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Authors: Tamura, Noriko, Ito, Mutsumi, and Hayashi, Fumio

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Original paper

Different responses of endemic and alien tree squirrels to tree seed chemicals

Noriko Tamura[1,*], Mutsumi Ito[2,3] and Fumio Hayashi[2]

[1] *Tama Forest Science Garden, FFPRI, 1833-81 Todori, Hachioji, Tokyo 193-0843, Japan*
[2] *Department of Biology, Tokyo Metropolitan University, 1-1 Minamiosawa, Hachioji, Tokyo 192-0397, Japan*
[3] *Conservation Biology Laboratory, Nippon Veterinary and Life Science University, 1-7-1 Kyounan, Musashino, Tokyo 180-8602, Japan*

Abstract. *Quercus* acorns and coniferous cones are the main food sources for tree squirrels, and the chemical contents of seeds, such as tannins and terpenes, are predicted to affect the squirrels' food preference. The tolerance of squirrels to chemical contents may vary between species that have evolved in different environments. The consumption of artificial food containing tannic acids or terpenes were compared between the endemic *Sciurus lis* and two alien species, *S. vulgaris* and *Callosciurus erythraeus*. *Sciurus lis* ate foods containing up to 4% tannic acid, whereas *S. vulgaris* and *C. erythraeus* ate foods containing 8% tannic acid. *Sciurus lis* and *S. vulgaris* could eat food containing a high concentration of terpenes (up to 0.3% α-pinene and 2% limonene), whereas the food consumption by *C. erythraeus* decreased drastically with increasing terpene concentrations. The two alien squirrels are highly resistant to tannins and thus the acorns of most *Quercus* species may be available for them. *Sciurus lis* exhibits a lower tolerance to tannins, but can consume foods with high concentrations of terpenes, suggesting a strong adaptation to coniferous forests, but not to *Quercus* forests. More attention must be paid to conserving suitable habitats for *S. lis*, an endemic relict species.

Key words: *Callosciurus erythraeus*, food preference, *Sciurus lis*, tannins, terpenes.

Quercus acorns and coniferous cones are the main food sources for tree squirrels, and under the predatory pressure of these squirrels, physical and chemical defenses may have evolved in these seeds (Steele 2008). Squirrels also exhibit behavioral and physiological adaptations to plant defenses, and these interactions are an example of coevolution (e.g., Smith 1970; Stapanian and Smith 1978). The coevolutionary patterns can be understood by investigating how the physical and chemical defenses of tree seeds affect the squirrels' tolerance to and preference for the seeds available in their habitats.

For *Quercus* acorns, the relationships between seed characteristics and squirrel feeding behavior have been thoroughly studied. Tree squirrels are predators of *Quercus* acorns, but they are also seed dispersers like other rodent species (Steele and Smallwood 2002). Chemical concentration, nutritional value, seed size, and perishability are important decision-making factors for feeding or caching by squirrels (e.g., Smith and Stapanian 2002; Steele 2008). For example, the group of red oaks (*Erythrobalanus*), which remains dormant and contains a high level of tannins and lipids, tends to be stored more frequently than the group of white oaks (*Leucobalanus*), which exhibits precocious germination and includes a low level of tannins and lipids (e.g., Smith and Stapanian 2002; Steele 2008). It is also known that the tannin content varies greatly depending on the species of acorn, the individual acorn, and the parts in the individual acorn (Steele et al. 1993; Shimada and Saitoh 2006; Shimada et al. 2015). Experimental studies revealed that squirrels sensitively responded to differences in tannin content when selecting food (Smallwood and Peters 1986; Chung-MacCoubrey et al. 1997; Barthelmess 2001). Eastern grey squirrels (*Sciurus carolinensis*) eat foods with a tannin content of up to 8%, and the tannin tolerance increases with the amount of lipids in the food (Chung-MacCoubrey et al. 1997). It is also believed that their decision to use or not use tannin-containing food

**To whom correspondence should be addressed. E-mail: haya@ffpri.affrc.go.jp*

depends on the availability of other food in the habitat. Therefore, the tolerance of tree squirrels to tannins may differ between species that have adapted to different habitats, but such interspecific differences in tannin tolerance have not been investigated in detail.

As with acorns, coniferous cones have attracted much attention in seed–squirrel interactions. The cones are covered with hard woody scales, which means that the squirrels need time to eat the inner seeds (Molinari et al. 2006). To increase the feeding efficiency, squirrels discriminate between the cones based on the number of seeds per cone, the size of the cones, the hardness of the cone scales, and the shape of the cones (Smith 1970; Elliott 1974; Benkman 1995; Talluto and Benkman 2011). Certain conifer species such as *Pinus contorta* and *P. flexilis* have a sticky resin around the cones, which decreases the feeding efficiency of squirrels (Smith 1970; Benkman 1995). The resin on the cones and the seeds themselves contain terpenes, which also affects the food choice by squirrels (Farentinos et al. 1981; Snyder 1992; Rubino et al. 2012). Terpenes are the components of the major chemical defenses of conifers; there is a negative correlation between the degree of herbivory by cone insects and the terpene concentration (e.g., Keefover-Ring and Linhart 2010). Among the terpenes, α-pinene and limonene are the most dominant contents of coniferous cones (Rubino et al. 2012). However, the direct effects of such terpenes on seed preference or avoidance by tree squirrels have yet to be examined.

