

# **Human and wildlife in the Anthropocene:**

The multidimensional approach to urban ecology in Eurasian red squirrels

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## Abstract

In this Anthropocene, human activities negatively affect ecosystems. Urbanization, one of the features in the Anthropocene, has reduced natural habitats for wildlife, provided anthropogenic disturbance, and consequently induced the loss of biodiversity. At the same time, urban environments work as alternative habitats for wildlife by providing anthropogenic resources such as food and refugia. Actually, numerous species altered their behavioral and ecological traits to live in urban environments. As those modifications were well investigated in these few decades, it was found that factors facilitating their urban lives are species and region-specific. To understand urban adaptation of wildlife and conduct evidence-based conservation in urban environments, urban ecology should be conducted in various species and regions. However, studies of urban ecology are biased, with studies in specific taxa and Western countries being predominantly accumulated. Here we compared behavior, life history patterns (diet, body weight, reproductive output), and population genetic structure of Eurasian red squirrels *Sciurus vulgaris* in urban and rural areas in Obihiro area, Hokkaido, Japan.

In chapter 2, I investigated the composition and seasonality of diets in urban and rural squirrels. Squirrels strongly rely on tree seeds (e.g., walnuts and pine nuts) which bear only in fall. So, it is predicted that rural squirrels would have the seasonal difference of seed use. On the other hand, urban squirrels would mitigate the seasonal difference of seed use due to the

supplemental feeding from citizens. To test these ideas, I conducted behavioral observation and stable isotope analysis for comparing the diet between urban and rural squirrels. As a consequence of dietary change, I compared body weight between urban and rural squirrels. I found that urban squirrels had a higher intake of tree seed than that of rural ones throughout the year. Also, I found that urban females increased more weight in the spring than rural ones.. These results suggest that urban squirrels utilizing SF changed their diet composition and increased body mass (but only in females). Since heavy body weight in females is usually associated with reproductive success, urban females might have better reproductive condition than that of rural ones.

In chapter 3, I compared female reproductive conditions between urban and rural squirrels and tested whether the increase in female body mass in urban areas resulted in high reproductive success. We investigated six proxies for their reproductive condition: (1) rates of pregnant females, (2) the age of initial pregnancy, (3) the maximum age of reproduction, (4) birth date, (5) the number of weaned litters, and (6) whether females attended multiple breeding. I found that the urban population had a higher rate of pregnancy and an earlier breeding season than rural populations. Urban females had earlier initial pregnancy (one year old) and longer maximum age of reproduction (five years old) than rural females (two and four years old respectively). Although the number of weaned young did not differ between rural and urban females, urban females attended multiple breeding more frequently than rural females. My

results suggest that urban squirrels have better reproductive conditions than rural ones, which possibly due to the advantages from SF. Overall, those suggest that squirrels seemed to obtain benefits from living in urban areas.

Finally, in chapter 4, I investigated the population genetic structure of squirrels in the Obihiro area. The squirrels in Obihiro city have been settled in large residential lots with a high risk of vehicle collisions for about 30 years, raising a possibility that the urban population would be genetically isolated from nearby rural populations and may have rapidly decreased genetic diversity. In this study, I analyzed the population genetic structure, genetic distance, and nucleotide diversity by using genome-wide SNPs extracted by MIG-seq. I used 12 populations with different degrees of urbanization, which were divided into “urban”, “border”, and “rural” areas. Principal component analysis and ADMIXTURE showed that the urban and rural populations formed distinct clusters with an intermediate of the urban and rural boundary populations. The genetic distances ( $F_{ST}$ ) between urban and other populations were higher than the other pairs. In addition, the nucleotide diversities ( $\pi$ ) were lower in urban populations than in the other populations, suggesting limited gene flow between urban and other populations possibly due to urbanization. The difference in the genetic characteristics of urban squirrel populations in this short period (i.e., 30 years) demonstrates the strong impact of urbanization.

Overall, my thesis found that urban lives had both positive (diet, body weight, and female’s reproductive success) and negative (population genetic structure) impacts for squirrels.

Urban environments would be suitable habitat at the individual level and in the short timescale, but not at the population level and in the long timescale. My thesis suggested that urban ecology of wildlife should be conducted not only for multi-species and regions, but also for multidimension of animal ecology and at multi-levels from individuals to populations.

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# Chapter 1. General introduction

## 1.1 Anthropocene

Humans are living in a unique era, so called “Anthropocene”. Crutzen and Stoermer (2000) suggested this new term for a new geological epoch because humans exert an outsized influence on ecosystems. For instance, the accelerating human population growth and human activity changed landforms and climate, and also decreased biodiversity (Corlett 2015, Lewis & Maslin 2015). Corlett (2015) summarizes the basic concept of the Anthropocene as “*humans are now a major geological and environmental force, as important as, or more important than, natural forces*”. Therefore, in order to maintain the ecosystem on the earth in which humans (or non-humans) can live in the future, we need to rethink the impact of human activity on ecosystems and, furthermore, human-nature relationships.

The environmental modification from natural to urban landscapes, known as urbanization, is one of the features in the Anthropocene. Urbanization is occurring globally at unprecedented rates, with the forecast that two-third of the human population will be living in urban areas in 2050 (United nation 2018). While urbanization has provided convenient habitats for humans, it also works as new habitat for wildlife (Lowery et al. 2013; Shutt & Lees 2021). For example, waterfowls have been observed resting on the shore in the city park (Murray et al. 2018), passerines and squirrels foraging for food in home gardens (Plummer et al. 2019,

Shutt & Lees 2021), and carnivores roaming in the human residential areas (Bateman & Fleming, 2012; Sugden et al. 2021).

## **1.2 Wildlife in urban environments**

Historically, research fields of ecology had been focused on “natural” environments which were not affected by human activity. However, as urbanization globally expanded around the world and wildlife intruded into urban environments, ecologists started to consider urban environments as the novel ecosystem. Compared to natural environments, urban environments differ in terms of the presence of humans, impervious surfaces, and anthropogenic disturbances (e.g., light, noise, and air pollution), and also lack of natural resources. How does wildlife living in urban environments (here after: urban wildlife) overcome those environmental differences and succeed to live in? Since the 1990s, ecology in the city – namely urban ecology – has been developed to understand their adaptation in urban environments. Also, urban ecology is motivated to use the outputs for wildlife conservation and management in urban environments.

Urban wildlife research has been well conducted in the last few decades (Collins et al. 2021), and ecological and behavioral characteristics that related to their urban adaptation were found. In some species or individuals, inherent traits possibly related to their urban lives such as having an omnivorous diet, innovatively (e.g., high learning ability, tolerance to novel objects, reducing neophobia), and bold temperament (Lowery et al. 2013). Also, life history

patterns such as fast life history strategies were considered as the one of possible factors which induce wildlife flourishing in urban areas (Santini et al. 2019). As other traits, or characteristics which are induced by inherent traits, behavioral flexibilities had been suggested as important characteristics for urban lives. For example, human activity such as human presence and traffic are common in urban environments. Urban wildlife changes their activity patterns to avoid humans such as a more nocturnal lifestyle and modifying temporal habitat use (Dawding et al. 2010; Haigh et al. 2017). In addition, behavioral flexibility, whether wildlife could use anthropogenic resources or not, would be one of the keys of urban adaptation. As the refugia, urban wildlife has been observed to exploit artificial structures such as the inside of buildings and nest boxes as dens and shelters (Lowery et al. 2013, Sudyka et al. 2022). As the food resources, urban wildlife has been observed to forage anthropogenic food from waste, bird feeders, and roadside vegetation, instead of natural food (Shutt & Lees 2021; Rimbach et al. 2023).

As studies of urban ecology have been accumulated in the last few decades, it was found to be difficult to generalize the findings of a given species to others or find a single syndrome of their urban adaptation. Urban adaptations are strongly affected by species inherent traits. Regarding taxon-specific traits, Hahs et al. (2023) analyzed data to examine ‘urban traits syndrome’ (i.e., the specific trend that related to urban adaptation) for using six territorial taxa (amphibians, bats, bees, birds, carabid beetles and reptiles) in 379 cities on 6 continents. They

found the types of urban adaptation could be divided into four types, but those were affected by each species' inherent characteristics, especially, by their diet and mobility. Also, Santini et al. (2019) found that body size, diet diversity, and large litter size were important for urban adaptation in mammals, but not in other taxonomic groups. Therefore, urban ecology should be conducted by using various taxa to understand urban adaptation. However, most of urban ecology was conducted in plants and avian species. In the case of mammals, few studies have been conducted except for carnivores.

In addition to the species-specific traits, complexity of urban ecosystems also contributes to the diversity of wildlife adaptation in urban areas. Urban ecosystems were constructed by human activities such as including human population density and socioeconomic levels in each region and county (Albelti et al. 2020, Hassell et al. 2021). Those differences make different types and degrees of anthropogenic disturbance such as noise, air pollution, and human activities, consequently, induce different effects for urban wildlife in each region. For instance, the genetic study of urban mice showed that constructions of population genetic structure were strongly affected by human activity and socioeconomic, and those effects differed between countries (Combs et al. 2018, Fidino et al. 2021). In addition, regional diversity in religions and culture induce the difference of human attitude for nature and wildlife (e.g., supplemental feeding for wildlife, Schell et al. 2021). Those differences would induce different consequences of animal adaptation in urban areas. Therefore, to understand the pattern

of urban adaptation and the effects of urban lives on wildlife, urban ecology in various regions should be studied. However, 85 % of studies were conducted in Western countries (i.e., North America, Europe, and Australia; Collins et al. 2021).

Urbanization is expanding around the world, not only in Western countries, but also not only in plants and avians. Therefore, frameworks that are constructed by biased studies toward specific regions or limited species may induce misinterpretations of urban adaptation of wildlife. Also, these biases may result in misguided conservation and management approaches that do not fit regional and species characteristics. To understand urban adaptation of wildlife, it would be important to promote case studies in diverse regions and animal species, and integrate the accumulated evidence or recontextualize in each region.

### **1.3 Urban Eurasian red squirrels**

Arboreal squirrels, especially Eurasian red squirrels *Sciurus vulgaris* are the representative urban wildlife. Since they abundantly distribute in both natural and urban environments in Eurasian countries, various aspects of their urban ecology have been investigated in each region (Fingland et al. 2022). In Europe, squirrels are considered an endangered species and urban environments become new habitats for them. Most of previous studies concluded that urban environments would be suitable habitat for red squirrels such as in terms of food availability, predation pressure of raptors, and reproduction (Fingland et al. 2022) As important factors

which support urban population, the presence of green space in urban areas (including habitat connectivity and quality) have been commonly reported (Verbeylen et al. 2003, Kopij 2009, 2014, Stirké 2019).

In most cases, the presence of anthropogenic food, that is, SF was mentioned as the critical factor which supports urban squirrel's population. SF for squirrels is a ubiquitous citizen activity in urban parks and home gardens in various countries. Motivation of SF varies, such as for conservation (especially in the UK) and daily entertainment of citizens (Krauze-Gryz et al. 2021; Takahata et al. 2023). SF are usually conducted with tree seeds (e.g., walnuts and hazelnuts) which is considered to be the squirrels' main diet. Also, SF provides food abundant and stable across seasons. So, squirrels are considered to gain benefit from SF. For example, abundance of squirrels was correlated with food abundance due to SF (Reher et al. 2016, Jokimäki et al. 2017, Turner et al. 2017). Also, successful reintroduction of squirrels in urban parks in the UK were mentioned as owing to the SF (Bertram and Moltu 1986, Wauters et al. 1997). To understand whether SF contribute squirrel's urban population, the carryover effects on their life history patterns (from dietary modification to fitness components such as reproduction) is needed. However, there is no research about fitness components which are based on qualitative dietary analysis.

SF has been also suggested to induce potential detrimental effects for urban squirrels. For instance, SF induces high squirrel's population density, high rates of competition for

resources, high risk of disease/parasite transmission, and reproductive suppression (Chantrey et al. 2014, Thomas et al. 2018, Stirké 2019). In addition to SF, urban squirrels would be affected by numerous urban factors such as urban predators and human activity on their behavior and life history patterns. To understand supporting/inhibiting factors of urban population, investigating multiple aspects of their urban ecology in the same regions is needed. However, most of previous studies had been conducted in each region with specific aspects (e.g., dispersal in Finland; Selonen et al. 2018, activity patterns in Poland; Kojima 2009, 2014, anti-predator behavior in Japan; Uchida et al. 2016, 2020). Therefore, it is still unclear how urban lives affected the multiple aspects of their life history patterns.

#### **1.4 Objectives and structure of my thesis**

In this thesis, I investigated multiple aspects of urban ecology of Eurasian red squirrels in Hokkaido, Japan, by comparing between rural and urban populations. In this study, I defined rural and urban areas as described below (see also, Supplementary Data SD1 in chapter 2). We selected rural sites as small-forested areas which were surrounded by agricultural lands, rarely visited by humans, and located at least 10 km from the central Obihiro city. For example, we used shrines and abandoned horse-riding parks as the rural sites. Forests in rural sites were mainly dominated by conifer trees such as Japanese larch *Larix kaempferi* and Korean pine nuts *Pinus koraiensis*, and some of deciduous trees such as Japanese walnuts *Juglans*

*mandshurica var sieboldiana* and white birch *Betula platyphylla var. japonica*. As urban study sites, we selected urban parks which were surrounded by residential lots and traffic roads, commonly visited by humans, and located in Obihiro city. Since the composition of forests were similar between urban and rural sites, the availability of natural food resources for squirrels was almost the same. However, in urban areas, supplemental feeding for squirrels was commonly conducted by citizens.

My thesis is organized into 5 chapters: this chapter for general introduction, three chapters for empirical studies (chapter 2, 3, 4), and the final chapter for the general discussion.

In chapter 2, I investigated diet composition and seasonality to understand how urban squirrels modified their diet to live in urban environments. Many previous studies in Europe have suggested that SF possibly supports urban populations (Fingland et al. 2022). However, qualitative diet analysis had not been conducted, making it unclear how urban lives modify urban squirrels' diet and whether SF works as a benefit for urban lives. Therefore, in addition to dietary analysis, I compared body weight between urban and rural populations.

In chapter 3, I investigated the reproductive conditions of females as fitness components. I examined female's reproductive conditions by using six proxies of female reproductive success and compared conditions between rural and urban populations.

In chapter 4, I investigated population genetic structure to understand whether urban environments work as a suitable habitat for urban squirrels in the long-term timescale. Also, by

investigating genetic diversity and genetic distance in each site and sex, we discuss the factors which might induce negative effects on their population.

Finally, in chapter 5, I synthesize my findings and discuss limitations of my thesis.

Also, I discuss some implications for urban ecology and wildlife conservation/management in urban environments.

## **Chapter 2. Diet composition and body weight**

### **2.1 Introduction**

In the past few decades, natural habitats for wildlife have decreased due to urbanization (Grimm et al. 2008; Santini et al. 2019). Urban environments function as alternatives to natural environments, providing anthropogenic resources for wildlife including garbage as a food source and buildings as nest sites and refugia (Robb et al. 2008a; Chamberlain et al. 2009; Lowry et al. 2013; Johnson et al. 2020). Some species have modified their ecological characteristics and now use anthropogenic resources (Robb et al. 2008b; Uchida et al. 2016; Santini et al. 2019). In particular, diet modification may enable wildlife to persist with or flourish in urban environments (Lowry et al. 2013; Shutt and Lees 2021). Provisioning food from anthropogenic resources, such as garbage, garden plants, and supplemental feeding (SF) is both stable and abundant across seasons (Robb et al. 2008a; Lowry et al. 2013). Anthropogenic food often has a high calorific value and is particularly useful for generalist species (Widdows and Downs 2018; Thatcher et al 2020). Identifying dependencies on anthropogenic food resources and seasonality will improve our understanding of wildlife acclimation to urban landscapes.

Previous studies have investigated dietary changes associated with urban habitat use in various species (O’Leary and Jones 2006; Krauze-Gryz et al. 2021). Although these studies

analyzed food composition by direct observation and fecal and gut analysis, the methods were qualitative (González-Solís et al. 1997; Newsome et al. 2015; Swan et al. 2020) in that consumed items were detected but not quantified. For example, while food items can be identified by direct observation, there is observational bias associated with some foods that are difficult to identify (Holechek et al. 1982). Moreover, although direct observations can estimate daily energy intake by assessing activity patterns (Holechek et al. 1982; Wauters and Dhondt 1987; Wauters et al. 1992, 2001) this may be difficult for some species because of weather, visibility, or other environmental conditions. Fecal and gut analyses provide a snapshot of dietary intake, but reflect only short-term dietary trends. In addition, these analyses quantify food residues, resulting in bias towards undigested food components.

Stable isotope analysis (SIA) offers a potential solution to these issues (Inger and Bearhop 2008; Robb et al. 2011). SIA has been used to reveal both foraging ecology and trophic structure (Rubenstein and Hobson; 2004; Crawford et al. 2008; Inger and Bearhop 2008) based on stable nitrogen and carbon isotope ratios. Stable isotopes in food items are sequestered in consumer tissue (keratinized tissue, plasma, bone, collagen, etc.). The difference in isotope ratio between diet and consumer tissue is the trophic discrimination factor (TDF). Using TDF, we can estimate the intake of each food item in the diet. In addition, tissue isotope ratios change during growth (Tieszen et al. 1983; Crawford et al. 2008), and tissue specific turnover rates vary widely (e.g., blood plasma, few days; red blood cells, several months; hair and vibrissae,

period from hair formation until molt; Tieszen et al. 1983; Hilderbrand et al. 1996; Bearhop et al. 2002). Difference in isotope turnover rates enables an examination of diet across several timescales. Thus, SIA may improve our understanding of wildlife diets in various environments, including urban landscapes. Although SIA can be used to quantify proportions of food items consumed, it cannot determine species identity or the specific food part consumed. Thus, direct observations of foraging and assessment of available food items together with SIA may provide more reliable results.

Because Eurasian red squirrels are commonly observed in urban parks and use anthropogenic food items including SF throughout the year (Jokimäki et al. 2017; Mori et al. 2018; Krauze-Gryz et al. 2021; Uchida et al. 2021) they are suitable models for analysis of diet modification in urban environments. Additionally, because of their diurnality and small size, behavioral observation and the collection of hair and fecal samples using non-lethal methods is straightforward. Consequently, changes in behavior and ecology in urban settings have been well-studied including behavioral responses to humans (Uchida et al. 2016, 2019), personality (Uchida et al. 2020), natal dispersal (Fey et al. 2016; Hämäläinen et al. 2019), cognition (Chow et al. 2021), and stress responses (Shimamoto et al. 2020). Although their diets are diverse, they rely on conifer seeds and nuts over winter (Hayashida 1989; Shuttleworth 2000; Krauze-gryz and Gryz 2015; Krauze-gryz et al. 2021). These seeds have high caloric content and durability within caches. Thus, squirrels cache seeds during fall in preparation for low food availability

during winter (Wauters et al. 1995; Wauters and Casale 1996). Body condition and survival are influenced by tree seed availability under natural conditions (Wauters et al. 2007). In urban environments, anthropogenic foods—including seeds—are available throughout the year via SF, creating a high-quality habitat for squirrels (Reher et al. 2016). Although SF for squirrels can affect both diet and life history, there has been no quantitative dietary analysis of urban squirrels.

