies comparing plant-eating behaviors in short-haired and long-haired domestic cats revealed that both groups consumed plants with similar frequency (B. L. Hart et al., 2021). This suggests that plant eating in felids is not primarily for expelling hairballs, challenging the traditional understanding of this behavior.

Regarding the self-medication hypothesis, the findings in Chapter 2 were consistent with it. The chapter suggests that smaller species, which have a higher metabolic rate relative to their body size, may benefit more from the physical or chemical reduction of parasites through plant ingestion. Chapter 4 further supports this hypothesis by demonstrating that wild snow leopards in Kyrgyzstan uniquely and frequently consume the genus Myricaria, which includes medicinal species (*Myricaira bracteata*) (J. B. Liu et al., 2015). Plant consumption by felids serves purposes beyond addressing parasites or diseases; it may aid in maintaining the normal functioning of their digestive systems. Fecal metabarcoding analysis of wild snow leopards in Kyrgyzstan revealed they might have consumed plants when their digestive tracts were empty as described in Chapter 4. This behavior is not exclusive to snow leopards. A similar pattern was noted in leopards in West Africa, where fecal samples often contained grass and few bone fragments (Hoppe-Dominik, 1988), suggesting plant consumption during periods of digestive tract emptiness. Hoppe-Dominik proposed that ingested grasses might help keep the digestive system functional during prolonged starvation (Hoppe-Dominik, 1988).

This series of studies was not designed to obtain information about the food source hypothesis, which was developed to explain the presence of fruit in felid feces. Chapter 2 did not include information on fruit content due to the scarcity of data. Chapter 4 focused solely on DNA found in feces, making it challenging to ascertain the inclusion of fruit. However, since *Myricaria spp.*, frequently consumed by wild snow leopards as shown in Chapter 4, does not bear fruit and is often consumed for its branches (as depicted in Figure 1.1), it suggests that plants are less likely to be consumed as a food source, at least by wild snow leopards. Nonetheless, it is plausible that plants frequently consumed by felids may offer supplementary nutrition, such as minerals. For instance, geophagy, or soil eating, observed in several herbivores and omnivores, is thought to serve as a mineral supplement (Krishnamani & Mahaney, 2000). While herbivores and omnivores in nutritionally variable landscapes adjust their diet to optimize nutrient intake, nutrient balancing in carnivores was previously deemed unnecessary, as their prey's nutrient content is believed to align well with their requirements (Kohl et al., 2015). However, laboratory studies have indicated that carnivores from various taxa have evolved mechanisms for nutrient intake balancing (Kohl et al., 2015), suggesting that plant consumption could compensate for certain nutrients that may be sometimes deficient in a carnivorous diet.

5.2.2. Evolution

The results of Chapter 2 indicated that frequency of plant occurrence tended to be higher in Panthera and Caracal lineage, which are basal to extant felids. However, this effect was limited. Since extant felids are believed to have diverged over a relatively short period (Johnson et al., 2006; G. Li et al., 2016; Morris, 2002), the influence of phylogeny within the Felidae might have been ambiguous. Ancestral Carnivora are believed to have been omnivores (Bradshaw, 2006) and this order includes herbivores such as pandas and omnivores such as fox. While felids have become highly adapted to a strictly carnivorous diet as introduced in Chapter 1 (Kim et al., 2016; McGeachin & Akin, 1979; Morris, 2002), the consumption of plants may persist as a trace of their omnivorous ancestral traits if plant-eating behavior has neutral effect for their fitness. Although Kerr et al. (2013) reported that a 2% and 4% cellulose intake inhibit digestion in felids, as introduced in Chapter 1, a small amount of intake might not affect the energy intake. Recent technological advancements, such as stable isotope analysis, have facilitated the estimation of dietary traits from fossils, exemplified by studies on Scimitar-toothed cat (*Homotherium serum*), an extinct sister species to the remaining cats (DeSantis et al., 2021). However, given that plants were unlikely a major nutritional source for felids, investigating the presence of plant-eating behavior in fossil ancestors of felids remains challenging.