The Japanese squirrel, *Sciurus lis* Temminck, 1844, is endemic to Honshu and Shikoku, two main islands of Japan. Adult *S. lis* ranges in body mass from 250 to 310 g (Tamura 2015a). According to the molecular phylogenetic analysis, *S. lis* diverged from the Eurasian red squirrel, *S. vulgaris* Linnaeus, 1758, 4.0–5.2 Myr ago (Oshida and Masuda 2000). *Sciurus vulgaris* is widely distributed in Eurasia from Iberia, Great Britain, the Mediterranean and Black Seas to Kamchatka, Sakhalin, northern Mongolia, northeast China, Korea, and the northern island Hokkaido of Japan (Thorington et al. 2012). The body mass ranges from 235 to 480 g, slightly heavier than that of *S. lis* (Thorington et al. 2012). The main food source for *S. vulgaris* is various species of coniferous seeds and oak acorns (Krauze-Gryz and Gryz 2015), whereas *S. lis* mostly uses pine seeds and walnuts, and rarely uses oak acorns (Kato 1985; Yatake et al. 1999). The different food habits of these two *Sciurus* species might be explained by the vegetation of the habitats to which they had allopatrically adapted. And thus, the tolerance to tannins in *Quercus* acorns and/or terpenes in coniferous seeds is expected to differ between *S. lis* and *S. vulgaris*.

The forest environment in Japan has undergone major changes from the Tertiary (Pliocene) period to the present (Momohara et al. 2017; Nishiuchi et al. 2017). The current forest is formed through such geohistorical climate changes, and *S. lis* is now endemic only to Honshu and Shikoku of Japan. In recent years, the vegetation has been changing rapidly due to human activities and global warming (Yasuda 1974). Furthermore, alien squirrel species have become established in various parts of the world, and there are concerns about their impact on endemic species and ecosystems (e.g., Palmer et al. 2007; Mori et al. 2016). In the 2000s, *S. vulgaris* was detected in an isolated forest on the border of Tokyo and Saitama in Honshu, Japan (Shigeta et al. 2000; Kasahi 2011; Tamura et al. 2017). Although the exact origin is still undetermined, the cold and temperate forest may be a typical habitat of this alien squirrel. Pallas's squirrels, *Callosciurus erythraeus* (Pallas, 1779), were introduced and released in the 1930s, and are now established in at least 17 places on Honshu and Kyushu of Japan (Tamura 2015b). This squirrel has a body mass ranging from 286 to 375 g, and was originally distributed in Bhutan, India, Myanmar, southern China, Laos, Cambodia, Vietnam, Thailand, Malaysia, and Taiwan (Thorington et al. 2012). The Japanese populations consist of individuals from Taiwan and Southeast Asia (Ikeda et al. 2011; Kuramoto et al. 2012) and their original habitats may be the subtropical to tropical forests. They use a variety of foods including plant seeds, flowers, tree sap, bark, insects, and bird eggs (Ozaki 1986; Tamura et al. 1989; Torii 1993; Seki and Yasuda 2018). By clarifying the differences in food selection between the endemic and alien squirrel species, especially their resistance to plant repellent substances, future expansion/reduction in the distribution of endemic and alien species can be predicted and used for conservation measures.

In the present study, we compared the consumption rates of artificial foods with different concentrations of tannic acids and terpenes between native *S. lis* and alien *S. vulgaris* and *C. erythraeus* introduced to central Honshu, Japan. Tolerances to phenolic compounds including tannins and terpenes were assessed to clarify the adaptation to *Quercus* acorns and coniferous seeds, respectively. These are the two main food sources for tree squirrels, but their relative abundance differs significantly between the cold, temperate, subtropical, and tropical

zones. Therefore, we also estimated the actual concentrations of these two chemicals for various species of *Quercus* acorns and coniferous seeds available in the field. Finally, we discuss the possibility that the distribution and speciation patterns of these three species of squirrels are restricted by the food distribution.

Materials and methods

Field experiments

The food choice of *S. lis* was investigated by a cafeteria-style feeding experiment at two study sites about 60 km apart from each other. One was located at the eastern foot of Mt. Takao, western Tokyo, Honshu (183–287 m in altitude), dominated by *Abies firma* and *Castanopsis sieboldii* (Katsuki et al. 2010). The other was at the northeastern foot of Mt. Fuji, Yamanashi, Honshu (1100–1200 m in altitude), dominated by *Quercus crispula* and *P. densiflora* (Tamura et al. 2007). A feeding device (150 × 100 × 250 mm^3 wire mesh cage) was installed on a tree trunk at a height of approximately 2 m, and the animals visiting the device were monitored by sensor cameras (Fieldnote DUO, Marif Co. Ltd., Yamaguchi, Japan). The mean home range length and the home range area (estimated by the 70% kernel method) were 624.4 m and 7.92 ha in males and 409.3 m and 4.21 ha in females at Mt. Fuji (Kataoka et al. 2010). At each site, ten devices were placed at least 200 m apart, preventing the same individual from accessing multiple devices. Prior to the experiment, *Juglans mandshurica* unshelled walnuts were placed in the device to attract squirrels for a period of two weeks. When the squirrels consumed the provided walnuts, we placed experimental foods in the feeding devices: ten *Q. serrata* acorns, ten *Q. crispula* acorns, ten *P. densiflora* cones, and ten *J. mandshurica* walnuts. The *Q. serrata* acorns and *J. mandshurica* walnuts were collected in western Tokyo, and the *Q. crispula* acorns and *P. densiflora* cones were collected in Yamanashi Prefecture. To prevent germination at the experimental sites, the pine cones and walnuts were stored at –20°C for 7–10 weeks, and the acorn embryos were removed before using. The seeds carried by the squirrels were counted for each species within six days after placement. The experiments were conducted from November to December in 2008 both at Mt. Takao and Mt. Fuji.