We examined diets of Eurasian red squirrels living in urban and rural areas of Hokkaido, Japan, using direct observation and SIA. We assessed diets during spring and fall because of seasonal difference in abundance of natural tree seeds and nuts. Eurasian red squirrels in Hokkaido rely on Japanese Walnut *Juglans mandshurica var sieboldiana* and Korean Pine Nut *Pinus koraiensis* (Hayashida 1989; Uchida et al. 2016) which can be naturally acquired in both urban and rural areas. In rural areas, squirrels forage for tree seeds during fall and other food items in spring because cached seeds are often depleted in spring. In urban areas, humans provide these tree seeds supplementally throughout the year (Uchida et al. 2016, 2021). Thus, urban squirrels have access to tree seeds from SF in addition to food from nature. Consequently, urban squirrels may have a larger proportion of seeds in their diet and lower dietary seasonality than rural squirrels. To test this, we compared (1) the degree to which squirrels consumed tree seeds and (2) the seasonal variation in diet for rural and urban squirrels. To examine diet seasonality, we performed a SIA on squirrel hair because molting coincides with seed scarcity

in spring and fruit-bearing in fall. Finally, we explored the effects of dietary differences by comparing (3) the body weights of rural and urban squirrels.

## **2.2 Materials and Methods**

### ***Study area***

Our study was conducted in Obihiro City, Hokkaido, Japan (42°49'N, 143°02'E), the largest city in the central Tokachi region with a population of approximately 160,000. We conducted field surveys in spring (May–June) and fall (September–November) from 2017 to 2019. We selected four natural forests that were located at least 10 km from central Obihiro city as rural sites (mean and *SE* for area of study sites was  $2.2 \pm 0.5$  ha; Supplementary Data SD1). These sites were rarely visited by humans, indicating the absence of SF. In addition, squirrels in rural sites could not access any SF from houses with bird feeders, because these sites were surrounded by agricultural lands.

We selected four urban parks in Obihiro City as urban sites (mean and *SE* for area of urban park areas:  $14.3 \pm 10.4$  ha). There were on average  $5.5 \pm 11.1$  human visitors hourly in the daytime. Data were collected during a 30 min line transect census (see below). Urban sites were surrounded by buildings and roads and managed by the Obihiro City government. Park characteristics (tree composition and park age) were generally the same. Supplemental food in urban areas was usually provided via feeders, temporary feeders made by people, and direct

feeding from human hands throughout the year (Uchida et al. 2021). Supplemental food offered to urban squirrels consisted mainly of Japanese walnuts and lesser amounts of Korean pine nuts. These were obtained from natural forests and house gardens. Supplemental foods sometimes included novel items such as sunflower seeds, peanuts, and squash seeds. Our observations confirmed that human activities and SF occurred almost daily except on rainy, windy, or heavy-snow days.

Juvenile squirrels typically disperse to new habitats at around four months of age (Wauters et al. 1994). In order to study dietary and body weight difference among populations, we chose isolated study sites separated by at least 1 km and focused on adult squirrels that had completed dispersal. We observed only one instance of a marked individual (see below) moving from one study site to another.

### *Field surveys*

We trapped squirrels from 4:00 am to 10:00 am from May to June and October to November between 2017 and 2019. Each study site was trapped at least three times, or until all squirrels were captured. Trapping sessions were at least three days apart. Squirrels were captured using box traps (Model RB-2, Sakae Industry Co., Ltd., Niigata, Japan; 290 x 290 x 650 mm) baited with walnuts and pine nuts. We checked traps every 1 – 1.5 h in rural sites. Traps at urban sites were monitored continuously to minimize stress on captured squirrels and human conflict.

Captured squirrels were weighed using a spring balance and ear-tagged (KN-295-A, Natsume Seisakusho Co., Ltd., Tokyo, Japan). Additionally, we attached collars (plastic cable ties with small beads, 3 g) to facilitate individual identification (Uchida et al. 2020). We collected 30 mg of shoulder hair for SIA and immediately stored the samples in a freezer (-30 °C). To avoid pseudoreplication in the SIA, hair samples were collected only once for each individual in each season. Samples were collected within 15 min to minimize stress, and squirrels were released at the point of capture. All procedures involving the capture and handling of squirrels followed the guidelines of the American Society of Mammalogists (Sikes et al. 2016). This study was approved by the Institutional Animal Care and Use Committee of the National University Corporation Hokkaido University (license number: 15-0121) and the Institutional Animal Care and Use Committee of the Graduate University for Advanced Studies, SOKENDAI (license number: SKD2020AR002).

During field surveys we noted food items foraged by squirrels based on 694 observational events (Table 1) recorded during daily visits to each site. Field observations were conducted from 5:00 am to 10:00 am when squirrels were most active in spring and fall. All observations were recorded during 30 min line transect censuses (see below). The routes traversed locations where marked squirrels were captured to increase the probability of encountering these squirrels. When marked squirrels were encountered on the line transects, we recorded their behavior as well as the food items foraged or held. Whenever possible, we

recorded whether the food items were provisioned by humans (e.g., food was not naturally available, or we observed direct food provisioning by people). In addition, we recorded the number of humans encountered. In 2019, we only recorded the food items consumed by marked squirrels to avoid observational bias resulting from higher encounter rates for a few individuals. Individual squirrels were observed on three successive days to collect information on three foraging events for each individual. To minimize the effects of observer presence, we used binoculars and maintained a distance of at least 30 m from the squirrel. In general, a distance of 30 m did not elicit a flight response (Uchida et al. 2016). The food items foraged by squirrels were collected and stored in a freezer (-30 °C) for SIA.

Foraged items were categorized as tree seeds (walnuts and pine nuts), plants (e.g., shoots, flowers, leaves, fruits, and moss), animal matter (e.g., insects and animal bones), fungi, and naturally unavailable items (e.g., sunflower seeds, squash seeds, and peanuts). In the category of tree seeds, we included only walnuts and pine nuts because these are the main tree seeds foraged by squirrels in Hokkaido (Hayashida 1989; Uchida et al. 2016) and the food most commonly provisioned for squirrels as SF in urban areas (see above). Nutritional content (proteins and lipids) is very similar between walnuts and pine nuts, and both have very high caloric value compared to other food items (Chung et al. 2013). These food items also exhibit low perishability on the ground and can be cached for long periods (Hayashida 1989). Although squirrels forage for natural walnuts and pine nuts in both rural and urban sites, urban squirrels

forage for additional seeds from SF. Therefore, we could not determine whether the walnuts and pine nuts originated from nature or SF by only using SIA because the isotope ratios of those seeds were the same. Thus, we recorded the origins of walnuts and pine nuts as much as possible during our observations. We used these food item categories for the SIA.

### *Stable isotope analysis*

Squirrel hairs were cleaned using a 2:1 chloroform:methanol rinse for lipid extraction and air-dried overnight. Samples were cut into small fragments, and 2 mg of hair was placed in tin boats. Food items were dried completely in an oven at 60 °C and pulverized with a mortar and pestle. Using the same lipid extraction protocol, 10 mg powdered plant tissue samples and 2 mg animal tissue samples were placed in tin boats. The samples were then analyzed for carbon and nitrogen isotope ratios using an elemental analyzer (vario MICRO cube, Elementar, Germany) connected to isotope ratio mass spectrometers (isoprime100, Elementar, Germany) at the Atmosphere and Ocean Research Institute, University of Tokyo. The stable isotope ratio was expressed in  $\delta$  notation per mil unit (‰), according to the following equation:

$$\delta X = [(R_{\text{sample}} / R_{\text{standard}}) - 1] \times 1000$$

X refers to  $^{13}\text{C}$  or  $^{15}\text{N}$  and  $R_{\text{sample}} / R_{\text{standard}}$  represents the isotope ratio of  $^{13}\text{C}/^{12}\text{C}$  or  $^{15}\text{N}/^{14}\text{N}$ .  $R_{\text{standard}}$  represents the R of Vienna Pee Dee Belemnite for  $\delta^{13}\text{C}$  and  $\text{N}_2$  in the air for  $\delta^{15}\text{N}$ . The isotopic compositions of the samples were calibrated against commercial standards

AZ100SS09 ( $\delta^{13}\text{C}$ : -19.6 ‰;  $\delta^{15}\text{N}$ : 8.72 ‰; Shoko Science, Kanagawa, Japan) and AZ101SS13 ( $\delta^{13}\text{C}$ : -19.6 ‰;  $\delta^{15}\text{N}$ : 13.7 ‰; Shoko Science, Kanagawa, Japan). For accuracy, both IAEA-CH-6 (sucrose) and IAEA-N-1 (ammonium sulfate) were analyzed. The average value ( $\pm 1SD$ ) of the measured isotope ratios (IAEA-CH-6:  $\delta^{13}\text{C}$ : -10.6 ( $\pm 0.3$ )‰,  $n = 4$ ; IAEA-N-1:  $\delta^{15}\text{N}$ : 0.43 ( $\pm 0.24$ )‰,  $n = 5$ ) were almost identical with the certified value (IAEA-CH-6:  $\delta^{13}\text{C}$ : -10.449 ‰; IAEA-N-1:  $\delta^{15}\text{N}$ : 0.43 ‰).

Keratinized tissues such as hair, whiskers, and claws are metabolically inert after tissue synthesis and sequester the isotopes in the diet of the animal at the time of tissue synthesis, such as in molting (O'Connell and Hedges, 1999; Williams et al., 2011). Because *Sciurus* species have both spring and fall molts, each of which lasts about one month (Layne, 1954; Sharp, 1958; Keith, 1965), the isotopic ratio of squirrel hair can be used to infer their diet during these molting periods. Derbridge (2018) used this assumption to determine stable isotope ratios for Abert's Squirrel *S. aberti* and the Mount Graham Red Squirrel *Tamiasciurus fremonti grahamensis*. Eurasian red squirrels also molt twice yearly, spring (April–May) and fall (October–November). Thus, hair collected before the spring molt was classified as a fall coat, and that collected prior to the fall molt as a spring coat.

### ***Dietary analysis using stable isotope ratio***

To quantify diet isotopic range (isotopic niche size) in the four area and season categories (rural spring; rural fall; urban spring; urban fall), we calculated the total area (TA) and standard ellipse area corrected for small sample sizes (SEAc) from stable isotope values of the hair samples using the ‘Stable Isotope Bayesian Ellipses’ package in R (SIBER; Jackson 2021). Sample sizes were not balanced. The SEAc for each area and season category contained approximately 40% of the data and is regarded as the most appropriate percentage for an unbiased sample size (Jackson et al. 2011). Therefore, we used only SEAc to provide a robust comparison among categories (Jackson et al. 2011). We calculated the SEAc overlap between seasons for rural and urban populations to quantify diet seasonality. A visual inspection of plotted data suggested that dietary variability of females and males was similar. Thus, we pooled the data for males and females in our SIA.

To quantify the diet composition of squirrels from stable isotope values, we used the ‘Stable Isotope Bayesian’ mixing model in R (SIAR, Parnell et al. 2010; Ver. 3.6.2.: R Development Core Team 2020). SIAR is a statistical method to estimate the consumption of multiple food sources in the diet of a consumer using Bayesian estimation and has been used for a variety of species (Polito et al. 2011; Ito et al. 2012; Jansen et al. 2013). This estimation is based on simulations that combine stable isotope ratios of carbon and nitrogen in multiple food sources in various patterns to converge on stable isotope ratios in consumers. In this study, two SIAR models with multiple food items for rural and urban areas were used to estimate the

diet composition for each area and season category, using the  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values of squirrel hair and the TDF (see below). In the rural model, we constructed four food item categories (seeds, plants, fungi, and animal matter). In the urban model, naturally unavailable items were added to all four food item categories. We estimated proportions of food sources consumed by the each of the four area and season categories with 185 squirrel hairs and five food item categories (Fig. 2 and 3, Table 4, Supplementary Data SD2, SD3 and SD4). We estimated the mean and 95% confidence intervals for these proportions.

TDF is an important metric for SIAR analysis, although TDFs of diet-based hair values have not been reported for *Sciurus* species because of difficulties associated with laboratory studies on squirrels. Previous studies estimated squirrel diets based on hair using TDFs derived from other animals (Hobbie et al. 2017; Derbridge 2018). However, large differences in laboratory-derived TDFs for mammalian hair exist among species (Crawford et al. 2008), especially for  $\delta^{13}\text{C}$ . In this study, we minimized bias by using three different TDFs (see below) for SIAR analysis: TDF1, 1.0‰ for  $\delta^{13}\text{C}$  and 3.0‰ for  $\delta^{15}\text{N}$ , the approximate diet-tissue values in mammals (Crawford et al. 2008); TDF2, -1.1‰ for  $\delta^{13}\text{C}$  and 3.0‰ for  $\delta^{15}\text{N}$ , diet-hair values in *Mus musculus*, the species most closely related to the *Sciurus* species group amongst species which published TDF values (DeNiro and Epstein 1978, 1981); TDF3, 2.6‰ for  $\delta^{13}\text{C}$  and 3.5‰ for  $\delta^{15}\text{N}$ , calculated the value of Eurasian Red Squirrel hair using the SIDER package in R (Healy et al. 2018). SIDER provides an estimated TDF of the target species using a

phylogenetic regression model, including diet, tissue type, and habitat information which could improve the fit to the actual TDF value (Goodwin et al. 2020; Swan et al. 2020). Subsequent analysis revealed that estimated consumption of each food source using these TDFs were similar (Supplementary Data SD3 and SD5). Thus, we used TDF1 (diet-tissue values in mammals) as the representative TDF to estimate diet consumption by SIAR.

### *Statistical analysis*

Using observational data from 2019, we investigated whether rural and urban squirrels exhibited seasonal differences in diet composition. Three food items foraged by each individual were classified into five categories (seeds, plants, fungi, animal matter, and naturally unavailable items; note that naturally unavailable items applied only to urban populations). We then counted the number of items foraged by each individual (0–3 counts for each of the five categories). In the animal matter category, we observed only two foraged items (one each in rural and urban areas). Because of sample size, we did not analyze these statistically. These counts were fitted to generalized linear mixed models (GLMM) using package ‘lme4’ (Bates et al. 2015) in R version 3.6.2 (R Development Core Team 2020), and separated into four food item category models. In each of the four models, the number of foraged items per individual was the dependent variable. Area (rural and urban), season (spring and fall), sex, and interaction terms were used as independent variables. We did not include naturally unavailable items in

the model because these were not observed in rural habitats. Individual animals and study sites were treated as random effects. We used a Poisson distribution for the error term and  $P$ -values were calculated using likelihood ratio tests (ANOVA function in the ‘car’ package). Conditional and marginal  $R^2$  values were calculated to check goodness of fit with and without the random effects (Nakagawa and Schielzeth 2013) using the ‘MuMIn’ package (Bartoń 2022). Because three-way interactions were not significant ( $P > 0.05$ ) in all models, they were removed and the model was refitted.

Using 338 body weights from 178 individuals between 2017 and 2019, we fitted a Linear Mixed Model (LMM). We separated the data based on sex to simplify the results as females gain weight in spring because of pregnancy. Body weight was the dependent variable in both female and male models. Area (rural or urban), season and the area by season interaction were set as independent variables. Individual animals, study sites and study years were treated as random effects. Significance was assessed using  $F$  tests. Conditional and marginal  $R^2$  values were computed as above. An interaction between season and area was not significant in the male model. Thus, we removed the interaction and refitted the models.

## **2.3 Results**

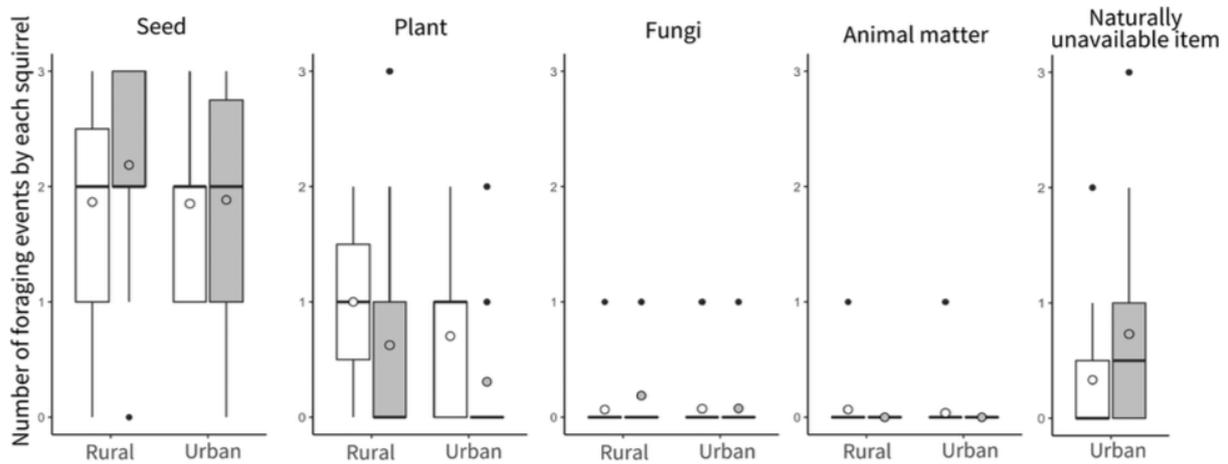
### ***Diet compositions from direct observation***

We captured 246 squirrels, of which 87 (19 rural and 68 urban), 83 (16 rural and 67 urban), and 76 (14 rural and 62 urban) were tagged in 2017, 2018, and 2019 respectively. Using these marked individuals, we observed three events of foraging behavior by 84 males and females in 2019 (rural:  $n = 13$  and  $n = 18$ , urban:  $n = 29$  and  $n = 24$ , respectively; for a total of 252 observations).

Squirrels foraged various food items including insects, animal bones, and leaves (Table 1), but their diets consisted mainly of walnuts and pine nuts (Table 1, Fig. 1). In all three food-item category models (seed, plant, and fungi), we did not find a significant difference between rural and urban squirrels (Table 2). Seasonal differences were apparent only for the plant food item model (Table 2, Fig. 1) and there were no differences associated with sex for any food item model. The seasonal difference in the plant model indicates that squirrels in spring consume plants more often than in fall (Table 2, Fig. 1). In urban squirrels, 50 of 85 walnuts (58.8 %) and 9 of 15 pine nuts (60.0%) were provided by humans (Table 1). As with other food items from SF, squirrels acquired various naturally unavailable items. In particular, they took sunflower seeds, peanuts, and pumpkin seeds (Table 1). Of 159 food items foraged by urban squirrels, 87 (54.7%; Table 1) were taken from SF (including seeds from SF and naturally unavailable items).