5.2.3 Causation

Based on data from Chapter 4, this study introduced that plant-eating in felids may be related to hunger. This aligns with the findings in Chapter 2, where it was observed that smaller felids tend to consume plants more frequently. This is attributed to their higher metabolic rates relative to body size, which increases the likelihood of experiencing hunger. Notably, snow leopards often consume branches, which are physically harder than leaves. Grasses, most commonly reported in felid diets (Yoshimura et al., 2021), contain silica and are tougher and more abrasive than woodland plants (Sanson, 2023). The link between availability of food and ingestion of alternative items was reported in domestic cat (Demontigny-Bédard et al., 2016). Therefore, felids may consume hard plants driven by appetite. However, as reported in domestic cats (B. L. Hart et al., 2021) and observed in captive snow leopards in Chapter 3, captive individuals often eat plants even when they are fed daily. Thus, hunger is probably not the sole driver of plant-eating behavior.

Although the taste sense of felids is generally thought to be unresponsive to plant sugars (P. Jiang et al., 2012; Kim et al., 2016), which motivate herbivores to eat plants, other senses, like olfaction, might encourage them to consume plants. Cats have highly developed olfactory systems and are capable of detecting volatile stimuli as well as pheromones (Bol et al., 2017). Volatile compounds from plants like catnip (*Nepeta cataria*) and silver vine (*Actinidia polygama*) are known to attract several cat species, including lions and bobcats (Uenoyama et al., 2021) and the effect of catnip is caused exclusively by its smell rather than its taste (Bol et al., 2017). Some of the plants consumed by felids may be preferred because they produce volatiles that happen to have a euphoric effect on cats. During the observation of captive snow leopards in Chapter 3, sometimes they rubbed cheek and head before eating plant. Activation of μ-opioid receptors elicits the rubbing response for catnip and silver vine (Uenoyama et al., 2021). Some plant volatiles possibly activate the neurophysiological mechanism and promotes rubbing behavior and biting behavior. However, plant-eating behavior does not always accompany rubbing behavior, and this alone does not adequately explain the diverse range of plant taxa consumed by felids, from grasses to bushes.

5.2.4. Development

Past experiences have lifelong influences on behavior (Villalba & Provenza, 2009). This study did not monitor the individual life histories of animals, thus providing limited insights into the development of plant-eating behavior. Given that the youngest snow leopard observed in Chapter 3 was one year old, it can be inferred that snow leopards begin consuming plants before they reach independence (Johansson et al., 2020). Domestic cats younger than one year old are reported to eat plants as well (B. L. Hart et al., 2021). It would be interesting to investigate whether cubs born in environments without plants eventually eat plants after being moved to facilities where plants are available.

Herbivores exhibit the ability to learn which plants to eat, when to consume them, and in what quantities to effectively manage plant secondary metabolites while obtaining sufficient nutrition (Villalba & Provenza, 2009). Since carnivores will be more susceptible to plant toxins, how they develop the capacity to distinguish suitable plant to eat is an interesting topic. Herbivores learn by themselves based on post-ingestive feedback from nutrients and secondary metabolites (Provenza et al., 1990; Villalba & Provenza, 1999), and through social learning from their mothers or pioneering animals in the group (Huffman, 2001; Mirza & Provenza, 1990, 1992). Given that most felids are solitary (Kleiman, 1973), they may learn from their mother or by themselves. Evaluating similarity of plant preference between mother and cub needs to be conducted. Observation in Chapter 3 demonstrates that the cub (Male 3) showed higher frequency of plant-eating behavior than other five individuals in the same zoo. Although the species of the plant eaten was not recorded, cubs may try more plants and learn based on post-ingestion effects. Hart et al. (2021) reported that domestic cats below one year of age more frequently ate non-grass plants than older cats.

5.3. Limitations and future directions

Chapter 2 explored the effect of large-scale environmental factors. Further finer scale investigation may lead to findings the unrevealed relationship between plant-eating behavior and external factors. It will be interesting to explore the relationships between morphological or physiological traits such as the size of colon. The study included feral cats, which, though not originally native to regions like Oceania and Madagascar, have become widely distributed across the world due to human factors. It possibly affected the result of environmental factors.