Laboratory experiment 1 (tannins)

Five matured individuals of *S. lis* (two males and three females) were caught in July 2016 at the eastern foot of Mt. Takao, western Tokyo, Honshu, and another five matured *S. lis* individuals (three males and two females) were caught in July 2017 at the northwestern foot of Mt. Fuji, Yamanashi, Honshu. In January 2017, five matured individuals of *S. vulgaris* (two males and three females) were caught at their introduction site, Sayama Hill located at the boundary of Saitama and Tokyo, Honshu (Tamura et al. 2017). Sayama Hill (3500 ha) is isolated from urban areas and covered mainly by *Q. serrata*. In July 2016, five matured males of *C. erythraeus* were caught in Yokohama, Kanagawa, Honshu. This introduced squirrel lives in small wooded areas constituted by *Cryptomeria japonica*, *Cerasus jamasakura*, and *Q. serrata* in Yokohama urban areas (Tamura 2020).

Captured squirrels were individually reared in cages measuring 1 m wide, 1 m deep, and 2 m high. Five or more tree branches were placed in the cages for the squirrels to move up and down freely. One or two wooden boxes were set as nests at a height of 1.7 m in the cages. The rearing room temperature was adjusted to 15 and 25°C by air-conditioning in the cold and warm seasons, respectively. Although sunlight came through the room windows, auxiliary lights were also used and adjusted to match the natural day length.

Water was always available and food was provided every morning at 0800–0900 h. The regular food consisted of bananas, apples, sweet potatoes, peanuts, dried sardines, and green vegetables. The experimental food was a powder containing wheat flour, sugar, and *J. mandshurica* walnuts. In tree squirrels, tannin tolerance increases with the amount of lipids in foods, but is less affected by the amount of protein (Chung-MacCoubrey et al. 1997; Barthelmess 2001). Therefore, we prepared three types of foods with different amounts of lipid with a similar amount of protein by changing the proportion in fresh weight: Food A (50 g flour, 3 g sugar, and 70 g walnut); Food B (100 g flour, 3 g sugar, and 20 g walnut); and Food C (119 g flour, 3 g sugar, and 1 g walnut). Analysis of lipid and crude protein concentrations performed by Japan Food Research Laboratories, Nagayama, Japan showed that Foods A, B, and C included 35%, 11%, and 2% lipids, and 18%, 16%, and 14% proteins in dry weight (n = 1 in all cases). Tannic acid ($C_{76}H_{52}O_{46}$, Sigma-Aldrich, St. Louis, USA) was added to these three types of foods to 0%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, and 8% by fresh weight, referring to the known tannin contents of *Quercus* acorns (Smallwood and Peters 1986; Chung-MacCoubrey et al. 1997;

Table 1. Feeding schedule in experiments 1 and 2

Experiments	Food types	*Sciurus lis*	*Sciurus vulgaris*	*Callosciurus erythraeus*
Ex. 1 (tannins)	Food A	10 g (54 kcal)	13 g (70 kcal)	12 g (65 kcal)
	Regular food	46 kcal	70 kcal	55 kcal
	Food B	10 g (41 kcal)	13 g (53 kcal)	12 g (49 kcal)
	Regular food	59 kcal	87 kcal	71 kcal
	Food C	10 g (36 kcal)	13 g (46 kcal)	12 g (43 kcal)
	Regular food	64 kcal	94 kcal	77 kcal
Ex. 2 (terpenes)	Food D	5 g (34 kcal)	7 g (47 kcal)	6 g (40 kcal)
	Regular food	66 kcal	93 kcal	80 kcal

The total calories for two consecutive days of the given experimental and regular foods were set at 100 kcal for *S. lis*, 140 kcal for *S. vulgaris*, and 120 kcal for *C. erythraeus*. Foods A, B, and C were experimental foods composed of wheat, sugar, and crushed walnuts with tannic acid. Food D was crushed walnuts with terpenes. Regular food consisted of fruits, peanuts, sunflower seeds, green vegetables, and dry sardines.

Barthelmess 2001; Shimada and Saitoh 2006). To prevent chemical modification with time, the tannins were added each time the experimental diet was given to the squirrels.

At the start of experiment, body mass of individual squirrels ranged from 245 to 290 g (mean ± *SD*: 267.1 ± 15.4, $n = 15$) in *S. lis*, from 415 to 450 g (434.0 ± 21.9, $n = 5$) in *S. vulgaris*, and 350 to 370 g (354.0 ± 11.4, $n = 10$) in *C. erythraeus*. The experimental foods were given in an amount equivalent to approximately 3% of the body mass of each species per day: 10 g for *S. lis*, 13 g for *S. vulgaris*, and 12 g for *C. erythraeus* (Table 1). We did not change the amount of food according to the individual body mass because the individual difference was not remarkable. To avoid a short-term overdose of tannins, the experimental foods and the regular foods were given every other day (Table 1). In rodents, the food requirements (Y kJ/day) is proportional to the body mass (X g) under the same conditions; $Y = 10.5\ X^{0.507}$ (Nagy 1987). In order to prevent the influence of food shortage, the caloric content was set to be 20% higher than the energy requirement calculated by the above formula. That is, the total calories for two consecutive days were set to 100 kcal for *S. lis*, 140 kcal for *S. vulgaris*, and 120 kcal for *C. erythraeus* (Table 1). After acclimatization for ca.10 days after capture, Food A of each tannin concentration was given four days (4 × 9 concentrations), followed by Food B (4 × 9 concentrations) and Food C (4 × 9 concentrations). Thus, in this experiment, it was necessary to give each squirrel the experimental food for 108 days and the regular food for 108 days. The amount of experimental food consumption per day was calculated as the difference in the fresh weight of the food before and after 24 h exposure and thus average for four values obtained for each concentration of tannins in Food A, B, or C. Each squirrel's body mass was monitored at intervals of eight to 20 days by video-monitoring a scale placed on the cage floor, on which approximately ten sunflower seeds were placed to attract the squirrel. If the squirrel left more than half the experimental food, or lost more than 15% of its body mass, further experiments were discontinued and subsequent consumption were analyzed as zero. The experiment was started in July 2016 and finished in February 2018. Upon completion of the experiments, all *S. lis* individuals were released to their capture sites after a recovery period of two weeks during which sufficient regular food was provided. The holding period of ten individuals of *S. lis* was 104–112 days. However, the alien squirrels remained in the laboratory for the rest of their lives.