**Table 1.**—List of food items observed in 2017-2019 and the number of foraging events in 2019.

Species	Parts	Foraged number in 2019	
		Rural	Urban
<b>Seed</b>			
Japanese Walnut ( <i>Juglans mandshurica</i> var. <i>sachalinensis</i> )	Seeds	15	85 (50 from SF)
Korean Pine ( <i>Pinus koraiensis</i> )	Seeds	48	15 (9 from SF)
<b>Plant</b>			
Locust tree ( <i>Robinia pseudoacacia</i> )	Flowers	1	—
Sakhalin Spruce ( <i>Picea glehnii</i> )	Shoots	4	—
Japanese Yew ( <i>Taxus cuspidata</i> )	Fruits	—	2
Annual Bluegrass ( <i>Poa annua</i> L.)	Leave	3	—
Painted Maple ( <i>Acer pictum</i> Thunb. ex Murray)	Shoots	—	2
	Seeds	—	1
Japanese Emperor Oak ( <i>Quercus dentata</i> )	Shoots	2	—
	Flowers	1	—
	Leave	—	—
	Acorn	4	—
Japanese Larch ( <i>Larix kaempferi</i> )	Shoots	—	—
	Corn	—	1
Empress tree ( <i>Paulownia tomentosa</i> )	Shoots	—	—
	Syrup	—	1
Japanese Chestnut ( <i>Castanea crenata</i> )	Seeds	—	1
Kobushi Magnolia ( <i>Magnolia kobus</i> )	Buds	—	1
	Flowers	—	—
Korean Pine ( <i>Pinus koraiensis</i> )	Shoots	—	1
	Unmatured corn	1	—
North Japanese Hill Cherry ( <i>Cerasus sargentii</i> (Rehder) H.Ohba)	Buds	—	1
	Fruits	1	4
Japansene White Birch ( <i>Betula platyphylla</i> )	Syrup	—	1
Dandekion ( <i>Taraxacum officinale</i> )	Leave	2	4
Moss ( <i>Bryophyte</i> )	Moss	3	2
Norway Spruce ( <i>Picea abies</i> )	Corn	—	—
Japansese Horse Chestnut ( <i>Aesculus turbinata</i> )	Flowers	—	—
Japanese Elm ( <i>Ulmus davidiana</i> var. <i>japonica</i> )	Shoots	—	1
Weeping Willow ( <i>Salix babylonica</i> )	Buds	—	—
	Leave	—	1
Aino Mulberry ( <i>Morus australis</i> )	Fruits	3	2
Fungi	Fungus	4	4
<b>Animal matter</b>			
Aphid ( <i>Aphididae</i> )	Insects	—	—
Cicada ( <i>Terpnosia nigricosta</i> )	Insects	—	—
Froghopper ( <i>Aphrophoridae</i> )	Caterpillars	—	1
Ichuworm ( <i>Geometridae</i> )	Caterpillars	—	—
Some medium mammal	Bones	1	—
Some small bird	Bones	—	—
<b>Naturally unavailable item</b>			
Japanese Pumpkin ( <i>Cucurbita moschata</i> )	Seeds	—	4
Corn ( <i>Zea mays</i> L.)	Seeds	—	—
Pellets for hamsters	Pellets	—	—
Bread	Bread	—	—
Peanuts ( <i>Arachis hypogaea</i> )	Seeds	—	9
Sunflower ( <i>Helianthus annuus</i> )	Seeds	—	15
Common Hazel ( <i>Corylus avellana</i> )	Seeds	—	—



**Fig. 1.**—Tukey box plots (Tukey 1977) of the number of foraging events by each squirrel for five food item categories. Data for spring are shown as white boxplots, and data for fall are shown in grey. Means were shown as a circle in each box. Horizontal lines represent medians, quartiles and interquartile ranges (IQR). The upper and lower whisker extends from the hinge to the largest and smallest value no further than  $1.5 \times \text{IQR}$  from the hinge.

**Table 2.**—GLMM results for number of squirrel foraging events for each of four food item categories as well as marginal and conditional  $R^2$ . Significant  $P$  values are shown in bold.

	Estimate	SE	$\chi^2$ , d.f. = 1	$P$ -value
<b>Seed</b>				
Area <sup>a</sup>	-0.09	0.16	0.32	0.57
Season <sup>b</sup>	0.06	0.16	0.16	0.69
Sex <sup>c</sup>	-0.05	0.16	0.11	0.74
Marginal $R^2$	0.01		Conditional $R^2$	0.01
<b>Plant</b>				
Area <sup>a</sup>	-0.38	0.28	1.85	0.17
Season <sup>b</sup>	-0.58	0.29	3.84	<b>0.05</b>
Sex <sup>c</sup>	0.57	0.31	3.48	0.06
Marginal $R^2$	0.13		Conditional $R^2$	0.13
<b>Fungi</b>				
Area <sup>a</sup>	-0.67	0.72	0.85	0.36
Season <sup>b</sup>	0.42	0.74	0.32	0.57
Sex <sup>c</sup>	-0.69	0.75	0.83	0.36
Marginal $R^2$	0.02		Conditional $R^2$	0.02
<b>Naturally unavailable item</b>				
Season <sup>b</sup>	0.70	0.41	2.87	0.09
Sex <sup>c</sup>	-0.45	0.41	1.16	0.28
Marginal $R^2$	0.09		Conditional $R^2$	0.17

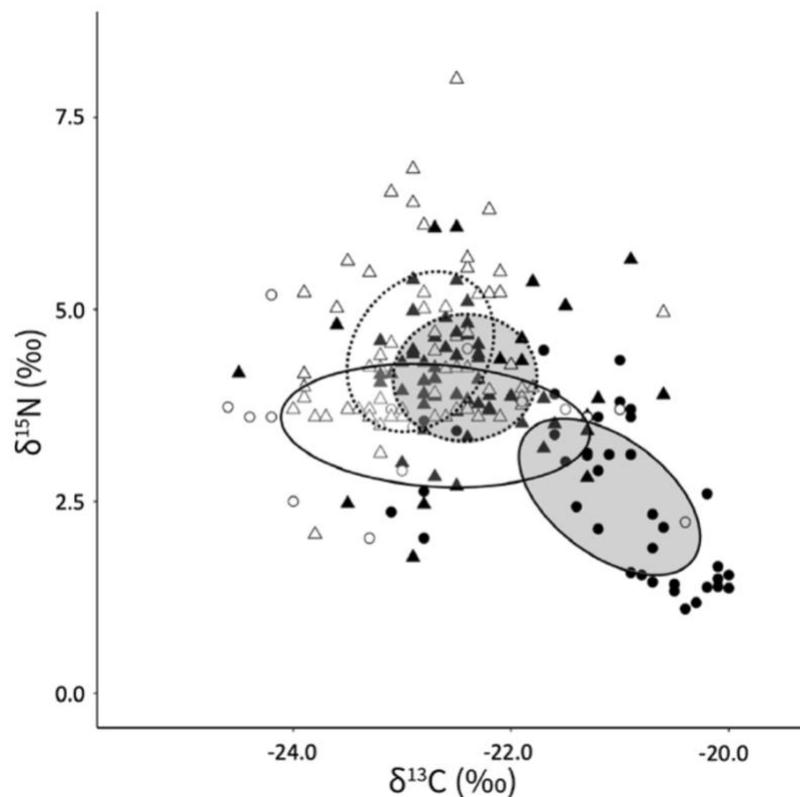
<sup>a</sup> rural = 0; urban = 1. <sup>b</sup> spring = 0; fall = 1. <sup>c</sup> female = 0; male = 1.

### ***Diet widths and seasonal changes by SIBER***

Both TA and SEAc proxies for diet widths were larger in rural areas than in urban areas (Table 3, Fig. 2). Additionally, SEAc overlap area among seasons was larger in urban areas than in rural areas (rural, 10–17%; urban, 46–57%; Table 3). Dietary width and seasonal changes were smaller in urban squirrels than in rural squirrels, indicating that urban squirrels rely strongly on specific food items, and their diets do not change appreciably across seasons.

**Table 3.**—Stable isotope values for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  and diet widths of squirrel hair in each area and season. Diet widths were estimated by Stable Isotope Bayesian Ellipses in R (SIBER) and shown in different estimations; total area (TA) and standard ellipse area corrected for small sample size (SEAc).

	<i>n</i>	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$	TA	SEAc	SEAc overlap area ( $\text{‰}^2$ )
	(male:female)	(Mean $\pm$ SD $\text{‰}$ )	(Mean $\pm$ SD $\text{‰}$ )	( $\text{‰}^2$ )	( $\text{‰}^2$ )	(Overlap%)
Rural						
Spring	16 (9:7)	-22.34 $\pm$ 0.98	3.88 $\pm$ 1.15	8.39	3.63	0.37 (10-17)
Fall	40 (15:25)	-22.21 $\pm$ 1.00	3.87 $\pm$ 1.26	6.68	2.27	
Urban						
Spring	63 (33:30)	-22.36 $\pm$ 0.98	3.89 $\pm$ 1.13	10.24	2.13	0.98 (46-57)
Fall	66 (31:35)	-22.31 $\pm$ 0.95	3.96 $\pm$ 1.25	10.63	1.72	



**Fig. 2.**—Isotopic distributions of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  and standard ellipse areas of rural and urban squirrel hair in spring and fall. Rural squirrel hairs were plotted as circles with solid ellipses, and those of urban squirrels were plotted as triangles with dotted ellipses. Hairs collected in spring were plotted as filled shapes ( $\bullet$ : rural,  $\blacktriangle$ : urban) and gray ellipses, and those collected in fall were plotted as open shapes ( $\circ$ : rural,  $\triangle$ : urban) and white ellipses.

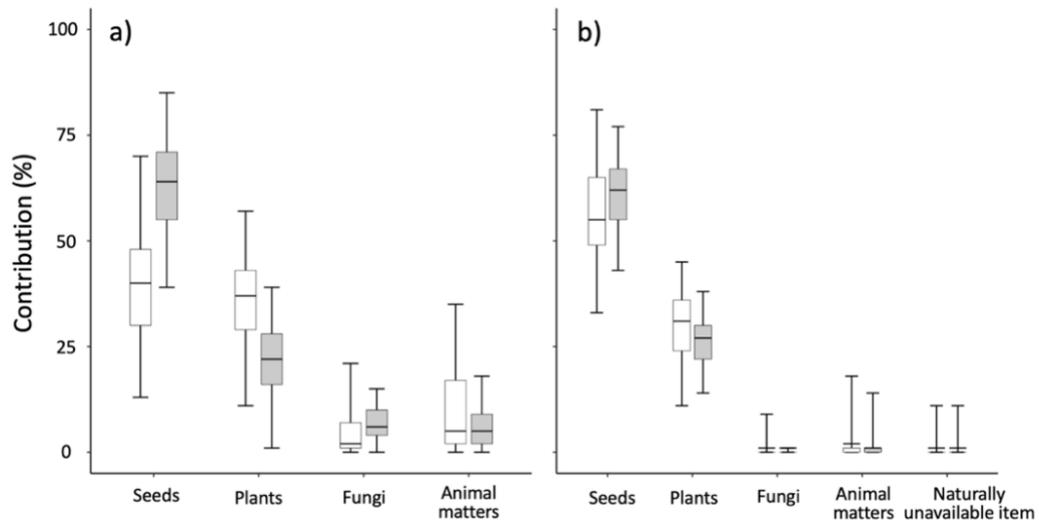
### *Estimated proportions for each resource by SIAR*

Stable isotope analysis showed that urban and rural squirrels used mainly seeds and plants (Table 4, Fig. 3). In rural populations, the proportion of seeds consumed in spring was lower than in fall (mean: 41% vs. 62%; Table 4, Fig. 3). The plant proportion of the diet was higher in spring than fall (mean: 35% vs. 22%; Table 4, Fig. 3). Fungal and animal matter consumption was rare in both spring and fall (Table 4, Fig. 3).

Consumption of seeds was lower in spring than fall for urban squirrels (mean: 57% vs. 60%; Table 4, Fig. 3). Urban squirrels consumed seeds more often than rural squirrels in spring (mean: 57% vs. 41%), but not in fall (mean: 60% vs. 62%; Table 4, Fig. 3). Seasonal difference in seed consumption in rural areas was higher than that in urban areas (21% vs. 3%; Table 4, Fig. 3). Additionally, the seasonal differences in plant consumption in rural areas was greater than that in urban areas (13% vs. 3%; Table 4, Fig. 3). The proportions of fungi, animal matter, and naturally unavailable items were also low in urban areas (Table 4, Fig. 3). The results of SIAR analysis using the other two TDFs are shown in Supplementary Data SD3 and SD5.

**Table 4.**—Estimated proportions for each food item category and those of seasonal differences in rural and urban populations of squirrels. Estimated proportions were shown as mean and 95% confidence interval (95% CI) which is calculated by TDF1 of representative diet-tissue values in mammals ( $\delta^{13}\text{C}$ : 1.0‰ and  $\delta^{15}\text{N}$ : 3.0‰ from Crawford et al. 2008) using Stable Isotope Analysis in R (SIAR). Seasonal differences are absolute values of mean differences between spring and fall.

Category	Estimated proportion (95% CI)		Seasonal difference (%)
	Spring	Fall	
<b>Rural</b>			
Seed	41 (13-70)	62 (39-85)	21
Plant	35 (11-57)	22 (1-39)	7
Fungi	8 (0-20)	8 (0-15)	0
Animal matter	16 (0-35)	8 (0-18)	8
<b>Urban</b>			
Seed	57 (33-81)	60 (43-77)	3
Plant	29 (11-45)	26 (14-38)	3
Fungi	3 (0-9)	3 (0-1)	0
Animal matter	7 (0-18)	6 (0-14)	1
Naturally unavailable item	4 (0-11)	5 (0-11)	1



**Fig. 3.**—Estimated contributions for each food item category in the diet of rural and urban squirrels using stable isotope analysis. a) Estimated contribution in rural and b) urban squirrels. Spring diets are shown as white, and fall diets are shown as grey. Medians are shown as lines within the boxes, 50% confidence intervals are shown as boxplots and 95% confidence intervals are shown as whiskers.

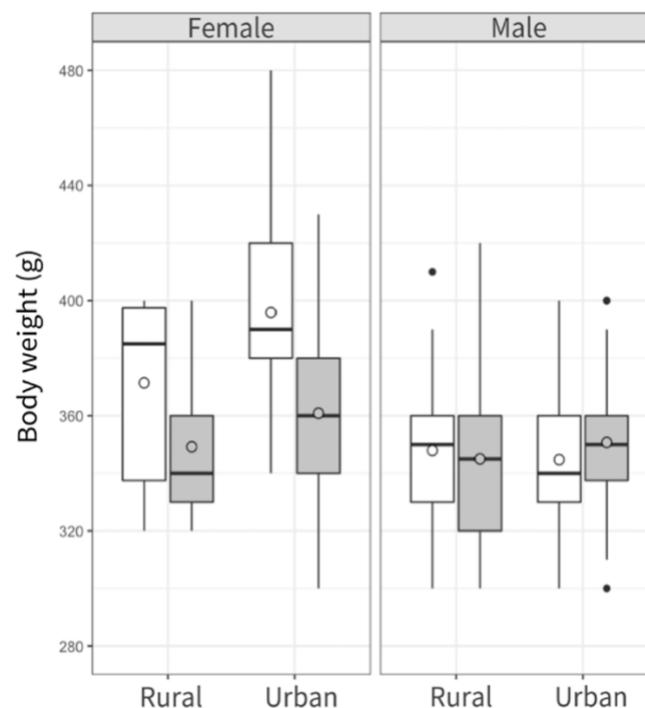
### ***Body weight comparison***

Female body weights were significantly larger in the spring, and there was a significant area by season interaction (LMM; Table 5). The significant interaction between area and season shows that urban females were significantly heavier in the spring, while rural females were not (Fig. 4).

There were no differences in body weights for rural and urban males (LMM; Table 5, Fig. 4). Neither area nor season differences existed amongst the weights of males (Table 5). The large difference between marginal and conditional  $R^2$  values (Table 5) indicates that random effects (individual ID, study site, and year) strongly affected model fit in both females and males.

**Table 5.**—The effects of area (rural vs. urban), season (spring vs. fall), and their interactions on female and male body weight of squirrels using LMM, and marginal and conditional  $R^2$ . Significant values are shown in bold.

	Estimate	SE	<i>d.f.</i>	<i>F</i>	<i>P</i> value
<b>Female</b>					
Area <sup>a</sup>	23.80	11.97	1, 6.56	1.01	0.35
Season <sup>b</sup>	-13.87	8.83	1, 105.61	44.45	<b>&lt;0.001</b>
Area x Season <sup>c</sup>	-21.66	10.07	1, 134.19	4.46	<b>0.04</b>
	Marginal $R^2$		0.24	Conditional $R^2$	0.57
<b>Male</b>					
Area <sup>a</sup>	-3.46	8.22	1, 5.54	0.17	0.70
Season <sup>b</sup>	4.13	3.19	1, 141.57	1.54	0.22
	Marginal $R^2$		0.24	Conditional $R^2$	0.57



**Fig. 4.**—Tukey box plots (Tukey 1977) of body weights of female and male squirrels in each area. Data for spring are shown as white boxplots, and data for fall are shown in grey. Means are denoted as a white circle in each box. Boxplots illustrate medians and quartiles, and the upper and lower whisker extends from the hinge to the largest and smallest value no further than  $1.5 \times \text{IQR}$  from the hinge.

## 2.4 Discussion

We assessed dietary differences and seasonal effects for urban and rural red squirrels. Both direct observations and SIA showed that tree seeds (walnuts and pine nuts) were the most-foraged food items. Trees mast during fall (Hayashida 1989; Goto and Hayashida 2002) resulting in seed abundance in fall and scarcity in spring. While fall seed consumption did not differ between rural and urban squirrels (Table 4, Fig. 3), in spring urban squirrels consumed more seeds than rural squirrels (Table 4, Fig. 3). Rural squirrels consumed a greater proportion of plants in spring (Table 4, Fig. 3) perhaps indicating some compensation for a reduction in seed availability. The high rates of seed consumption by urban squirrels during spring and fall (Table 4, Fig. 3) may be consequence of availability via year-round SF. This reduction in seasonality of urban seed availability may affect squirrel behavior and life history patterns, resulting perhaps in a longer breeding season and reducing selection for caching behavior. The consequence of these changes may include altered population dynamics (Wauters and Dhondt 1990). The dietary and seasonal differences we found between urban and rural squirrels are likely the consequence of anthropogenic food (SF). Clearly, an investigation of SF effects on fitness-related traits is required.

Unexpectedly, fall seed consumption did not differ between rural and urban squirrels (Table 4, Fig. 1 and 3). Even with greater access to seeds via SF, seed use by urban squirrels reaches a maximum of approximately 70% (Table 4, Fig. 1 and 3). The diets of urban and rural

Eurasian red squirrels in Poland are comprised of approximately 87% and 70% seeds, respectively (Krauze-Gryz et al. 2021). Although seeds are calorie rich, squirrels may forage for other foods including leaves and fruits to balance nutritional micro-components (including calcium and sodium). Pregnant and lactating females must consume adequate sources of energy, calcium, and sodium to rear offspring (Havera 1979).