Chapter 3 targeted only snow leopards, therefore it is important to also test the hair evacuation hypothesis with other felid species, particularly those outside the Panthera lineage. Experiments should ideally be conducted in more natural environments, such as enclosures with vegetation similar to the species' original habitat.

Assessing the nutritional value of preferred plants will shed light on the food source hypothesis, while individual monitoring, although challenging, will help evaluate nutrient-specific foraging. The findings from Chapter 4 will underpin of these assessments. However, a comprehensive vegetation survey of the felids' environment is imperative beforehand to comprehend their plant preferences. Some plants consumed by carnivores have been found to contain medicinal compounds like anti-inflammatory agent (e.g. *Myricaria bracteata*) (J. B. Liu et al., 2015). However, it remains to be verified whether these compounds are effective in felids and how they impact fitness. Studying the physical characteristics of plants is also important, as they can physically remove parasites as observed in leopard cat (Lee et al., 2014) and domestic cat (B. L. Hart et al., 2021). Conventional fecal analysis can assist in distinguishing the parts of plants consumed, thereby facilitating the assessment of physical characteristics. It will provide quantitative information to evaluate the preference of plants as well. Lastly, even if a plant does not contain such chemicals, its ingestion could still influence the gut microbiome, potentially affecting the individual's health through substances produced by fermentation (Sommer & Bäckhed, 2013; Tremaroli & Bäckhed, 2012).

Animal-oriented studies aimed at understanding which individuals consume plants and how they do so encompass various aspects that are yet to be fully investigated. There exists variation in the frequency of plant-eating among different individuals. Uncovering the characteristics of individuals who eat plants can shed light on the drivers and functions of this behavior. The behavior itself also varies; felids sometimes eat plants after rubbing their cheeks and heads against them, while other times they eat without this cheek and head rubbing. This variation could indicate different contexts in which plant-eating occurs. Analyzing the sequence of actions before and after plant-eating can offer insights into the contexts of this behavior.

5.4. Concluding remarks

This series of studies has involved a range of feline individuals, from those in captivity to those in the wild. Its findings can contribute to animal welfare in captivity.

Environmental enrichment, a fundamental aspect of modern captive animal management, aims to modify an animal's environment to stimulate psychological and physiological processes that enhance wellbeing (Shepherdson, 1998). Given that plant-eating behavior is widespread among wild felids, introducing plants into enclosures could effectively evoke their natural behavior and improve the welfare of captive felids. This approach could be particularly beneficial for species that exhibit a higher frequency of plant consumption in the wild (Chapter 2). Additionally, incorporating plant species frequently consumed in their natural habitats, such as *Myricaria spp.* for snow leopards, would be appropriate whenever possible.

Although the impact of plant-eating on the fitness of felids has yet to be thoroughly investigated, this series of studies indicates that it may be beneficial to consider vegetation in the conservation planning for felids. All species are part of complex ecosystems, engaging in both antagonistic (e.g., predator-prey) and mutualistic (e.g., pollinator-plant) relationships, and preserving these network structures is crucial for ecosystem health (Tylianakis et al., 2010). Traditionally, in ecosystem networks involving plants, the focus has been on the links between plants and their herbivores or pollinators. However, the findings of Chapter 4 indicate that wild felids engage with plant species that are seldom consumed by herbivores or omnivores. Therefore, incorporating the felid-plant relationship into conservation strategies could lead to a reassessment and potential enhancement of ecosystem conservation plans.

This series of studies, by questioning established paradigms and preparing the groundwork for future inquiries, serves as a pivotal influence in wildlife science. The research illuminates intriguing behaviors previously unnoticed in felid behavioral ecology. It links the plant-eating behavior of felids to various disciplines, including behavioral ecology, genomics, and physiology. To decipher the intricate factors driving plant consumption in feline carnivores, additional exploratory research and hypothesis testing, as previously detailed, are essential.

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