Laboratory experiment 2 (terpenes)

This experiment was conducted from March 2018 to January 2020. Two matured individuals (one male and one female) and three matured individuals (two males and one female) of *S. lis* were caught at the study sites of western Tokyo and northeastern Yamanashi, respectively, from May to June 2019. We used three (one male and two females) of the five *S. vulgaris* individuals from Experiment 1, who survived in captivity until March 2018. Five matured males of *C. erythraeus* were captured in Yokohama City, Kanagawa Prefecture in October 2019.

The basic food used in Experiment 2 was crushed walnuts with a food processor running for 2 min. The oil-soluble monoterpenes α-pinene ($C_{10}H_{16}$, 0.855–0.862 g/mL, Wako Pure Chemical Industries Ltd., Tokyo, Japan) and limonene ($C_{10}H_{16}$, 0.840–0.850 g/mL, Wako Pure Chemical Industries Ltd., Tokyo, Japan) were added to the crushed walnuts (Food D) to 0, 0.5, 1.0, 1.5, and

2.0 μL/g fresh weight and 0, 4, 8, 16, and 20 μL/g fresh weight, respectively, referring to Rubino et al. (2012). This experimental food was given in an amount equivalent to approximately 2% of the body mass of each species per day: 5 g for *S. lis*, 7 g for *S. vulgaris*, and 6 g for *C. erythraeus*. Experimental and regular foods were given every other day to avoid a short-term overdose of terpenes. The total calories for two consecutive days were set to 100 kcal for *S. lis*, 140 kcal for *S. vulgaris*, and 120 kcal for *C. erythraeus* by adjusting the amount of regular food to 66–93 kcal per day (Table 1).

The experimental foods with each α-pinene concentration were given four days (4 × 5 concentrations), after which the foods with each concentration of limonene were given four days (4 × 5 concentrations). Thus, it was necessary to give each squirrel the experimental foods for 40 days and the regular food for 40 days. Other procedures were the same as those for Experiment 1. All five individuals of *S. lis* were released to their capture sites after a recovery period of two weeks during which sufficient regular food was provided. Their holding period was 101–104 days in total. However, the alien squirrels remained in the laboratory for the rest of their lives.

Chemical analyses of experimental and natural foods

Acorns of all 14 species of *Quercus* on mainland Japan were used for the analyses of total phenolic contents. Six species were deciduous (*Q. acutissima*, *Q. aliena*, *Q. crispula*, *Q. dentata*, *Q. serrata*, and *Q. variabilis*) and eight species were evergreen (*Q. acuta*, *Q. gilva*, *Q. glauca*, *Q. hondae*, *Q. myrsinifolia*, *Q. phillyraeoides*, *Q. salicina*, and *Q. sessilifolia*). Mature fresh acorns were collected on the ground, discriminating between individual trees, and the acorns were stored at –30°C before analyses. The total concentration of phenolic compounds in acorn cotyledons was measured using the Folin–Ciocalteu method (Nurmi et al. 1996; Ito et al. 2016). The shells, seed coats (astringent skins), radicles, and plumules were completely removed using tweezers, and only the cotyledon parts were milled into a fine powder, using a mortar and pestle. The powder was dried at 60°C overnight, after which approximately 50 mg was transferred to a 2.0 mL tube and weighed to the nearest 0.1 mg. Then, the powder was mixed with 1 mL 70% aqueous acetone. The homogenate was sonicated for 10 min and centrifuged at 2500 *g* for 10 min. The supernatant was stored in a 15 mL tube. This procedure was repeated three times, and the entire 3 mL of aqueous acetone extract was fully evaporated under low pressure at 40°C. The residue was dissolved in 1 mL distilled water. After being diluted 60 times, 0.3 mL of the solution was mixed with 0.3 mL of 2N Folin reagent (Folin–Ciocalteu's phenol reagent, Sigma-Aldrich, Steinheim, Germany) in a 2.0 mL tube, allowed to stand for 5 min, and then mixed with 0.6 mL 20% sodium carbonate (Na_2CO_3, Wako Pure Chemical Industries Ltd., Osaka, Japan). After 10 min incubation at room temperature, the mixture was centrifuged at 1500 *g* for 8 min, and the absorbance at 730 nm was measured using a spectrophotometer (DU 640, Beckman Instruments, Fullerton, USA). The standard curve was prepared with six concentrations of 0–50 mg/L gallic acid ($C_7H_6O_5$, MP Biomedicals, Solon, U.S.A), and the phenolic contents were expressed as the gallic acid equivalent in mg/g dry weight. Three acorns from the same tree were used for this analysis and their mean value was calculated for each tree.