Quantitative analyses of diets are important when assessing the effects of anthropogenic foods on the life histories of urban wildlife. While direct observation may reveal specific food items and whether they are natural or anthropogenic, qualitative methods are limited in their ability to quantifying diet composition. Visibility, squirrel sensitivity to humans (Uchida et al. 2016), and food item size can result in observational bias towards conspicuous foraging behaviors and consequent overestimation of the proportion of anthropogenic foods consumed relative to SIA (Table 4, Figs. 1 and 3). Previous studies have focused on the life histories of urban wildlife utilizing anthropogenic foods (Støstad et al. 2019) without conducting quantitative dietary analyses. Even when there is an apparent dependency on anthropogenic foods, qualitative observations may overestimate anthropogenic food use and the consequent effects on life histories.

Results derived from SIA may exhibit less bias than those derived from direct observation. SIA has been used in numerous diet studies across taxa (Goodwin et al. 2020; Meyer et al. 2020). Our results derived via SIA were similar to those of direct observation.

However, assumptions associated with SIA include TDF and tissue turnover rates (Crawford et al. 2008; Stephens et al. 2022). Additionally, it is necessary to know key dietary components based on observations before using SIA. It is difficult to investigate diet composition using SIA alone, particularly in animals where turnover and TDF in tissues are unknown. To investigate accurately the diet composition of wildlife, it is beneficial to use multiple methods, including direct observation and SIA.

Body weight comparisons allowed us to assess the effect of dietary differences on rural and urban squirrels. Female urban squirrels were heavier than rural squirrels in the spring. This may be a consequence of high seed abundance from SF in spring. While the proportion of seed use in spring was higher in urban squirrels, urban squirrels increased plant use in the same manner as rural squirrels. This is consistent with the idea that females should forage for other food items to increase intake of nutritional micro-components. Because urban females are heavier, they can allocate additional energy to reproduction. Urban females that consume abundant seeds may breed earlier, prolong the breeding season, and potentially produce additional litters.

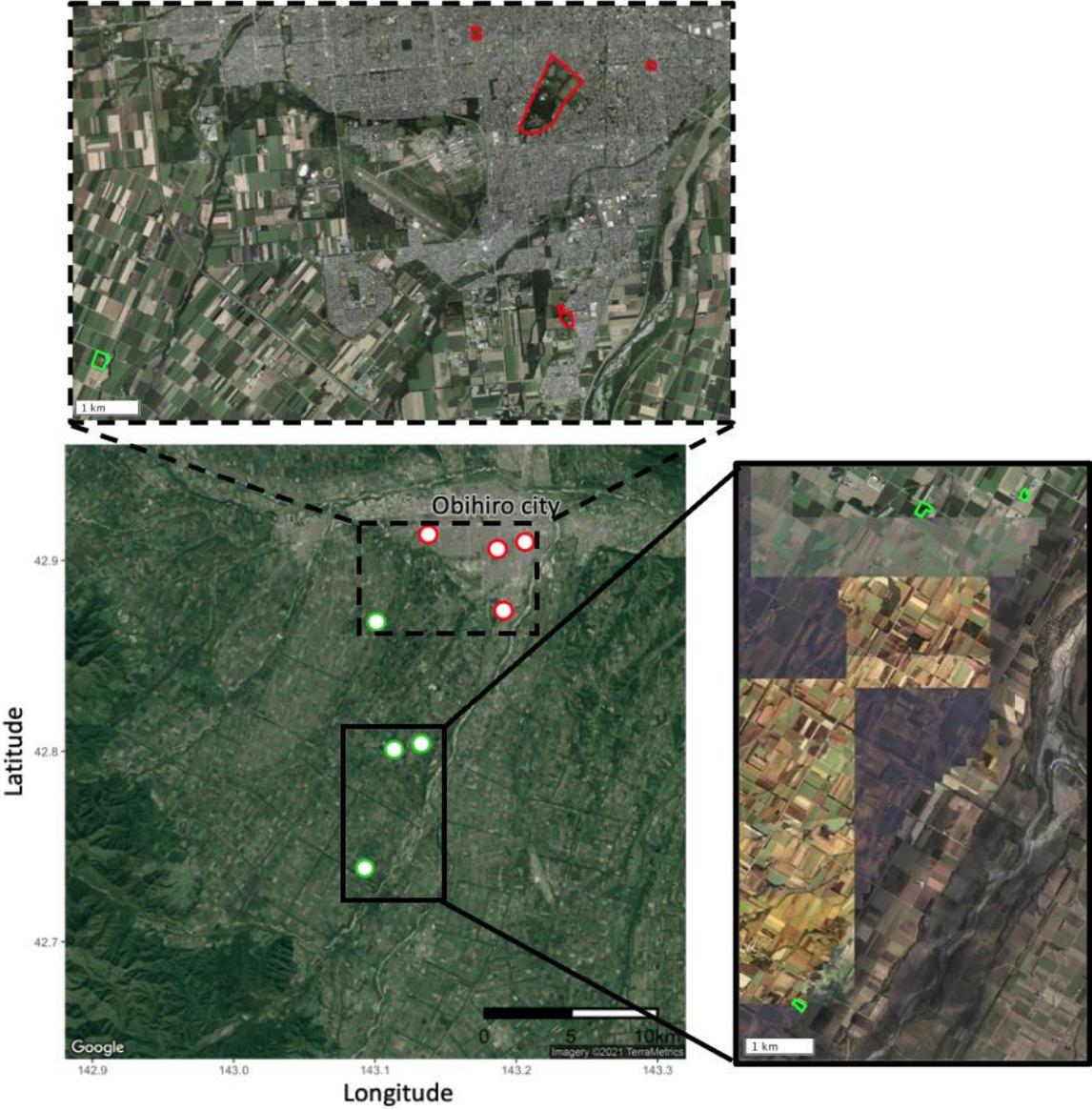
We found no difference in body weights of urban and rural males. This may be a consequence of energy expended on intense male–male competition in urban environments. Here, SF attracts multiple individuals with a consequent increased in local population density (Starkey and Delbarco-Trillo 2019) and a greater likelihood of male-male interactions. Females

usually compete only for food and territories (Wauters and Dhondt 1992, 1993). Because red squirrels are promiscuous (Wauters et al. 1990; Wauters and Dhondt 1992), males compete for food and access to mates. We observed large aggregates of squirrels at feeding sites, and male-male interactions were frequent in urban areas. We suspect that male-male competition at urban feeding sites may increase energetic expenditures by urban males and thus reduce difference in body weights between urban and rural males.

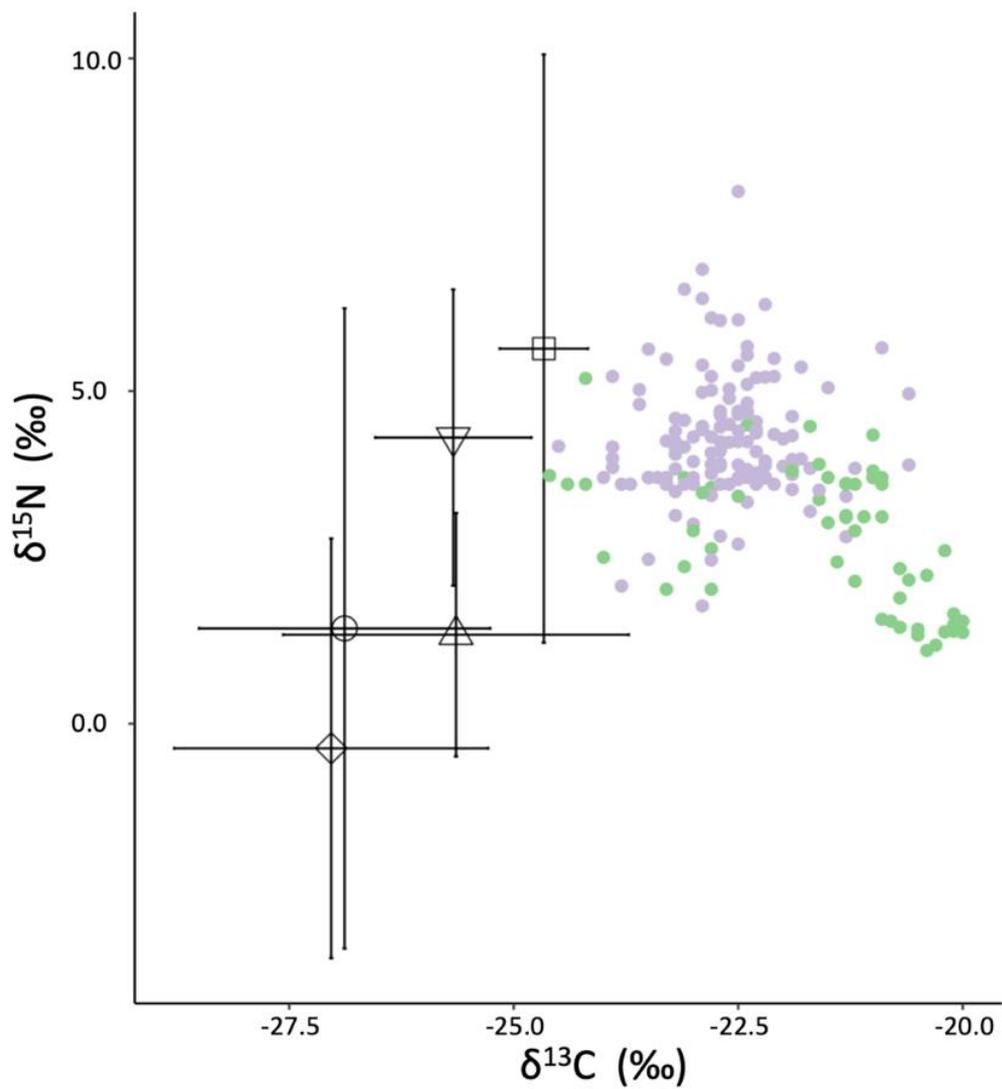
Male–male competition in urban areas may result in selection for some behavioral traits, including aggression or boldness. Red squirrels exhibit a dominance hierarchy when aggregated (Wauters et al. 1990). Male dominance is determined by body weight and age, with heavier, older males dominant (Wauters et al. 1990). Dominant males often exclude others at feeding sites and monopolize food by SF, which can result in increased body weight and improved reproductive success. Thus, urban environments may select for aggressiveness. Additionally, urban squirrels are exposed to more human disturbance (Uchida et al. 2019) and SF likely selects for increased boldness (Uchida et al. 2021). Boldness is often positively correlated with aggressiveness (“behavioral syndrome”; Sih et al. 2004), so increased boldness induced by SF may also increase aggressiveness. If true, SF could result in individual variations in life history traits such as increased reproductive and survival rates, potentially affecting population characteristics. The evolutionary consequences of these behavioral changes on life history traits have not yet been adequately explored.

Urban squirrels showed less dietary seasonality than rural squirrels. They consumed seeds with high caloric value at a high rate throughout the year, likely because of availability through SF. Supplemental foods provided by humans may result in increased female body weight and contribute to a robust growing population. Increased reproductive success and survival as a result of SF, with consequent population growth, has been reported for other species (Robb et al. 2008b; Plummer et al. 2019). However, there are examples of possible detrimental effects caused by SF, including reduced reproductive success (Robb et al. 2008b; Plummer et al. 2013). Unbalanced nutritional content available through SF is associated with decreased immunity and reproduction (Plummer et al. 2013; Strandin et al. 2018). It encourages wildlife to associate humans with food, inducing an increased likelihood of disease transmission (Pisanu et al. 2014), injury, and mortality in both humans and wildlife (Cox and Gaston 2018; Strandin et al. 2018; Lawson et al. 2018). Moreover, these negative effects may also appear in non-target species and may affect interspecific relationships and consequently, community structure (Reed and Bonter 2018; Meyer et al. 2020; Shutt and Lees 2021). The anthropogenic component of foods for wildlife should be considered more fully.

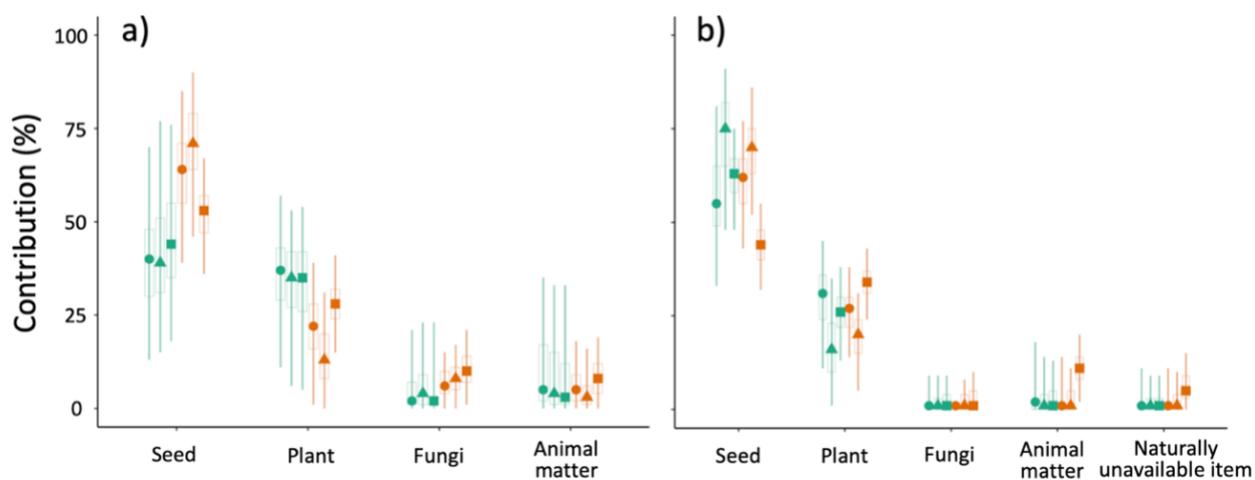
**Supplementary Data SD1.**—Map of study sites and enlarged maps for urban and rural sites. The urban study sites were located in a residential area (white area) in Obihiro City. Rural sites were located on agricultural land (shallow green areas). Four urban study sites are plotted as red, and rural sites are plotted as green. All study sites were isolated 1 km from neighboring study sites.



**Supplementary Data SD2.**— $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  stable isotope ratios in squirrel hairs and food sources. Each food source is shown in shape. Triangle, seed; diamond, plant; square, fungi; circle, animal matter; inverted triangle, naturally unavailable food item. Hairs of urban squirrels are shown as purple circles, and those in rural areas are shown as green circles. Hairs are row isotopic values that are not adjusted by TDFs.



**Supplementary Data SD3.**—Estimated contributions of each food item category in squirrel diet using three TDFs. Estimated contributions of each food item category: a) urban squirrels and b) rural squirrels using SIAR. Spring diets are shown as green plots, and fall diets are shown as orange plots. Each shape indicates TDFs, circle: TDF1 ( $\delta^{13}\text{C}$ :1.0‰ and  $\delta^{15}\text{N}$ : 3.0‰ from Crawford et al. 2008), triangle: TDF2 ( $\delta^{13}\text{C}$ : -1.1‰ and  $\delta^{15}\text{N}$ : 3.0‰ from diet-hair values in Mouse, DeNiro and Epstein 1978; DeNiro and Epstein 1981), and square: TDF3 ( $\delta^{13}\text{C}$ :2.6‰ and  $\delta^{15}\text{N}$ :3.5‰ from SIDER estimation). Each plot is shown as median, and the 50% and 95% CI as boxplots and whiskers, respectively.



**Supplementary Data SD4.**—Summary of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  stable isotope ratios for five food item categories.

	n	Mean $\delta^{13}\text{C}\pm\text{SD}$ (‰)	Mean $\delta^{15}\text{N}\pm\text{SD}$ (‰)
Animal matter	3	-26.18 $\pm$ 1.82	1.67 $\pm$ 3.19
Fungi	3	-24.66 $\pm$ 0.49	5.64 $\pm$ 4.42
Plant	21	-26.33 $\pm$ 1.86	1.11 $\pm$ 3.47
Seed	16	-26.00 $\pm$ 2.14	0.85 $\pm$ 1.97
Naturally unavailable item	5	-26.14 $\pm$ 2.00	1.43 $\pm$ 3.36

**Supplementary Data SD5.**—The result of SIAR from three TDFs. SIAR results from three TDFs, including estimated mean and median, and 50% and 95% CI of contributions for each food category.

TDF	area	season	category	mode	mean	95% CI	50% CI
TDF1	Urban	Spring	Seed	55	57	33-81	49-65
			Plant	31	29	11-45	24-36
			Fungi	1	3	0-9	0-0
			Animal matter	2	7	0-18	0-1
			Naturally unavailable item	1	4	0-11	0-0
	Rural	Spring	Seed	62	60	43-77	55-67
			Plant	27	26	14-38	22-30
			Fungi	1	3	0-1	0-0
			Animal matter	1	6	0-14	0-1
			Naturally unavailable item	1	5	0-11	0-0
		Fall	Seed	40	41	13-70	30-48
			Plant	37	35	11-57	29-43
			Fungi	18	8	0-20	1-1
			Animal matter	5	16	0-35	2-17
TDF2	Urban	Spring	Seed	64	62	39-85	55-71
			Plant	22	22	1-39	16-28
			Fungi	6	8	0-15	4-10
			Animal matter	5	8	0-18	2-9
			Naturally unavailable item	1	3	0-9	0-3
	Rural	Spring	Seed	75	70	48-91	65-82
			Plant	16	18	1-35	10-23
			Fungi	1	3	0-9	0-3
			Animal matter	1	5	0-14	0-4
			Naturally unavailable item	1	3	0-9	0-3
		Fall	Seed	70	69	52-86	63-75
			Plant	20	19	5-31	15-24
			Fungi	1	4	0-8	0-4
			Animal matter	1	5	0-11	0-5
Rural	Spring	Naturally unavailable item	1	4	0-10	0-4	
		Seed	39	44	15-77	31-51	
		Plant	35	32	6-53	27-42	
		Fungi	4	9	0-23	1-9	
		Animal matter	4	15	0-33	1-15	

		Seed	71	69	46-90	64-79
	Fall	Plant	13	16	0-31	8-20
		Fungi	8	9	0-17	5-11
		Animal matter	3	7	0-16	1-6
		Seed	63	62	48-75	58-67
	Spring	Plant	26	26	13-38	22-30
		Fungi	1	4	0-9	0-4
		Animal matter	1	5	0-13	0-5
	Urban	Naturally unavailable item	1	4	0-9	0-3
		Seed	44	44	32-55	40-48
		Plant	34	34	24-43	31-37
	Fall	Fungi	1	4	0-10	1-5
		Animal matter	11	11	2-20	8-14
		Naturally unavailable item	5	7	0-15	3-9
TDF3		Seed	44	46	18-76	35-55
	Spring	Plant	35	32	5-54	26-42
		Fungi	2	9	0-23	1-1
		Animal matter	3	13	0-33	1-12
	Rural	Seed	53	52	36-67	47-57
		Plant	28	28	15-41	24-32
	Fall	Fungi	10	11	1-21	7-14
		Animal matter	8	10	0-19	4-12

## **Chapter 3. Female's reproductive condition**

### **3.1 Introduction**

Urbanization is expanding worldwide and has negative impacts on wildlife, such as habitat loss and anthropogenic disturbances (e.g., light pollution, noise, and metal exposure, Grimm et al. 2008; Senzaki et al. 2020). However, many species invade and survive in urban environments because of their inherent characteristics such as boldness and their ability to be omnivorous (Lowry et al. 2013). Alternatively, some species succeed while living in urban environments by modifying their ecological and behavioral traits, such as personality traits and resource use, including nesting sites and food (Robb et al. 2008; Chamberlain et al. 2009; Lowry et al. 2013; Johnson et al. 2020). In particular, food resource use is crucial for urban life because energy intake strongly affects reproduction and survival. In urban environments, many types of anthropogenic food resources are accessible to wildlife, such as garbage, garden plants, and sometimes kleptoparasitism of human food (Goumas et al. 2019; Shutt and Lees 2021).

Supplemental feeding (SF) is a common activity in many countries for various purposes, including conservation, tourism, and entertainment (Robb et al. 2008; Murray et al. 2016). In particular, SF of garden wildlife is the most common activity in many countries, and target species were mainly passerines and rodents. In addition, food from SF (e.g., tree seeds and grains) usually has high calorific value and is available across seasons (Robb et al. 2008;

Murray et al. 2016). Thus, many species depend on and benefit from SF, such as improved body conditions, reproductive success, and winter survival rates, consequently inducing population growth (Robb et al. 2008; Murray et al. 2016). Therefore, SF is believed to contribute to wildlife living in urban environments and has been encouraged as an animal conservation activity (Murray et al. 2016). However, recent studies have demonstrated that SF also has negative effects on wildlife, such as increasing the possibility of disease transmission within species, decreasing the vigilance for predators, and inducing intra- and inter-specific competition (Shutt and Lees 2021). Therefore, SF policies are becoming controversial (Baverstock et al. 2019), and more data on the effects of SF on wildlife are required.

Reproductive output is one of the most important methods for understanding the effects of SF because it strongly reflects population growth and urban adaptation. Many studies have demonstrated that SF contributes to reproductive output (Murray et al. 2006; Robb et al. 2008). For example, in avian species, the Mauritian echo parakeet, *Psittacula eques* increased the number of fledglings produced per brood (Tollington et al. 2018). In mammals, the American red squirrel, *Tamiasciurus hudsonicus* prolonged the breeding season, and some females had more than one litter (i.e., attended multiple breeding, Sullivan 1990). However, negative effects on the reproductive output have also been observed. For example, the growth rates of the parasitic jaeger, *Stercorarius parasiticus*, are negatively correlated with the intake of SF (Davis et al. 2005). In the blue tit, *Parus caeruleus*, fed individuals birthed chicks having

a low weight, small body size, and low survival (Plummer et al. 2013). Therefore, the effects of SF on reproductive output are not always positive, and are species- and context-specific. Although the effects of SF on reproduction may vary between avians and mammals, previous studies have mainly focused on the avian species. Therefore, the effects of SF on mammalian reproductive output remain unclear.

Eurasian red squirrels, *Sciurus vulgaris* are suitable for investigating the effects of SF on reproductive output. They are representative urban animals commonly fed by humans in Eurasian countries (Jokimäki et al. 2017; Mori et al. 2018; Beliniak et al. 2022; Takahata et al. 2023). Between winter and spring, they mate and give birth to an average of three young (anywhere between 1–7 individuals) in a single litter in early spring (Wauters and Lens 1995; Mari et al. 2008). Females are usually pregnant at two years of age (Wauters and Dhondt 1995). If females had a good body condition, they were pregnant at one year of age and had two litters in one year (i.e., multiple breeding, Wauters and Dhondt 1995). Although their urban ecology, such as the causes of mortality, diet composition, and population genetic structures, is well understood (Fingland et al. 2022), their reproductive conditions have been previously investigated in only one study. Beliniak et al. (2022) investigated the rates of pregnant females in Poland and found that these rates were higher in urban than in rural settings. Ideally, comparisons between urban and rural populations should also be performed for other proxies

of reproductive output, such as litter size, whether multiple breeding were accomplished, breeding season, and the age of initial pregnancy.

We studied the reproductive conditions of urban and rural populations in the Obihiro area of Hokkaido, Japan. Capturing and recording the actual number of young is ideal for assessing reproductive outputs. However, capturing young individuals induces squirrels to change their nests and induces mortality, such as attacks by crows during immigration. Therefore, we investigated their reproductive conditions using six proxies that could be directly observed: (1) rate of pregnancy, (2) age at initial pregnancy, (3) maximum age of reproduction, (4) birth date, (5) number of weaned litters, and (6) whether they had two litters. Our previous study in the Obihiro area showed that urban squirrels had a better diet composition than rural squirrels because of SF, since urban squirrels ate a large number of tree seeds throughout the year (Takahata et al. 2023). Possibly because of SF, in the spring, urban females had higher body weight gains than rural females (Takahata et al. 2023). In squirrels, a high body weight is correlated with lifetime reproductive success (Wauters and Dhonadt 1995). Thus, urban squirrels in Obihiro have better reproductive conditions than those in rural areas. Following these data, we predicted that: (1) pregnancy rates would be higher in urban populations rather than in rural populations; (2) age of initial pregnancy would be earlier in urban than in rural squirrels (i.e., more females would be pregnant at 1 year old in urban populations); (3) the maximum age of reproduction would be longer in urban than in rural squirrels, (4) birth dates

would be earlier in urban than in rural squirrels; (5) the number of weaned litters would be higher in urban than in rural squirrels; (6) urban squirrels were more likely to have two litters than rural squirrels.

### **3.2 Materials and Methods**

#### ***Study sites***

Our study was conducted in Obihiro, Hokkaido, Japan (42°49'N, 143°02'E), between 2020 and 2022. Six forests located at least 10 km away from central Obihiro city and surrounded by agricultural fields were selected as rural study sites (the area of study sites:  $2.8 \pm 0.7$  km<sup>2</sup> ( $\bar{x} \pm SE$ )). Although rural study sites included human-mediated sites, such as shrines or horse-riding parks, all sites were rarely visited by humans, and SF was not conducted. Forests mainly consisted of deciduous trees, such as the Japanese emperor oak, *Quercus dentata* and Japanese walnuts, *Juglans mandshurica var. sieboldiana*, and conifer trees, such as the Korean pine *Pinus koraiensis* and Sakhalin spruce *Picea glehni* (Fr. Schm) Masters.

Four urban parks located in residential areas were selected as urban study sites (the area of the study sites was:  $14.3 \pm 10.4$  km<sup>2</sup> ( $\bar{x} \pm SE$ )). Urban sites are surrounded by residential buildings and traffic roads, and frequently visited by humans (number of human visitors in an hour was calculated to be:  $5.5 \pm 11.1$  ( $\bar{x} \pm SE$ )). Planted trees in urban sites were similar to those in rural sites, whereas SF for wildlife was a common activity only in urban sites. Tree seeds

such as walnuts, Korean pine nuts, and peanuts are mainly provided as SF, and these kinds of food are abundantly provisioned every day via feeders or human hands. Thus, food availability for squirrels is more stable in urban areas than in rural study sites (Takahata et al. 2023)

### *Capturing squirrels*

Trapping of squirrels was conducted at all study sites between 2020–2022. The protocol for capturing squirrels at our study site was the same as that used in previous studies (Uchida et al. 2019; Shimamoto et al.2020; Takahata et al. 2023). Squirrels were captured using box traps (Model RB-2, Sakae Industry Co., Ltd., Niigata, Japan) from 4:00 am to 11:00 am in the months May–June and October–November. Box traps were checked every 1–1.5 hours in rural areas, whereas they were always monitored in urban areas. If squirrels were captured, we measured their body weights (analyzed in Takahata et al. 2023), attached ear tags and collar rings for individual identification, and checked their sex and maturity (juvenile, subadult, or adult). If the captured squirrels were juveniles, we recorded them as 0 years old and used the data to calculate their age in subsequent years. All protocols were completed within 15 min, and the squirrels were released back at the capture location. The capturing and handling of squirrels were ethically approved by the Institutional Animal Care and Use Committee of The Graduate University for Advanced Studies, SOKENDAI (license number: SKD2020AR002).

### ***Data collections by direct observation***

In both, rural and urban populations, squirrels engage in mating behavior from February until the end of May and have one (single breeding) or two litters (multiple breeding) per year. The first litter is usually born at the end of March–April, and the second litter between June–July (Beliniak et al. 2022). Thus, we conducted direct observations every day during the breeding season from which runs from the end of March to June, from 2020–2022. We conducted all observations during the 30 min line transect census, and this protocol was the same as that used in previous studies (Takahata et al. 2023). When marked females were encountered along the line transects, their reproductive conditions were recorded following the definition of reproductive status by Wauters and Gurnell (1999). Females are defined as “non-breeding” when they are anoestrus, small vulva, no longitudinal opening, and invisible nipples; as “breeding” when they display a large and swollen vulva and longitudinal opening, and an enlarged belly during pregnancy (Supplementary Information, Fig. S1). We observed the belly size of breeding females daily to record their first parturition date. When the squirrels gave birth, their belly size shrank (Supplementary Information, Fig. S2). Thus, birth data are easily measurable through daily observations. In addition, we observed the females after giving birth and recorded their nipple and milk extractions (Supplementary Information, Fig. S1) to confirm the presence of young, lactating, and weaning conditions. Before the juveniles were weaned (at 8–10 weeks, National Zoo of Wales 2019) and became independent, they followed their mother

for approximately one week near the nest sites. If possible, the number of juveniles around their nest sites was counted as “the number of young weaned individuals”. In addition, we continued to observe the female’s belly size, even after giving birth, to record whether they had two litters.

From the daily observations described above, we recorded the following reproductive conditions of each individual: (1) whether pregnant or not, (2, 3) age of the pregnant female, (4) birth date of the first litter, (5) number of young weaned, and (6) whether they bred multiple times. Although we tried to record the reproductive conditions for all marked females, we could not do so for some individuals because of their disappearance from the study sites. After excluding those data, we could record reproductive conditions of 40, 13, and 19 females in 2020, 2021, and 2022, respectively (a total of 773 observational events,  $7.5 \pm 4.3$  (mean  $\pm$  SE) observations in each female). In addition, because we spent more time and more effort on capturing squirrels than the observations in 2021 and 2022, we could not record the reproductive conditions of all females in the population. Therefore, we calculated (1) the rate of pregnancy in the population and (6) the number of females that had two litters using data only from 2020. For (2) age at initial pregnancy, (3) maximum age of reproduction, (4) birth data, and (5) number of young weaned individuals, we used data from 2020–2022.

### *Statistical analysis*

To compare the pregnancy rate between rural and urban populations, we performed Fisher's exact test. To identify factors that affect birth date, we conducted a general linear mixed model (LMM) using the "lme4" package in R (Bates et al. 2015; R Development Core Team 2023). In the model, the birth date was the objective variable. The area (rural or urban), study year (2020, 2021, or 2022), and their two-way interactions were the explanatory variables. Individual animals (squirrel ID) and study sites were treated as random effects. In addition, we used a generalized linear mixed model (GLMM) with a binomial error structure to examine the effect of the birth date of a female's first litter on whether that female had the second litter or not. In this model, multiple breeding (1 or 0) is used as the objective variable. Birth date (days from March 1st), age of female, and two-way interactions were the explanatory variables. Individual animals (squirrel ID) were treated as random effects. P-values were calculated using likelihood ratio tests (ANOVA function in the 'car' package). If two-way interactions were not significant ( $p > 0.05$ ) in the model, they were removed and the model was refitted. Conditional and marginal  $R^2$  values were calculated to check the goodness of fit with and without the random effects (Nakagawa and Schielzeth 2013) using the 'MuMIn' package (Bartoń 2022).

To compare the number of weaned young between rural and urban squirrels, we performed permutation tests because of the small sample size (Results section). First, we generated a null distribution of the difference in the mean number of weaned young between urban and rural squirrels (urban-rural). Data were generated 999 times by randomly replacing

the area rows (urban or rural). Number of 1000 data which were constructed from 999 randomized data points and one actual data point, were permuted, and the order of the actual data was checked. Data on the number of females that had two litters and the initial and maximum age of reproduction were not included in the statistical analysis because of the small sample sizes.

### **3.3 Results**

#### ***Rates of pregnancy in the population***

We observed female reproductive conditions in 40 individuals in 2020 (n=11 and n=29 in rural and urban areas, respectively). Rates of pregnancy were higher in the urban population (96.6%, 28 of 29 females) than the rural population (72.7%, 8 of 11 females, Fisher's exact test  $p=0.05$ ).

#### ***Reproduction and female age***

We recorded the pregnancy rates of 27 females (n=7 and n=20 in rural and urban areas, respectively) whose ages could be identified (Table 1). In rural areas, there were no females that were pregnant at one year old (0%, 0 of 1 female, Table 1). Half of rural females (two of four females) were pregnant when they were two years old and they were always pregnant after three years old (100%, two of two females, Table 1). On the other hand, 75% (six out of eight females) of urban females were pregnant when they were one year old, and they were always

pregnant after two years old (100%, 12 of 12 females, Table 1). Thus, the age at initial pregnancy was earlier in urban squirrels than in rural squirrels.

The maximum age of reproduction in rural females was four years old (Table 1), which could be caused by longevity, because we could not find any individuals that were four years old in rural areas. In contrast, we observed two urban females who were pregnant at five years of age. Therefore, female reproduction is longer in urban (1–5 years old) than in rural females (2–4 years old).

**Table 1.**—The rate of pregnancy at each age between 1-5 years old.

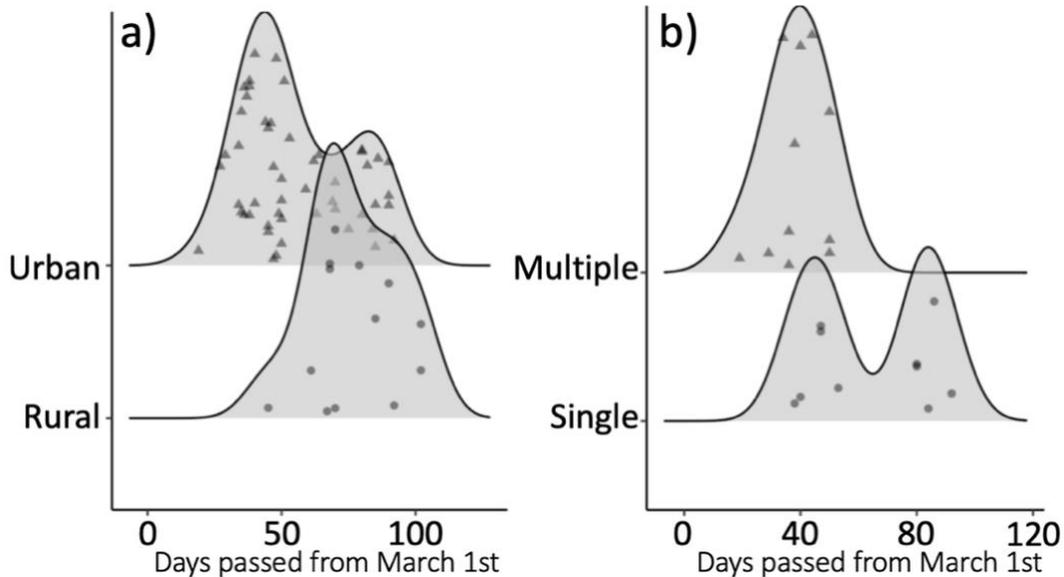
	Age of females				
	1	2	3	4	5
Rural (n=6)	0% (n=1)	50% (n=4)	100% (n=1)	100% (n=1)	-
Urban (n=20)	75% (n=8)	100% (n=4)	100% (n=3)	100% (n=3)	100% (n=2)

In rural sites, no females were observed which survived more than five years old.

### ***Birth date***

Birth dates were earlier in urban females than in rural females (Table 2), indicating that urban females had early breeding periods by about one month (Fig. 1a). There was no significant effect of the year (Table 2). The large difference between the marginal and conditional  $R^2$  values (Table 2) indicated that random effects (individual ID and study site) strongly affected the model fit.

The tendency for multiple breeding was affected by early birth dates (Fig. 1b, Table 3). Age did not have a significant effect (Table 3). The small difference between the marginal and conditional  $R^2$  values (Table 3) indicates that the random effect (individual ID) was small.



**Fig. 1.**—Density plots for the birth date. a) birth date for rural (circles) and urban (triangles) females. b) birth date of the first litter from females attended multiple (triangle) and single (circles) breeding in urban females. Days means passed days from March 1<sup>st</sup> (e.g., 25 means March 25). The number of dots means the sample size in each density plot.

**Table 2.**—LMM results for the effects of area (urban and rural) and year (2020, 2021, and 2022) on the birth date, and marginal and conditional  $R^2$ .

	Estimate	SE	$X^2$	$P$ value
Area*	-0.40	0.19	5.36 ( $df=1$ )	<b>0.02</b>
Year (2021)**	-0.01	0.07	1.75 ( $df=2$ )	0.42
Year (2022)**	0.07	0.07		
	Marginal $R^2$	0.18	Conditional $R^2$	0.81

Significant value was shown in the bold letters.

\*Rural=0; urban=1. \*\*2020=0; 2021=1; 2022=1.

**Table 3.**—GLMM results for the effects of birth date (the number of passed days from March 1st) and female age on the attendance of multiple breeding or not (1 or 0), and marginal and conditional  $R^2$ .

	Estimate	SE	$X^2$	$P$ value
Birth date	-0.16	0.02	11.08 ( $df=1$ )	<b>&gt;0.001</b>
Age	0.61	0.52	1.43 ( $df=1$ )	0.24
	Marginal $R^2$	0.77	Conditional $R^2$	0.79

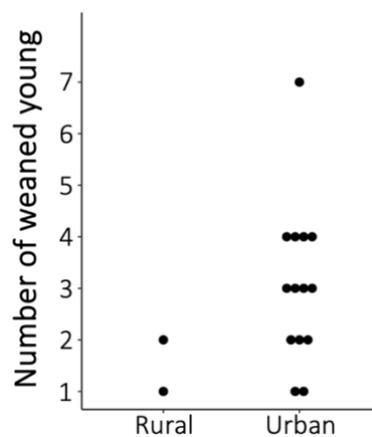
Significant value was shown in the bold letters.

### ***Multiple breeding***

Out of 40 females (n=11 and n=29 in rural and urban areas, respectively) observed in 2020, we observed 12 urban females (41.4%) had two litters. Of these, one female had two litters because of the failure to raise her young from the first litter (because we could not find any sign of lactation even 10 days after parturition). None of the females in the rural areas had second litters.