The edible parts of five *J. mandshurica* walnuts and two *P. densiflora* cones used in the field experiment and the three types of experimental foods (Foods A, B, and C) with the added 0–8% tannic acid were also analyzed for their total phenolic content for a comparison between the experimental and natural foods.

Food samples containing 0, 0.5, 1.0, and 2.0 μL/g α-pinene, and 0, 4, 12, and 20 μL/g limonene were prepared and the standard curves of added amounts and concentrations were created, respectively. The amount of α-pinene and limonene contained in Food D used in Experiment 2 was quantified as mg/g dry weight by Japan Food Research Laboratories, Nagayama, Japan, using gas chromatography–mass spectrometry. The same analyses were also conducted for a mass of fresh mature seeds (including seed coats) of seven common species of Japanese conifers; *C. japonica*, *P. densiflora*, *P. parviflora*, *Picea jezoensis*, *A. firma*, *A. mariesii*, and *A. sachalinensis.*

Statistical analysis

In the cafeteria-style experiment, the number of seeds removed by squirrels was compared between the two populations of *S. lis* by the Mann–Whitney *U*-test for each four species of seeds. In the laboratory feeding experiment, the amount of food consumption per day were compared between two populations of *S. lis* using the Mann–Whitney *U*-test. Due to the small sample size and not necessarily a normal distribution, the effects of the total phenolic and terpene concentrations on the food consumption were individually tested by the corresponding nonparametric tests. The Friedman test was used to

compare the values between three or more groups, and the Wilcoxon signed-rank test was used when two groups were compared. If there was a significant difference in the Friedman test, the Dunn–Bonferroni *post hoc* test was performed to determine which group combinations had a significant difference (type I error rate, $\alpha = 0.05$). To check whether insignificant results were due to small sample size, we conducted *post hoc* effect size (Cohen's *d*) and power analysis when β (type II error rate) was set at 0.20. The calibration curves between the added chemical and absolute chemical contents were checked by Pearson's correlation analysis. Statistical tests were performed using IBM SPSS Statistics, ver. 25, and GPower 3.1 program.

Ethics statements

The field experiment and capture of *S. lis* was approved by the Yamanashi Prefectural Government, the Onshirin Regional Public Organization, and the Tokyo Metropolitan Environmental Bureau. Two species of squirrels, *S. vulgaris* and *C. erythraeus*, were captured under the alien species countermeasure projects by the Ministry of the Environment and Yokohama City, respectively. The laboratory experiments were conducted from April 2016 to March 2020 under the approval of the Animal Experimentation Committee of the Forestry and Forest Products Research Institute (No. 16A-7, 17A-1, 17A-2).

Results

Field experiments

In the cafeteria-style experiment, the mean (± *SD*) number of walnuts taken by squirrels was 10.0 ± 0.0 at Mt. Takao and 9.8 ± 0.6 at Mt. Fuji, which did not differ significantly (Mann–Whitney *U*-test, $U = 45$, $P = 0.37$) (Fig. 1). The mean number of pine cones taken by squirrels was 9.3 ± 2.2 at Mt. Takao and 10.0 ± 0.0 at Mt. Fuji, which did not differ significantly ($U = 45$, $P = 0.37$) (Fig. 1). However, for *Q. serrata*, the mean number of seeds removed was significantly greater at Mt. Fuji (mean ± *SD*: 9.4 ± 1.9) than Mt. Takao (3.1 ± 3.0), ($U = 3$, $P < 0.01$) (Fig. 1). The mean number of *Q. crispula* seeds removed was also significantly greater at Mt. Fuji (9.2 ± 2.2) than Mt. Takao (1.5 ± 2.5), ($U = 1$, $P < 0.01$) (Fig. 1).

Experiment 1 (tannins)

The total phenolic content of the experimental foods increased linearly with the percentage of tannic acids

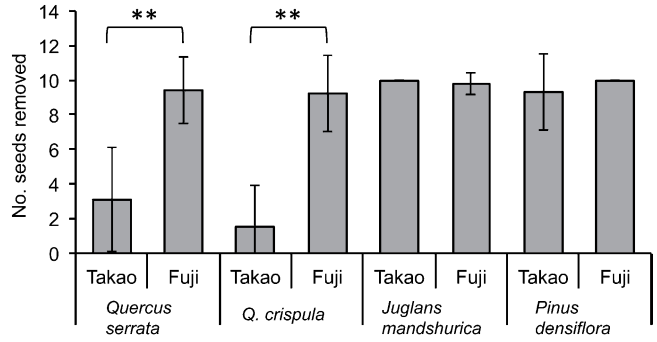

Fig. 1. Mean (± *SD*) number of four species of seeds removed by *Sciurus lis* during six days at ten feeding devices installed at Mt. Takao and Mt. Fuji, respectively. Mann–Whitney *U*-tests indicated that more seeds were removed at Mt. Fuji than at Mt. Takao (**$P < 0.01$).

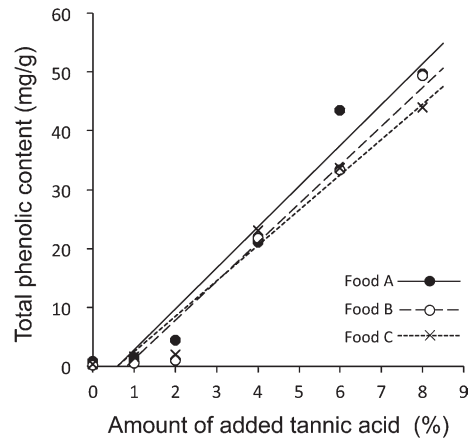

Fig. 2. Relationship between the percent of tannic acid added to the fresh experimental food and the total phenol content (mg/g dry weight) expressed as gallic acid equivalents. Three types of experimental foods with different lipid contents were used: Food A (35% lipid), Food B (11% lipid), and Food C (2% lipid). For the regression lines, see the text.

added to Food A ($y = 6.94x - 4.11$, $R^2 = 0.96$, $df = 4$, $P < 0.001$); Food B ($y = 6.58x - 5.33$, $R^2 = 0.96$, $df = 4$, $P < 0.001$); and Food C ($y = 6.00x - 3.49$, $R^2 = 0.96$, $df = 4$, $P < 0.001$) (Fig. 2). The intercept of regression lines became slightly high with the increasing content of walnuts. This is probably due to the phenolic content in the walnuts (see Table 5).

Consumption of the tannin-containing experimental foods revealed that *S. lis* was able to eat foods with less than 4% tannic acid (Fig. 3). As the amount of lipids decreased, the repellent effect of the tannic acid became stronger from Foods A to C (Fig. 3). There was no significant difference in the food consumption of each food type between the two study sites (Table 2). The Friedman and Wilcoxson signed-rank tests revealed that the food

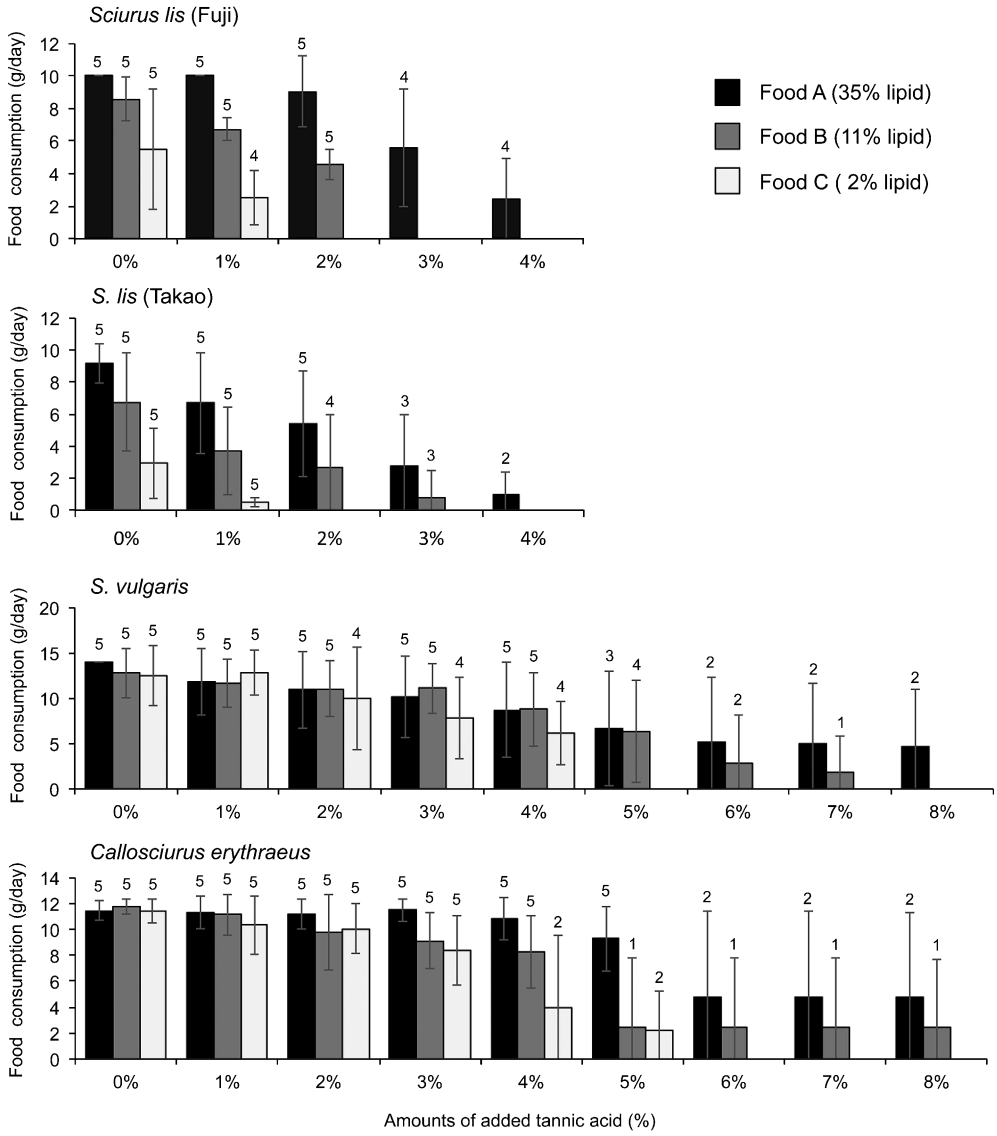

Fig. 3. Mean value (± *SD*) of consumption of 0–8% tannin-containing experimental foods by three species of squirrels, *Sciurus lis* at Mt. Fuji ($n = 5$) and Mt. Takao ($n = 5$), *S. vulgaris* ($n = 5$), and *Callosciurus erythraeus* ($n = 5$). Three types of experimental foods, A, B, and C with different lipid contents were used. The numbers above the bar indicate the number of individuals who participated in the feeding test for each type of food.

consumption of *S. lis* decreased significantly with increasing tannic acid concentrations in all food types (Table 3a). The Dunn–Bonferroni *post hoc* test indicated that significant difference was detected between 0/1% *vs.* 4% tannic acid in Food A and 0% *vs.* 2/3% tannic acid in Food B. The statistical power (1 – β) was more than 0.80 in all combinations between 0/1% *vs.* 3/4% in Food A, 0% *vs.* 2/3% in Food B, and 0% *vs.* 1% in Food C. Moreover, *S. lis* tended to consume less food with a lower lipid content (Food C) than a more lipid content (Food A), even at the same tannic acid concentration (Table 3b). The statistical power of all combinations among Foods A, B, and C was more than 0.80.