### ***The number of weaned young***

Urban females weaned an average of 3.0 young (1–7 young from 14 females) in a single litter (Fig. 2). Rural females weaned an average of 1.5 young (1–2 young from two females, Fig. 2). The difference in the mean number of weaned young between urban and rural squirrels (1.5) was not within the upper 2.5% range of the null distribution generated by randomization (values for 2.5% and 97.5% of the null distribution were 2.4 and -1.7, permutation test,  $p=0.11$ ).



**Fig. 2.** —The number of weaned young from rural (n=2) and urban females (n=14). The number of weaned young in a single litter were shown as each dot.

## Discussion

We investigated the reproductive conditions in terms of (1) rate of pregnancy, (2) age at initial pregnancy, (3) maximum age of reproduction, (4) birth date, (5) number of young weaned, and (6) whether they had two litters in rural and urban squirrels in Hokkaido, Japan. We found that the urban population had a higher pregnancy rate (Table 1) and an earlier breeding season than rural populations (Fig. 1a, Table 2). We also found that urban females were pregnant at one year of age (Table 1), which might have induced high rates of pregnancy in urban areas. In addition, urban females could breed multiple times, possibly because of the earlier onset of the breeding season (Fig. 1b, Table 3). Additionally, urban females could also reproduce at an older age than rural females. These findings suggest that urban females have better reproductive conditions than rural females. These better reproductive conditions in urban squirrels might be induced by an increase in body weight in spring (Takahata et al. 2023). In our previous study, combining behavioral observations of the food repertoire and stable isotope analysis, we concluded that this body weight increase in urban females was induced by better dietary conditions due to SF (Takahata et al. 2023). Therefore, urban squirrels may have gained benefitted from SF in terms of their reproduction via dietary changes and body weight increases.

Although the number of weaned young in the first litter did not differ between urban and rural females, urban females participating in multiple breeding raises the possibility that

the number of annual weaned young was larger for urban females than for rural ones. In addition, the long reproductive age of urban females suggests that the total weaned young age in their lifetime may be higher in them than in rural ones. As we could not count the number of young weaned from the second litters, future studies should employ prolonged survey periods to record and include this.

Although we focused on SF as the main factor affecting reproductive conditions of urban squirrels, other urban factors should also be considered. Early reproduction in urban females could be induced not only by the female's body condition but also by the high winter-spring temperatures in urban environments. Owing to impervious surfaces (i.e., asphalt) and human activity, urban environments often have higher temperatures and lower seasonal fluctuations than rural environments (so called, heat island effect, Chapman et al. 2017). Some studies have reported that urban environments accelerate the breeding season of wildlife (Selonen et al. 2016). In addition, urban environments provide alternative nesting sites, such as gaps between roofs and buildings, where the temperature is higher and more stable than in natural nests. In addition, they serve as refuges for wildlife. For instance, nests in gaps within buildings may be more durable than nests that are usually constructed using tree branches. Therefore, urban environments provide squirrels with suitable nesting sites for reproduction.

Predation pressure is defined as the environmental differences between urban and rural areas. In the case of squirrels, urban environments have alternative predators (or meso

predators), such as crows and domestic cats, in addition to natural predators, such as hawks and red foxes (Lee 2001; Fey et al. 2016; Blackett et al. 2018). Therefore, predation pressure on squirrels may be stronger in urban populations than in rural populations. However, the number of weaned young in urban areas (on average, 3 young) did not differ from that in rural areas (on average 1.5 young), and natural environments in other countries; an average of 3.2 young from N=81 in the UK (Tittensor 1977); an average of  $3.0 \pm 0.9$  from N=9 in Western Europe (Wauters and Lens 1995); an average of 3.5 from N=4 in Northern Italy (Mari et al. 2008). Thus, the effect of urban predators on the number of young, weaned individuals may not be critical. Nevertheless, it may be premature to conclude that differences in predation pressure, if any, had little influence on reproduction. Predation pressure may indirectly affect the reproductive success of wildlife (Creel et al. 2007, 2009). For example, predation pressure elevates glucocorticoid stress hormones in prey species, affecting their reproduction through effects on the immune and digestive systems (Sheriff et al. 2009). Another possibility is that predation pressure constrains foraging activity and efficiency, affecting reproduction owing to nutritional and energetic limitations (Creel et al. 2009). In addition, predator distribution might create a landscape of fear, inducing interspecific competition for limited resources, such as food and nests. In future studies, it would be fruitful to consider environmental differences (i.e., predation pressure, food availability, and squirrel density) in their reproductive outputs.

In summary, we found that urban females had better reproductive conditions than rural females, which may have been due to the use of SF. Although the effects of SF on wildlife are usually discussed inclusively, from birds to mammals, the critical differences between these taxa should be considered. In mammals, if mothers can access food and consume energy adequately, they can produce milk adequately for the young. Therefore, SF supports mammalian population growth and conservation. However, in avian species, especially insectivorous birds (e.g., great tits *Parus major* and blue tits), food from SF might not be an appropriate diet for chicks. Therefore, even if adults have access to food, such as tree seeds and grains from SF, chicks may still not get enough food (insects) in the presence of a scarcity of natural insects in urban environments (Seress et al. 2020). Thus, in some passerine species, SF may have negative effects on chick growth, even though they lay a large number of eggs and have high rates of hatching (Plummer et al. 2018, 2019). Therefore, if we are to conserve urban wildlife, feeding wildlife is not the only solution, and we need to consider the ecology of the target species.

**Supplementary fig. 1.**—Reproductive status and conditions. a) Definition of non-breeding and breeding females. b) Definition of lactation. Squirrels in photos were not same individual.

**a)**

<p><b>Non-breeding</b></p>  <p><b>Anoestrus</b></p> <ul style="list-style-type: none"> <li>• The vulva is small with a non-longitudinal opening</li> <li>• Small or invisible nipples</li> <li>• Non-enlarged belly during breeding season</li> </ul>	<p><b>Breeding</b></p>  <p><b>Oestrus and post-oestrus</b></p> <ul style="list-style-type: none"> <li>• The vulva is partly/ strongly swollen with a longitudinal opening</li> <li>• Visible nipples</li> <li>• Enlarged belly</li> </ul>
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(Wauters & Gurnell 1999)

**b)**

<p><b>Before lactating</b></p>  <ul style="list-style-type: none"> <li>• Small or invisible nipples</li> </ul>	<p><b>Lactating</b></p>  <ul style="list-style-type: none"> <li>• Large nipples</li> <li>• Around nipples are bald</li> <li>• Milk excretion</li> </ul>	<p><b>Weaned</b></p>  <ul style="list-style-type: none"> <li>• Large nipples</li> <li>• Darken and shrink</li> </ul>
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(Lee 2001)

**Supplementary fig. 2.**—Reproductive status and conditions of the same individual (R210, lived in MID site, urban area) with photographs.



ID: R210; observed 2 juveniles on 14 July; road killed on 16 July

## Chapter 4. Population genetic structure

### 4.1 Introduction

Over the past few decades, urban areas have dramatically expanded around the world, providing alternative habitats for wildlife to natural ones (Lowry, Lill, & Wong, 2013; Johnson, Lewis, & Breck, 2020). Some wildlife species have successfully flourished in urban areas by altering their ecological traits (Santini *et al.*, 2019; Shutt & Lees, 2021). However, even species living in urban areas experience maladaptive effects such as increased inbreeding and decreased genetic diversity (Schmid & Garroway, 2020). These negative impacts mainly result from impervious surfaces (e.g., urban infrastructure, roads, and buildings) in urban environments, which prevent dispersal and induce habitat fragmentation (Hulme-Beaman *et al.*, 2016; Munshi-South, Zolnik, & Harris, 2016), consequently inducing genetic differentiation between populations. Genetic differentiation caused by urban factors has been termed as the “urban fragmentation model”, and has been tested and supported in urban evolutionary studies (Crispo *et al.*, 2011; Miles *et al.*, 2019). Although the urban fragmentation model was the most commonly reported pattern of urban evolutionary consequence, an inverse pattern referred to as the “urban facilitation model” has also been reported (Björklund, Ruiz, & Senar, 2010; Miles *et al.*, 2018). For example, the urban populations of the Western black widow spider *Latrodectus hesperus* and the great tit *Parus major* have higher genetic diversity than forest

populations (Bjorklund *et al.*, 2010; Miles *et al.*, 2018). Therefore, the factors and conditions that affect genetic structure in urban environments are still unclear.

According to the review by Fusco, Carlen, and Munshi-South (2021) several factors must be considered when developing the field of population genetic structure in urban environments, such as molecular methods and environmental variations. Previous studies have used microsatellite markers to identify genetic differentiation (Rézouki *et al.*, 2016; Selonen, Fey, & Hämäläinen, 2018). However, this has low detectability of genetic differentiation because of the use of a small number of loci (McCartney-Melstad, Vu, & Shaffer, 2018; Fusco *et al.*, 2021). Thus, a genome-wide approach is more appropriate than the use of microsatellite markers. In addition, because geographic patterns and landscapes would differ between countries, there are differences in the effects on population genetic structure (Johnson & Johnson, 2017; Fusco *et al.*, 2021). Most studies have been conducted in North America and Europe, with fewer studies from other regions (e.g., Asia, Africa, and South America) with differing urban developments, socioeconomics, and human cultures. In addition, studies investigating the life history patterns and behavior of urban wildlife would help us understand the big picture of their population genetic structure in urban areas and the mechanism of urban effects. However, the urban ecology of few species has been understood from multiple perspectives.

Eurasian red squirrels *Sciurus vulgaris* are the appropriate animals to identify the urban factors that affect their population structures because their ecological modification in urban environments has been well understood in the last decade pertaining broad perspectives such as diet (Krauze-Gryz, Gryz, & Brach., 2021; Takahata *et al.*, 2023), cause of mortality (Blackett *et al.*, 2018), habituation to humans (Uchida *et al.*, 2019), dispersal (Fey, Hämäläinen, & Selonen, 2016; Hämäläinen, Fey, & Selonen, 2019), as well as population genetic structure (Rézouki *et al.*, 2016; Selonen *et al.*, 2018). Considering the natural history of urban squirrels described below, they might be vulnerable to urbanization, and genetic differentiation between urban populations could easily occur. Firstly, the presence of roads would prevent squirrel dispersal because the highest cause of mortality of urban squirrels occurs due to vehicle collisions (Blackett *et al.*, 2018, Fingland *et al.*, 2022). Secondly, landscapes and anthropogenic resources in urban environments would affect squirrel dispersal. For example, the dispersal distances and routes of squirrels are affected by the presence of open spaces (without tree canopy cover, i.e., a high risk of predation), such as agricultural fields in urban areas (Hämäläinen *et al.*, 2019). In addition, they are usually fed by citizens in the parks and private gardens (Krauze-Gryz *et al.*, 2021; Uchida *et al.*, 2021; Takahata *et al.*, 2023), and this supplemental feeding might affect their decision to disperse and the dispersal distances (Selonen *et al.*, 2018). Therefore, urban environments contain numerous factors that prevent

squirrel dispersal, induce habitat fragmentation, and increase genetic differentiation between populations.

Although habitat fragmentation and genetic differentiation are feasible in urban squirrels, previous studies investigating their population structure have shown little evidence of maladaptive effects. Selonen *et al.*, (2018) concluded that although genetic differentiation increased within urban populations in Finland, there was no indication that urban populations were isolated from rural populations. In addition, Rézouki *et al.*, (2016) concluded that urban populations in France were not affected by urbanization because the squirrel population had high genetic diversity and low inbreeding coefficients. However, these studies were conducted at urban sites where conditions that facilitated squirrel movements and dispersals were maintained. The Finnish study was conducted in a small city (Turku, approximately  $3 \times 4 \text{ km}^2$  area) surrounded by forests and numerous urban green spaces. Similarly, the French study was conducted in an urban green space surrounded by residential areas and adjacent to a large forest area. Although these study sites are located in urban areas, squirrels were studied at sites where they could easily disperse to other habitats and maintain gene flow. Therefore, the population genetic structure of urban squirrels could differ in environments with different degrees of urbanization and occupancy of green spaces. Alternatively, it is possible that the previous studies using microsatellite markers could not detect the genetic differentiation (Rézouki *et al.*, 2016; Selonen *et al.*, 2018).

The Obihiro area in Hokkaido, Japan is one of the regions where squirrels live in urban environments (Uchida *et al.*, 2019; Shimamoto *et al.*, 2020; Takahata *et al.*, 2023). In Obihiro City, large residential lots (approximately  $13 \times 7$  km<sup>2</sup> area) were constructed, and squirrel habitats such as urban parks, groves, and roadside trees were scattered throughout the urban areas. These habitats were at least 1 km apart from the others, and there were few forests that connected each squirrel habitat (Fig. 1). A previous study showed that squirrel dispersal was not affected by roads in Finland (Fey *et al.*, 2016). However, squirrels in Obihiro City, which is constructed of large residential lots, are frequently killed by vehicle collisions (Yanagawa, 2005). Therefore, urban squirrels in Obihiro City can be expected to be genetically different from those in adjacent populations in rural and natural areas. Genetic differentiation also occurs in urban populations. That is, squirrels in Obihiro City would have different evolutionary consequences for their urban lives compared to previous study findings (Rézouki *et al.*, 2016; Selonen *et al.*, 2018). However, the genetic structure of squirrels in the Obihiro region remains unknown.

In this study, we used genome-wide single nucleotide polymorphisms (SNPs) to investigate the population structure and genetic diversity of Eurasian red squirrels in the Obihiro region of Hokkaido, Japan. We obtained SNPs data by MIG-seq (Suyama & Matsuki, 2015) and focused on three aspects; (1) population structure: we used principal component analysis (PCA) and ADMIXTURE to investigate the population structure; (2) genetic differentiation:

we used pairwise  $F_{ST}$  values between populations (study sites) to find environmental factors affecting genetic differentiation; and (3) genetic diversity: we used nucleotide diversity ( $\pi$ ) evaluated from spatial-temporal standpoint as well as sex differences to investigate factors affecting their genetic diversity. In this study, we predicted that genetic differentiation would occur between urban and rural populations, as well as within urban populations, owing to decreased gene flow in urban populations. From a spatial standpoint of genetic diversity, we predicted that urban populations would have a lower nucleotide diversity than rural populations. From a temporal standpoint, we predicted that nucleotide diversity would decrease faster in urban populations than in rural populations. For sexual differences, we predicted that males would have a higher diversity than females because of the possibility of male-biased dispersal at our study sites (see below).

## **4.2 Materials and method**

### *Study sites*

Data sampling was conducted in the Obihiro area of Hokkaido, Japan (42°49' N, 143°02' E). Obihiro City is composed of large residential lots surrounded by agricultural land that was cultivated 140 years ago. Within the city, urban green spaces (such as urban parks, windbreak forests, and groves) are scattered and serve as habitats for squirrels (Fig. 1). Squirrels have

settled in urban green spaces as forests have matured (Ezorisu no kai, 1989), and a stable population has been observed over the past 30 years.

We selected 12 squirrel habitats located in the urban and rural areas of the Obihiro area as study sites. We defined squirrels that inhabit those study sites as “urban”, “border”, and “rural” populations in order to calculate land use percentages (Table 1; Fig. 1) using QGIS (QGIS Development Team, 2023) in this study. Land use data (100 × 100 m mesh map with 12 land use categories, such as residential lots, roads, agricultural land, forests, and rivers) were downloaded from the Ministry of Land, Infrastructure, Transport, and Tourism (MLIT 2016). In QGIS, we created a point at the center of each study site and a 1 km radius area of the center point. We then overlapped the land use data to 1 km radius areas and calculated the number of meshes belonging to each land use category in the number of meshes within the radius areas. We excluded the number of meshes belonging to the study sites because we calculated land use percentages to define the environment surrounding the study sites.

Six forests located at least 10 km far from central Obihiro city and surrounded by agricultural lands with few roads were selected as “rural” sites (mean and SE for area of study sites was  $2.75 \pm 0.65$  ha; Table 1; Fig.1). Although rural sites included human-mediated sites, such as shrines or horse-riding parks, all sites were rarely visited by humans. Because agricultural lands were mainly occupied in rural sites (Table 1), many windbreak forests

comprising Korean pine, *Pinus koraiensis* or Japanese larch, *Larix kaempferi* were distributed such that they connected those sites.

Two urban parks located within residential areas were selected as “urban” sites (mean and SE for area of study sites was  $25.83 \pm 17.42$  ha; Table 1; Fig.1). The urban sites were mainly surrounded by residential lots and roads (more than 90%), and there were few groves and forests (Table 1; Fig. 1). Thus, many squirrels were killed by vehicle collisions during dispersal or movement (Yanagawa 2005). Urban sites were frequently visited by humans (mean and SE for the number of human visitors in 30 min:  $9.83 \pm 0.09$ , see below). In addition, supplemental feeding of wildlife is a common activity in urban sites (Uchida *et al.*, 2021; Takahata *et al.*, 2023). Food availability for squirrels (particularly tree seeds) is more stable in urban than in rural study sites because of the presence of supplemental feeding (Takahata *et al.*, 2023).

Three urban parks and the Obihiro University campus, located in residential areas, were selected as “border” sites in this study (mean and SE for area of study sites was  $14.62 \pm 10.23$  ha; Table 1; Fig. 1). Border sites were similar to urban sites in terms of location, high land use proportion of roads and residential lots (Table 1), frequent visitation of humans (mean and SE for the number of human visitors in 30 min:  $4.02 \pm 0.05$ ), and presence of supplemental food. However, border sites were connected to neighboring sites or habitats by groves and windbreak forests, similar to rural sites. In addition, the proportion of agricultural land and

forest was higher than that in urban sites (Table 1). Therefore, although the border sites were located in residential areas, they exhibit the characteristics of both urban and rural sites.

**Table 1.**—Summary of study sites and its category, the number of sampled squirrels (N), rates of squirrels using each land category calculated by QGIS, and nucleotide diversity. Urban: residential lots and roads occupy most of the area, with few forests in the neighborhood. Border: residential lots and roads occupy most of the area, with many forests and a few agricultural lands in the neighborhood. Rural: there are few residential lots and no roads, and is surrounded mostly by agricultural lands.

Study sites	Urban		Border				Rural					
	MID	ODO	NIS	AZU	MAN	CHI	TAI	FUJ	EKI	KIY	HEI	UMA
N	115	25	32	3	28	13	4	7	4	10	13	10
Land use (%)*												
Residents and buildings	93.08	87.43	79.66	64.02	39.11	34.81	19.03	2.95	2.66	2.39	1.14	1.06
Roads	5.19	10.43	8.76	5.10	2.79	0.55	0.00	0.00	0.00	0.00	0.00	0.00
Agricultural lands	0.58	1.60	3.39	1.98	38.83	37.29	70.51	90.08	95.74	85.94	95.71	85.68
Forests	1.15	0.53	8.19	28.90	19.27	27.35	10.46	6.97	1.60	11.67	3.14	13.26
Nucleotide diversity ( $\pi$ )	0.17	0.17	0.18	NA	0.16	0.17	NA	0.19	NA	0.18	0.19	0.17



**Fig. 1.**—Map of Hokkaido and the Obihiro area with 12 study sites. Urban, border, and rural study sites are plotted as each shape and color (urban: blue circle, border: yellow square, rural: green triangle). Residential lots are shown as white, agriculture lands are shown as yellow, brown, and shallow green. Forests including windbreak forests are shown as deep green.