Table 2. Results of nonparametric test for differences in consumption of different concentrations of Foods A, B, and C between two populations (Mt. Fuji and Mt. Takao) of *Sciurus lis* (see Fig. 3)

Food types	Tannic acid added (%)	Mann–Whitney *U*-tests	
		U	*P*
Food A	0	7.5	0.44
	1	5.0	0.17
	2	4.0	0.05
	3	8.0	0.41
	4	7.5	0.33
Food B	0	9.0	0.53
	1	4.5	0.10
	2	5.0	0.15
Food C	0	7.5	0.34
	1	5.0	0.15

Table 3. Results of nonparametric test for the effects of tannin (a) and lipid (b) concentrations on the food consumption of experimental foods (see Fig. 3)

(a) Effects of tannin concentration

Species (site)	*Sciurus lis* (Mt. Fuji)	*S. lis* (Mt. Takao)	*S. vulgaris*	*Callosciurus erythraeus*
Food A	$\chi^2 = 19.22$, $df = 4$, $P < 0.01$	$\chi^2 = 18.67$, $df = 4$, $P < 0.01$	$\chi^2 = 36.93$, $df = 8$, $P < 0.01$	$\chi^2 = 28.99$, $df = 8$, $P < 0.01$
Significance	0–4%, 1–4%	0–4%	0–8%, 0–7%, 1–8%, 2–8%	
Power	0–4%, 0–3%, 1–4%, 1–3%, 2–4%	0–4%, 0–3%, 0–2%, 1–4%, 1–3%	0–8%, 0–7%	
Food B	$\chi^2 = 8.40$, $df = 2$, $P = 0.02$	$\chi^2 = 13.65$, $df = 3$, $P < 0.01$	$\chi^2 = 31.66$, $df = 7$, $P < 0.01$	$\chi^2 = 28.59$, $df = 8$, $P < 0.01$
Significance	0–2%	0–3%	0–7%, 0–6%, 1–7%, 0–7%	0–8%
Power	0–1%, 0–2%, 1–2%	0–1%, 0–2%, 0–3%, 1–3%	0–7%, 0–6%, 0–5%, 1–7%, 1–6%, 2–7%, 2–6%, 3–7%, 3–6%	0–8%, 0–7%, 0–6%, 0–5%, 0–4%, 0–3%,1–8%, 1–7%, 1–6%, 1–5%, 1–4%, 1–3%, 2–8%, 2–7%, 2–6%, 2–5%
Food C	$W = 2.02$, $df = 1$, $P = 0.04$	$W = 2.02$, $df = 1$, $P = 0.04$	$\chi^2 = 9.22$, $df = 4$, $P = 0.06$	$\chi^2 = 23.20$, $df = 5$, $P < 0.001$
Significance				
Power	0–1%	0–1%	0–4%, 0–3%, 1–4%, 1–3%	0–5%, 0–4%, 1–5%, 1–4%, 2–5%

(b) Effects of lipid concentration

Species (site)	*S. lis* (Mt. Fuji)	*S. lis* (Mt. Takao)	*S. vulgaris*	*C. erythraeus*
Tannic acid 0%	$\chi^2 = 7.11$, $df = 2$, $P = 0.03$	$\chi^2 = 9.58$, $df = 2$, $P = 0.01$	$\chi^2 = 1.00$, $df = 2$, $P = 0.61$	$\chi^2 = 3.85$, $df = 2$, $P = 0.15$
Significance	A–C	A–C		
Power	A–C, A–B, B–C	A–C, A–B, B–C		
Tannic acid 1%	$\chi^2 = 10.00$, $df = 2$, $P = 0.01$	$\chi^2 = 10.00$, $df = 2$, $P = 0.01$	$\chi^2 = 0.13$, $df = 2$, $P = 0.94$	$\chi^2 = 2.29$, $df = 2$, $P = 0.32$
Significance	A–C	A–C		
Power	A–C, A–B, B–C	A–C, A–B, B–C		
Tannic acid 2%			$\chi^2 = 0.44$, $df = 2$, $P = 0.80$	$\chi^2 = 1.20$, $df = 2$, $P = 0.55$
Significance				
Power				
Tannic acid 3%			$\chi^2 = 0.40$, $df = 2$, $P = 0.82$	$\chi^2 = 7.60$, $df = 2$, $P = 0.02$
Significance				
Power				A–C, A–B
Tannic acid 4%			$\chi^2 = 0.40$, $df = 2$, $P = 0.82$	$\chi^2 = 7.68$, $df = 2$, $P = 0.02$
Significance				
Power				A–C, A–B
Tannic acid 5%				$\chi^2 = 5.77$, $df = 2$, $P = 0.06$
Significance				
Power				A–C

Wilcoxon signed-rank tests (W) are used to compare between two related groups and Friedman tests (χ^2) are used between three or more groups. Significant effects ($P < 0.05$) are shaded. A combination of groups with a significant difference ($P < 0.05$) by the Dunn–Bonferroni *post hoc* test and a combination of groups with > 0.80 statistical power are also shown.