### ***Data collection***

Trapping of squirrels was conducted between 2015 and 2021. The protocol for capturing squirrels was the same as that used in our previous study (Takahata *et al.*, 2023). Squirrels were captured using box traps (Model RB-2, Sakae Industry Co., Ltd., Niigata, Japan) from 4:00 am to 11:00 am in May–June and October–November when squirrels were most active. Box traps were set on the ground and checked every 1–1.5 hours in rural sites, whereas they were always monitored in urban and border sites to avoid human conflict. When the squirrels were captured, we recorded their sex and maturity (juvenile, subadult, or adult) and attached individual identifiers (ear tags and collar rings). For the DNA experiment, 2 mm round tissue was taken from their ear by using the ear punch (Model KN-292, Natsume Seisakusho Co., Ltd., Tokyo, Japan) and stored in 99% ethanol in a deep freezer (−80 °C). All protocols were completed within 15 min to minimize stress, and the squirrels were released at the capture location. Squirrels that had previously undergone tissue sampling were immediately released. The capture and handling of squirrels was approved by the Institutional Animal Care and Use Committee of the Graduate University for Advanced Studies, SOKENDAI (license number: SKD2020AR002), and the Institutional Animal Care and Use Committee of the National University Corporation Hokkaido University (license number:15–0121).

### ***DNA extraction and MIG-seq***

Genomic DNA was extracted from tissue samples (half of 2 mm round tissue) using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

MIG-seq (Suyama & Matsuki, 2015) was used to obtain sequence data. This method amplifies regions flanked by microsatellite loci via a two-step PCR. In the first step of PCR, Inter-Simple Sequence Repeat (ISSR) regions were amplified using eight pairs of ISSR primers (minor modification of primer sequences from Suyama & Matsuki (2015); Table S1). The first PCR was conducted following Suyama & Matsuki (2015): initial activation at 94 °C for 1 minute, 25 cycles for denaturation at 94 °C for 30 seconds, annealing at 48 °C for 1 minute and extension at 72 °C for 1 minute, followed by a final incubation at 72 °C for 5 minutes. In the second PCR, we used the NEBNext Multiplex Oligos for Illumina (New England Biolabs Inc., Massachusetts, USA) for primer pairs: initial activation at 94 °C for 1 minute, 25 cycles for denaturation at 94 °C for 30 seconds, annealing at 50 °C for 30 seconds, and extension at 72 °C for 75 seconds, followed by a final incubation at 72 °C for 3 minutes. Two-step PCR was conducted using the TaKaRa PCR Thermal Cycler Dice Touch (Model TP350; Takara Bio Inc., Shiga, Japan). After the second PCR, equal amounts of PCR products from each individual were mixed and purified using AMPure XP (Beckman Coulter). Paired-end (2 ×150 bp) sequencing (59.9M bp–259.5M bp) was performed on the Illumina HiSeq X platform.

### *SNPs extraction*

Sequence reads from the genomic DNA libraries of 286 squirrels (rural populations, n=52; border populations, n=90; urban populations, n=144) were trimmed to remove adaptor sequences and mapped to the reference genome (mSciVull1.2) using the CLC Genomics Workbench ver. 20.0.4 (<https://www.qiagenbioinformatics.com/>). Reads showing high similarity (> 80% in > 80% of reads length) were mapped to the reference genome sequence and reads mapped to more than one position were removed (“ignore” option for reads mapped to multiple positions). The mapping data were exported in bam file format, sorted, and indexed using SAMtools (Li *et al.*, 2009). The duplicated reads in the bam files were marked using the MarkDuplicates algorithm implemented in GATK v4.2 (<https://gatk.broadinstitute.org/hc/en-us>). Genotype calling was performed for all individuals analyzed in this study using the HaplotypeCaller algorithm in GATK v4.2. The genotypes of all individuals were output in the gvcf format (-ERC GVCF option). All gvcf files were combined into a single gvcf format file using the CombineGVCF algorithm in GATK v4.2. The combined file was genotyped by the GenotypeGVCFs algorithm and filtered by FilterVcf in GATK v4.2 with parameters; --filter-expression “QD < 2.0” --filter-name “QD2” --filter-expression “QUAL < 30.0” --filter-name “QUAL30” --filter-expression “FS > 200.0” --filter-name “FS200” --filter-expression “SOR > 10.0” --filter-name “SOR10” --filter-expression “ReadPosRankSum < -20.0” --filter-name “ReadPosRankSum-20”. The genotyped vcf file was filtered using vcftools with the following

options: mac 3 --maxDP 1e+03 --minDP 3 --minGQ 20 --max-missing 0.8 (Danecek *et al.*, 2011).

### ***Genome-wide SNPs Analyses***

We performed a principal component analysis (PCA) using PLINK ver. 1.9 (Purcell *et al.*, 2007) with an option “--indep-pairwise 50 10 0.1” to examine the genetic relationship between urban (including border) and rural squirrels (Fig. 1). In addition, the ADMIXTURE ver. 1.3 (Alexander & Lange, 2011) was run on the same PCA dataset, assuming two to five clusters (K=2-5).

We calculated pairwise  $F_{ST}$  values using vcfTools v0.1.16 (Danecek *et al.*, 2011) to investigate genetic distances between population pairs (in total, 66 pairs, Table S2). To investigate the correlation between  $F_{ST}$  values and geographical distance, we used Mantel’s test. Geographic distances (km) between populations were measured as the shortest distance from one edge of the study site to another, using QGIS tools. We constructed a general linear model (GLM) to investigate the sites where genetic differentiation likely occurred. The pairwise  $F_{ST}$  value between populations was the dependent variable, and the geographic distances between populations and site category of pairs (urban-urban, urban-rural, rural-rural; n=15, 36, and 15, respectively) were explanatory variables. Additionally, genetic distances standardized by geographic distance (pairwise  $F_{ST}$  /geographic distance) were calculated for each site category.

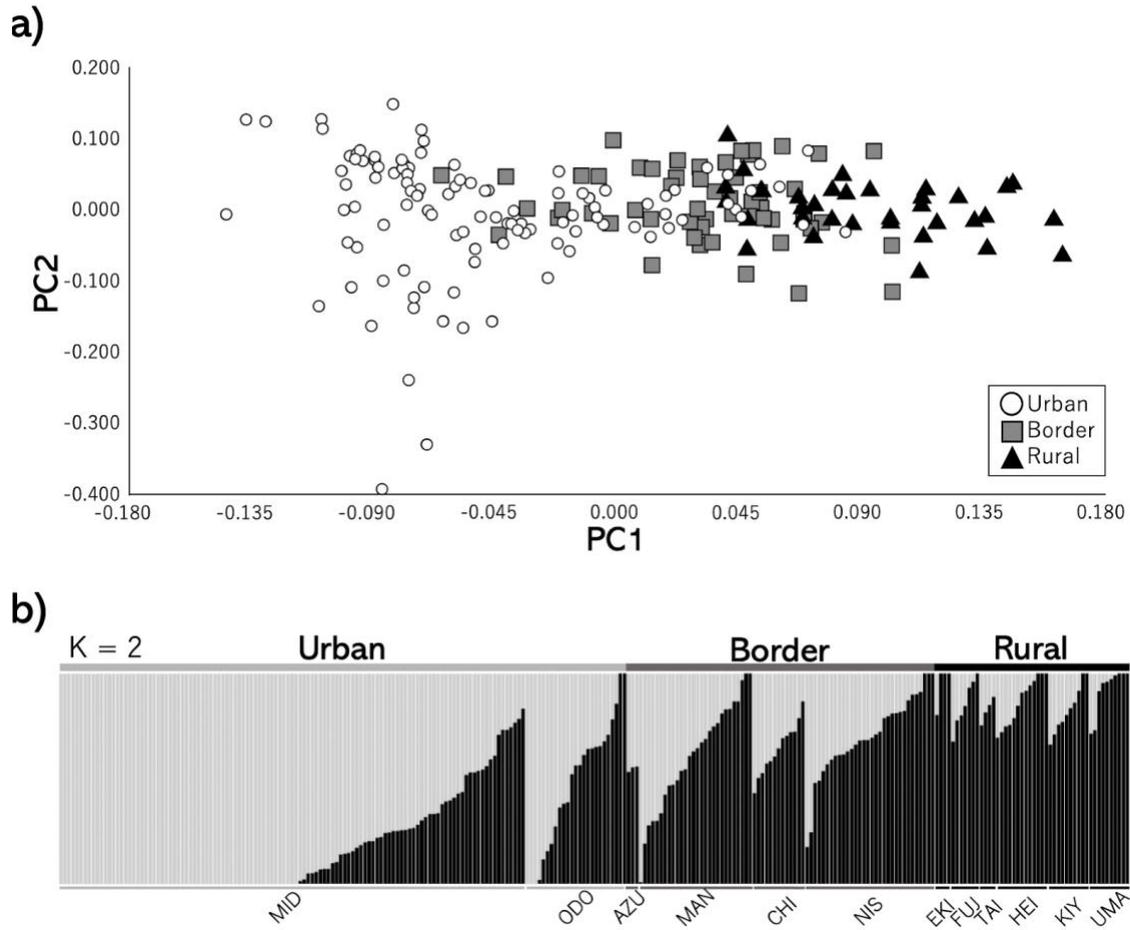
Although we wanted to investigate the effects of residential lots on genetic distance, we had only two urban populations (MID and ODO populations). Thus, border populations were included as urban populations in the model because border sites were located in residential areas similar to urban sites.

Nucleotide diversity was calculated for each population, as well as for combinations of populations between years and sex, using *vcftools* v0.1.16. To ensure data accuracy, populations or combinations with fewer than five samples were excluded. Because the sample size of all combinations of rural study populations and years was less than five, we combined them into a single rural area. To identify factors that affect nucleotide diversity (sites category, years, and sex), we conducted two separate GLMs for the sex (the sex model) and the year (the year model) using the “*car*” package in R (R Development Core Team, 2022). For the sex model, nucleotide diversity was the dependent variable, and sex (female or male) and site category (urban, border, or rural) were the grouping variables. For the year model, nucleotide diversity was the dependent variable, year (2015–2021) was the covariate, and the site category (urban, border, or rural) was the grouping variable. If we found significance in the site category, we performed a post-hoc test using Tukey’s multiple comparison test.

### **4.3 Results**

#### ***Population genetic structure of squirrels in the Obihiro area***

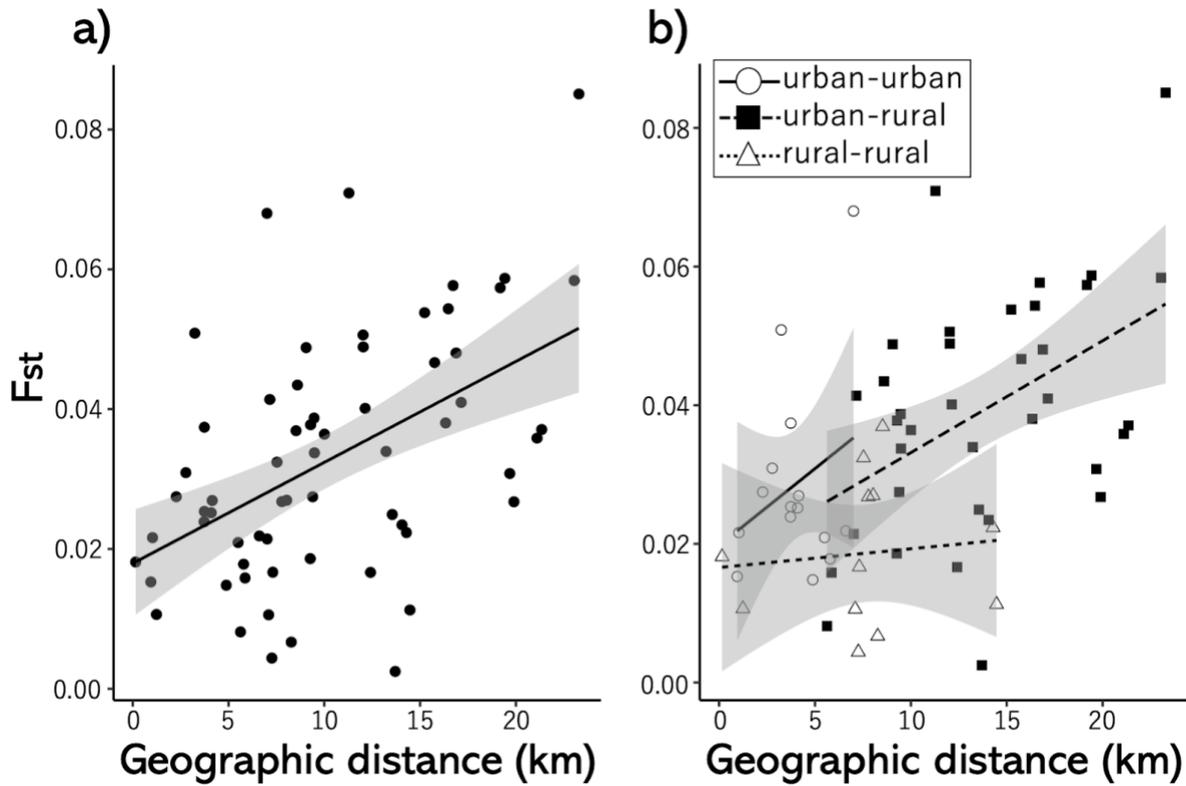
Initially, we investigated the population genetic structure of squirrels in the Obihiro region using PCA. As shown in the PCA results, urban and rural squirrels formed non-overlapping clusters (Fig. 2a). In addition, border squirrels were located between the clusters of urban and rural squirrels along the PC1 axis (Fig. 2a). These results suggest that urban and rural squirrel populations were genetically differentiated, with a limited extent of mixing through border populations. ADMIXTURE analysis divided all individuals into two genetic compositions: one predominant in the urban populations and the other predominant in the rural populations, with the lowest CV error of  $K=2$  (Fig. 2b). Although urban and rural populations had two genetic compositions with different ratios, they were genetically differentiated. The border populations were a mixture of the two compositions (Fig. 2b), indicating a mix of urban and rural populations (i.e., gene flow) through the border populations.



**Fig. 2.**—Population genetic structure of Eurasian red squirrels in Obihiro city. a) Principal components analysis (PC1 vs. PC2) of 199 samples based on 655 SNPs. Urban samples are shown as circles, border samples are shown as squares, and rural samples are shown as triangles. b) ADMIXTURE results based on SNPs data for K=2.

### ***Genetic distance between populations***

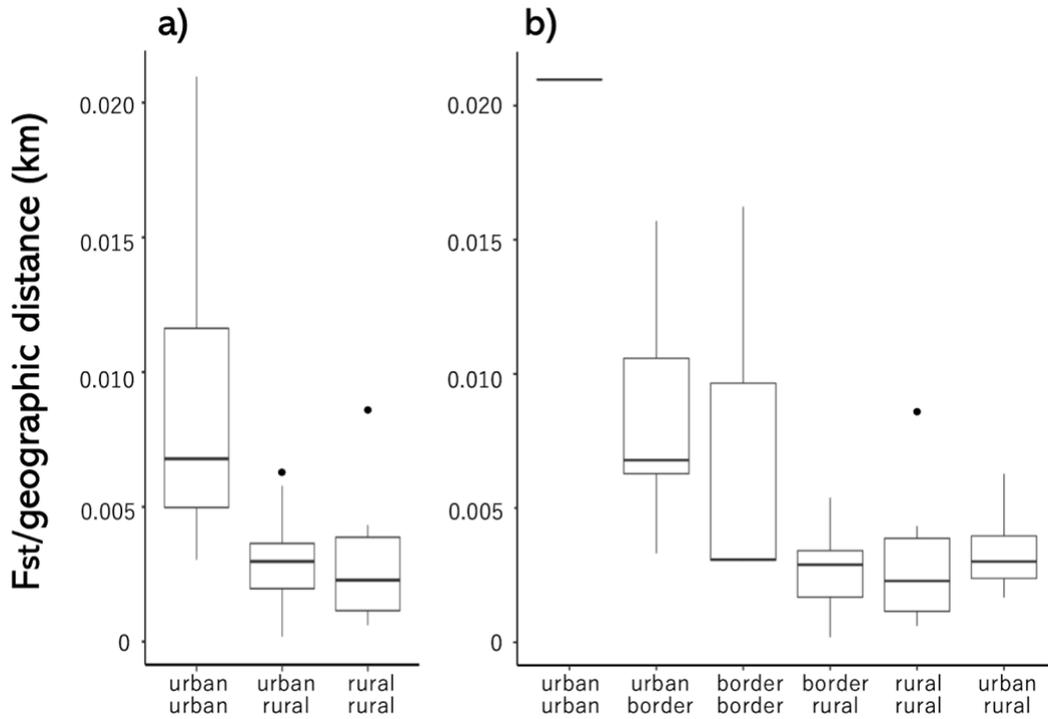
Next, we analyzed the genetic distance between populations in the Obihiro area. We used pairwise  $F_{ST}$  values between populations as genetic distances. We found that genetic distance values were significantly correlated with geographic distance (Mantel  $r = 0.58$ ,  $P < 0.001$ ; Fig. 3a), indicating that squirrels in the Obihiro area showed a pattern of isolation based on distance. The GLM showed that pairwise  $F_{ST}$  values were affected by the site category of pairs (Fig. 3b, Table 2), indicating that the urban-urban and urban-rural categories had larger genetic distances than the rural-rural category (Fig. 3b, Table 2). The standardized genetic distances of urban-urban pairs were significantly higher than those of rural-rural pairs (Fig. 4a, Table 2). For a closer comparison of the genetic distances, we classified the 12 areas into three categories: urban, border, and rural. Although we did not perform a statistical analysis because of the small sample size in the urban-urban category (only one pair), pairwise urban populations (urban-urban and urban-border sites) had higher standardized genetic distances than the other pairs (Fig. 4b). In addition, pairwise border populations (border-border sites) had higher standardized genetic distances than pairwise rural populations (border-rural–rural sites). These results suggest limited gene flow between urban populations and other populations and between border populations.



**Fig. 3.**—Relation between geographic distances and  $F_{ST}$  values. a) Relation between geographic distances and  $F_{ST}$  values between all site category pairs (66 pairs). b) Relation between geographic distances and  $F_{ST}$  values between each site category pairs (urban-urban: circles with solid line, urban-rural: squares with dotted line, rural-rural: triangles with dashed lines). 95% credible intervals are shown in grey in each plot.

**Table 2.**—Summary for the effects of geographic distance and site categories (urban-rural, rural-rural, and urban-urban) on genetic distance ( $F_{ST}$  values) between site category pairs. Significant values are shown in bold letters.

	Estimate	Std. Error	df	t	<i>P</i>
Distance	0.001	0.000	59	3.236	<b>0.002</b>
Site category*					
Urban-rural	-0.003	0.006	3	-0.505	0.615
Rural-rural	-0.015	0.006	3	-2.597	<b>0.012</b>

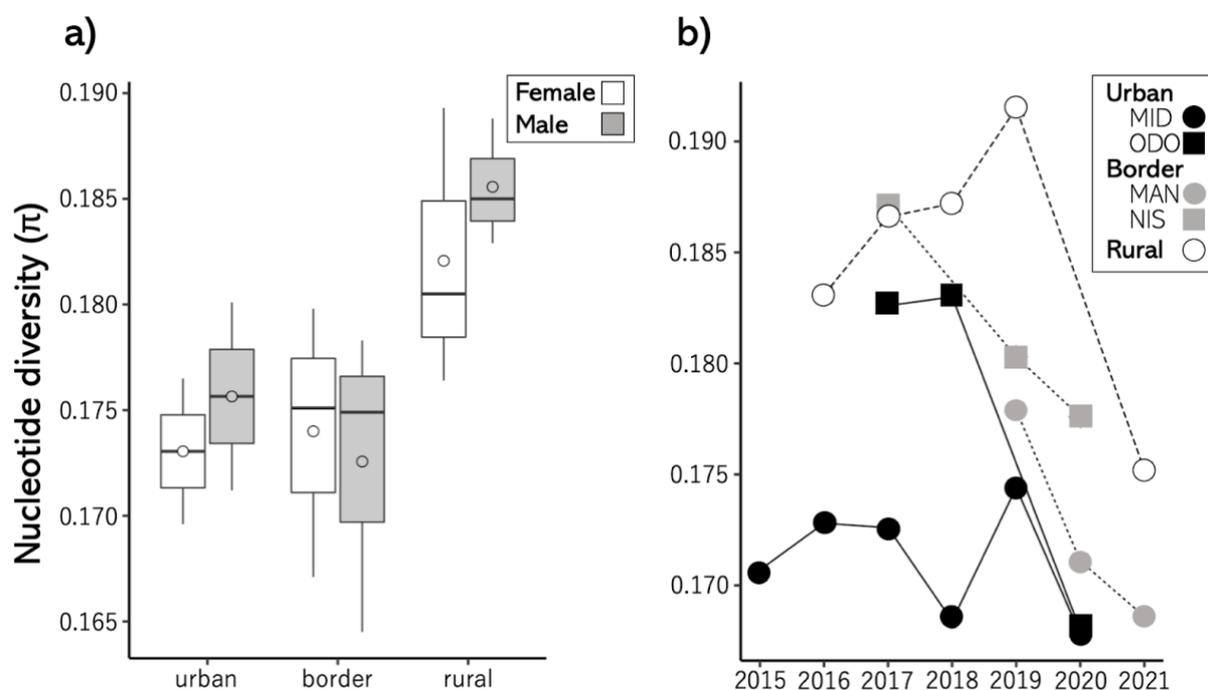


**Fig. 4.**—Standardized genetic distances ( $F_{ST}$ /geographic distance) between each site category. a) Box plots of standardized genetic distances between each site category pair (urban-urban, urban-rural, and rural-rural). b) Box plots of standardized genetic distances between each site category pair including border areas. Horizontal lines represent medians and interquartile ranges (IQR). The upper and lower whiskers extend from the hinge to the largest and smallest value no further than  $1.5 \times \text{IQR}$  from the hinge.

### *Nucleotide diversity*

The sex model showed that nucleotide diversity was affected by the site category (GLM,  $F_{2,12} = 6.19$ ,  $P = 0.01$ , multiple  $R^2 = 0.51$ ). From Tukey's multiple comparisons of means, we found that nucleotide diversity was higher in rural squirrels than in urban ( $P = 0.04$ ) and border squirrels ( $P = 0.01$ ). Diversity did not differ between the urban and border areas ( $P = 0.95$ ). In addition, we did not find any significant differences in sex ( $F_{1,12} = 0.26$ ,  $P = 0.61$ ; Fig. 5a).

The year model showed that nucleotide diversity was affected by the site category (GLM,  $F_{2,16} = 7.66$ ,  $P = 0.01$ , multiple  $R^2 = 0.53$ ). Using Tukey's multiple comparisons of means, we found that nucleotide diversity was higher in rural areas than in urban areas ( $P = 0.01$ ). There were no significant differences between rural and border areas ( $P = 0.13$ ) or between urban and border areas ( $P = 0.50$ ). In addition, nucleotide diversity was affected by year ( $F_{1,16} = 4.68$ ,  $P = 0.05$ ; Fig. 5b), indicating that diversity decreased with years.



**Fig. 5.**—Nucleotide diversity in each area, sex, and year. a) Box plots of nucleotide diversity in each area and sex. Females are shown as white, and males are shown as grey boxplots. Circles represent means, and horizontal lines represent medians and interquartile ranges (IQR). The upper and lower whisker extends from the hinge to the largest and smallest value no further than  $1.5 \times \text{IQR}$  from the hinge. B) Nucleotide diversity in each site category and year (2015–2021). Rural areas are shown as white shapes with the dashed line, border areas are shown as grey shapes with dotted lines, and urban areas are shown as black shapes with solid lines. Each shape with the same color indicates the same study sites.

#### 4.4 Discussion

We used a genome-wide approach to investigate the genetic structure and nucleotide diversity of Eurasian red squirrels distributed in rural to urban areas in the Obihiro area of Hokkaido, Japan. We found that squirrels in the Obihiro area were genetically differentiated between urban and rural populations (Fig. 2a, b) with gene flow via border populations. Genetic distances between urban populations were significantly greater than those between rural populations affected by isolation (Fig. 4a). This result indicates that urban factors limit the dispersal of

squirrels, and thus gene flow between urban populations and border and rural populations. In addition, genetic distance was remarkably higher in urban populations than in border populations (Fig. 4b), indicating that certain factors prevent squirrel dispersal and cause genetic differentiation in urban areas.

We also found that the nucleotide diversity in urban populations was lower than that in rural populations (Fig. 5a, b). This is possibly due to the decreased population size caused by limited inhabited areas. Another possible explanation for the lower genetic diversity in urban populations could be the different degrees of gene flow between urban and rural populations. Because Obihiro City is surrounded by forests (Fig. 1), the rural population has a gene flow with populations around Obihiro City, and the rural populations would have maintained genetic diversity. However, the genetic diversity of urban populations may have decreased because of restricted gene flow between the urban population and the populations around Obihiro City. Our results showed low genetic diversity and reduced gene flow in urban populations, indicating that urbanization has a negative impact on the genetic structure of squirrels. Which urbanization factors reduce gene flow in the Obihiro area? We consider the following three causes.

One possible reason for the prevention of squirrel dispersal is the presence of busy roads. In Obihiro City, many squirrels were killed in vehicle collisions (Yanagawa 2005), and our results showed that one of the populations (the AZU population) that was separated by the

arterial road had a greater genetic distance between neighboring populations (Table S2). Therefore, it is possible that roads act as barriers to squirrel dispersal, consequently increasing genetic differentiation between populations. This possibility is also supported by the evidence that the main cause of fatalities in urban squirrels is vehicle collisions (Blackett *et al.*, 2018, Fingland *et al.*, 2022). Fey *et al.*, (2016) showed that the dispersal behaviors of juvenile squirrels, such as crossing roads, were not affected by road traffic levels in Finland. They also found that traffic was not a major cause of juvenile squirrel mortality (11 dead juveniles were observed). However, it is possible that dispersal behavior and mortality differ between juvenile and adult squirrels.

Secondly, population connectivity, such as the presence of forests, is a factor that affects gene flow. Forests with connected populations were abundant between the rural and border populations (Table 1). In the Obihiro area, numerous Korean pines, Japanese larches, and Sakhalin spruce *Picea glehnii* have been planted to construct windbreak forests for agricultural purposes (Tsuji *et al.*, 2004, 2007). These windbreak forests aid squirrel dispersal from rural to urban areas by connecting habitats and providing food (tree seeds) for squirrels. Therefore, gene flow between populations in rural and border areas, and between populations in border areas, may be due to the dispersal of squirrels through connective forests. In contrast, the urban areas were mainly constructed with residential lots that prevented squirrel dispersal, and there were few forests or groves (Table 1). The dispersal of squirrels may be restricted

because of the lack of forests in urban areas. Although forests, including windbreak forests in the Obihiro area, might be a crucial factor for squirrel gene flow, windbreak forests have been cut in recent years for more efficient farming practices (Tsuji *et al.*, 2004, 2007). Further logging would result in even less gene flow between rural and urban populations, causing urban populations to become more isolated. To maintain individual movement and high genetic diversity, it is crucial to manage green spaces and plant trees in urbanized areas to conserve urban wildlife.

Thirdly, the abundance of supplemental food in urban areas may alter squirrels' decisions to disperse and the dispersal distance. Squirrels disperse when they encounter a high frequency of intraspecific competition for food and breeding resources (Wauters & Dhondt 1993). Therefore, abundant food from supplemental feeding possibly mitigates food scarcity and intraspecific competition and prevents their dispersal behavior. Additionally, if supplemental food is widely distributed in urban green spaces, squirrels do not need to disperse over long distances (Selonen *et al.*, 2016). Selonen *et al.*, (2016) showed that urban squirrels disperse over shorter distances than rural squirrels. As we surveyed only two urban and three border sites, we were unable to detect factors that affect genetic differentiation in urban populations. In future studies, it would be beneficial to collect samples from many urban habitats with variations in the presence of roads and forests, site connectivity, food availability, and distribution.

Overall, our results suggest that genetic differentiation between rural and urban squirrel populations occurred rapidly in Obihiro City, within 30 years of squirrels settling in urban environments. Considering the generation time of squirrels, this differentiation may occur within only 15–30 generations. Therefore, our results indicate that the genetic structure was strongly affected by urbanization (including human activities and man-made landscapes) over a relatively short timescale. Our results from the genome-wide approach suggest that although squirrels live in urban areas, the land use pattern in Obihiro City results in unsuitable habitat for urban squirrels.

We also investigated the effects of sex on the genetic diversity of urban squirrels. In Eurasian red squirrels, sexual bias in dispersal has not been reported (Wauters *et al.*, 2010; Hämäläinen *et al.*, 2019). However, they exhibit a sexual bias in their dispersal when they encounter frequent intrasexual competition for food resources and breeding (Wauters & Dhondt, 1993). In our study population, male-male competition was more frequently observed than female-female competition in both rural and urban areas (Takahata *et al.*, unpublished data). Therefore, we expected males to have higher nucleotide diversity than females in both urban and rural populations. However, we did not find any significant sex-related differences in nucleotide diversity (Fig. 5a). A possible reason for this is that the small sample size may have precluded the detection of sex differences. Alternatively, this may be due to the lack of sexual bias in the dispersal of squirrels in Obihiro, which could be caused by different routes between

rural and urban populations. In rural populations with low squirrel density, the frequency of intraspecific competition would be low, which did not facilitate sex differences in their dispersal or nucleotide diversity. In urban populations, urban factors such as the presence of roads and a lack of forests may strongly prevent squirrel dispersal, which may have resulted in similar nucleotide diversity between the sexes. To better understand the effect of sex, it would be useful to investigate the relationship between sexual biases in genetic diversity and behaviors, such as competition and dispersal, across many populations.

Our study findings suggest that urban squirrels are affected by urbanization over short timescales. In addition, based on the findings of previous studies on the urban ecology of Eurasian red squirrels, we gained insights into the factors that possibly affect their population genetic structure. In recent years, urban green spaces have been considered places to conserve wildlife and biodiversity. For instance, European countries conduct urban planning, such as arranging green infrastructure, including wildlife corridors and green roofs, that connect wildlife habitats and maintain their gene flow (Lundholm, 2015; Schell *et al.*, 2021). However, when we conduct urban planning as described above and evaluate the results from those efforts, knowledge about their urban ecology, such as dispersal behaviors, causes of mortality, and resource use, is necessary. To substantially conserve wildlife in urban environments, it is necessary to evaluate the effects on genetic structures with a deep understanding of the ecology

of urban wildlife, and to consider urban planning that could contribute to their urban lives from a comprehensive perspective.

## **Chapter 5. General discussion**

In my thesis, I investigated the urban ecology of Eurasian red squirrels in terms of diet composition, body condition, and female's reproduction by comparing between urban and rural populations. In chapter 2, I found that urban squirrels utilized a higher amount of tree seed and the diet seasonality was more stable in urban than in rural squirrels. Since abundant tree seeds were provided in addition to natural resources throughout the year in urban areas, diet modification of urban squirrels was possibly because of excessive seeds by SF. Additionally, in spring, urban females gained more body weight than those of the rural females. Considering the temporal overlap of capture and the peak of female's pregnant periods in both urban and rural areas (around April to June), an increase of body weight in females could possibly indicate the difference of body condition (store energy as fat) or reproductive success (litter size). Although the mechanisms of the weight difference between urban and rural females is unclear, both scenarios would suggest the reproductive advantage of urban females. Actually, in chapter 3, I found female's reproductive condition (in terms of parturition data and rate of pregnancy) were better in urban than rural squirrels. Finally, I investigated the population genetic structure including urban and rural populations in chapter 4. I found that urban and rural squirrel populations formed distinct clusters with intermediate urban and rural boundary populations. Genetic distances between the urban and other populations were greater than those between the

other pairs. In addition, nucleotide diversity was lower in urban populations than in other populations, suggesting limited gene flow between urban and other populations.

Overall, my thesis suggested that urban lives affected urban squirrels both in positive (for diet, body weight, and reproduction) and negative (population genetic structure) ways. At the individual levels, urban squirrels gained benefits from urban lives in terms of fitness components (i.e., heavy body weight and better reproductive condition). However, at the population levels, gene flow is limited in urban areas and possibly induced an increased inbreeding. If so, urban populations are becoming a vulnerable population. Thus, in the long run, urban environments might not be suitable habitats for squirrels. Most previous studies in urban ecology had investigated only specific aspects of life history or conducted short-term study. Therefore, studies investigating the impact of urbanization on their life history with multiple aspects are lacking. My thesis investigating multiple aspects and over the long-term period showed that urban lives were not generally appropriate for squirrels.

### **Factors that affected urban squirrel's ecology**

I initially expected that SF (supplemental feeding) would contribute for squirrels to live in urban environments rather than the other urban factors. Therefore, I investigated their life history patterns (body weight and reproduction) which would strongly be affected by the body condition. However, squirrel's urban ecology might be influenced by not only food resources

(abundance and distribution) but also other factors. For instance, chapter 2 showed that both females and males in urban areas gained adequate tree seed from supplemental feedings throughout a year. Although urban males possibly had heavier body weight than rural ones, there was no difference in body weights between urban and rural males (chapter 2). I supposed that this result was induced by the energy loss which might be caused by high squirrel density and frequent male-male competition at the feeding site. In addition, the results of female's reproductive condition suggested that urban females had earlier parturition data than rural ones (chapter 3). This would be caused by the better reproductive condition in urban females, while high temperature in urban environments might also affect their parturition date.

Other possible factors shaping the differences between urban and rural areas are squirrel's ecology (i.e., social and mating systems, and sexual differences, chapter 2 and 4), environmental characteristics of Obihiro city (i.e., occupancy and connectivity of forests, predation pressure, and availability of food, chapter 2, 3, 4), and anthropogenic factors (i.e., accessibility of supplemental feedings, occupancy of impervious surfaces, traffic levels, chapter 2, 3, 4). Various factors affect the ecological trait of wildlife, and those are difficult to detect without multifaceted study of their life history or deep understanding of their habits. Thus, to understand adaptation in urban areas, multidimensional study including their life history patterns with long-term study would be necessary. Moreover, it would be fruitful to investigate squirrel's urban ecology in many sites which have various environmental differences.

### **Difficulties of generalization in urban ecology**

I explained above that squirrels would be affected by numerous urban factors such as environmental and anthropogenic factors, and species-specific characteristics. It indicates the difficulty of generalization of the wildlife's urban adaptation. For example, as I mentioned in the general introduction (chapter 1), types and degrees of anthropogenic factors would differ between regions and countries due to the difference of human activity levels and socioeconomics levels. Also, environmental factors such as occupancy of forests and temperature differ, inducing differences in wildlife's distribution. In addition to those, species-specific characteristics (i.e., body size, cognitive ability, life history patterns) induce the difference in the consequence of urban adaptation. Therefore, even if researchers used the same species to examine urban effects, the outputs and consequences of urban adaptation would be different because of the interaction of those three factors.

Actually, in the population genetic studies, the French and Finish populations did not show detrimental effects, but my study showed that the Obihiro population had negative impacts. This was possibly induced by the methodological difference (microsatellite vs. SNPs), the environmental difference (i.e., connectivity of forests) and differences in anthropogenic factors (i.e., frequency and amount of food from SF, traffic levels). In addition, although I showed significant changes of their tree seed use owing to the SF in the Obihiro populations,

impacts from SF would be depending on each region and scales of the study site (e.g., prefecture and city). Therefore, it should be noted that my results reflected the specific consequence of urban adaptation in squirrels in Obihiro city, and we need to be cautious about applying our results in other regions and species. Although suggesting frameworks and generalization would be important, collecting evidence about urban ecology of wildlife in broad species and regions would be more important to understand urban adaptation of wildlife. Those local level' evidence would contribute to suggest an approach for conservation or management in specific species.

I discussed the importance of understanding urban adaptation by multidimensions. In addition to those, we should recognize the importance of understanding “human dimensions” which includes such as human attitude and consciousness for wildlife to conserve or manage wildlife in urban areas (Decker et al. 2001). As wildlife use urban environments as habitats, interface and conflict between human and urban wildlife would be induced simultaneously (e.g., property damage, disease transmission, and vehicle collisions; Schell et al. 2021). However, whether the circumstances are considered as “human-wildlife conflict” or not depends on the perception of wildlife by local citizens, which were constructed by background of local culture, religion, values for wildlife and nature, and economic value. Those factors from human dimensions would promote or inhibit wildlife conservation and management, and also make complexity and diversity of animal adaptation in urban areas. Therefore, urban ecology should

be collaborated with social science including such as psychology, sociology, and anthropology (i.e., human dimensions for wildlife management) in the future.

## **Conclusion**

Understanding of wildlife adaptation and conserving them in urban environments are necessary to maintain biodiversity in an urbanized world and to enhance human wellbeing. My Ph.D. thesis suggested the importance of conducting multifaceted studies for urban ecology from animal species, regions, ecological aspects, and levels in urban ecology. In addition, this thesis suggested that urban ecology should be developed as interdisciplinary studies in the future.

In the Anthropocene, the global environment and ecosystems are changing. Urban ecology could provide evidence how human activity affects nature, and suggest directions of wildlife conservation. Furthermore, urban ecology suggests opportunities of thinking about how humans will behave and become one of the organisms on the earth.

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