Sciurus vulgaris was able to eat foods containing up to 8% tannic acid (Fig. 3). Even for Food C (2% lipid), four individuals consumed some foods containing 4% tannic acid. Friedman tests revealed that the food consumption decreased significantly with increasing tannic acid in Foods A and B, and significant difference was detected between 0/1/2% *vs.* 7/8% in Food A and 0/1% *vs.* 6/7% in Food B (Table 3a). The statistical power between 0% *vs.* 7/8% in Food A and 0/1/2/3% *vs.* 6/7/% in Food B was more than 0.80. The response to Food C varied greatly among individuals, and no clear decrease in food consumption due to increased tannic acid was observed

(Table 3a). However, the statistical power of food consumption between 0/1% *vs.* 3/4% tannic acid in Food C was all more than 0.80. When compared at the same tannic acid content, the amount of lipid in the food did not affect the food consumption (Table 3b). Statistical power of all combinations among Foods A, B, and C was less than 0.30.

Callosciurus erythraeus also consumed foods containing up to 8% tannic acid. Even for Food C, two individuals consumed some foods containing 5% tannic acid (Fig. 3). Friedman tests revealed that the food consumption decreased significantly with increasing tannic acid in all food types (Table 3a). Due to the large individual differences, the *post hoc* test did not detect any significant difference in either combination in Food A and the statistical powers of all combinations were less than 0.80. In Food B, the food consumption with 8% tannic acid was significantly lower than that with 0% tannic acid (Table 3a). The power of all combinations between 0/1/2% *vs.* 5/6/7/8% were more than 0.80. In Food C, the *post hoc* test did not detect any significant difference. The power of all combinations between 0/1% *vs.* 4/5% were more than 0.80. When compared at the same tannic acid content, the amount of lipid in the food did not clearly affect the food consumption (Table 3b). The statistical power between Foods A and C were more than 0.80 in 3%, 4%, and 5% tannic acid, but less than 0.40 in 0%, 1%, and 2% tannic acid.

Experiment 2 (terpenes)

The α-pinene content of Food D (crushed walnuts) increased with the added amount of liquid α-pinene ($y = 1.20x + 0.40$, $R^2 = 0.89$, $df = 2$, $P = 0.05$) and the limonene content of Food D also increased linearly with the added amount of liquid limonene ($y = 1.27x + 0.36$, $R^2 = 0.99$, $df = 2$, $P < 0.01$) (Fig. 4). Based on the gas chromatography–mass spectrometry results, the food supplemented with 0.5, 1.0, 1.5, and 2.0 μL/g of α-pinene actually contained 1.0 (0.10%), 1.6 (0.16%), 2.2 (0.22%), and 2.8 mg/g (0.28%), respectively. The food supplemented with 4, 8, 16, and 20 μL/g of limonene actually contained 5.4 (0.54%), 10.5 (1.05%), 20.7 (2.07%), and 25.8 mg/g (2.58%), respectively.

Sciurus lis and *S. vulgaris* were able to eat foods with α-pinene concentrations up to 2.0 μL/g (Fig. 5). Friedman tests revealed that the decrease in the food consumption with increasing α-pinene concentration was not significant (Table 4). In contrast, the food consumption by *C. erythraeus* tended to decrease as the food contained 1.5 μL/g and higher α-pinene concentrations (Fig. 5, Table 4). The *post hoc* test did not detect any significant difference between the combination of different α-pinene content. The statistical power for all combination was less than 0.80 in three species (Table 4).

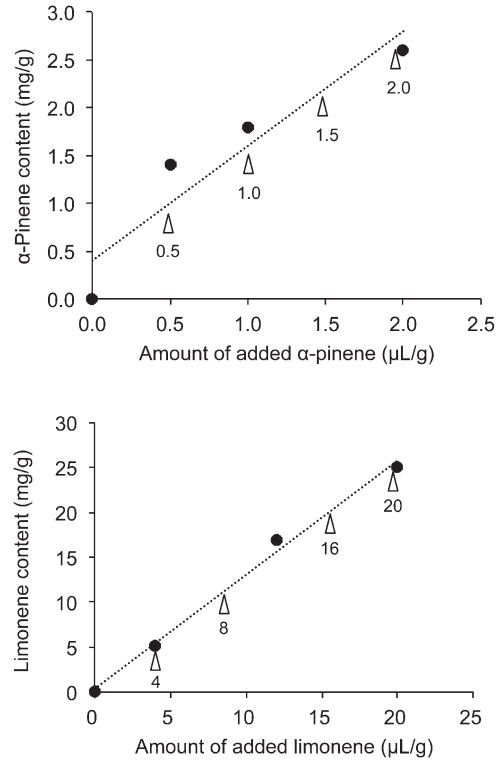

Fig. 4. Relationship between the amount of liquid α-pinene (upper) and limonene (lower) added to the experimental fresh food (Food D) and the terpene concentration (mg/g dry weight) measured by gas chromatography–mass spectrometry. Triangles indicate the terpene contents used in this study. For the regression lines, see the text.

The limonene content of foods significantly affected the food consumption in all species (Table 4). By the *post hoc* test, no significant difference was detected in *S. lis*, but a significant difference was detected between 0 μL *vs.* 20 μL in *S. vulgaris* and 0 μL *vs.* 16 μL in *C. erythraeus*. For both *S. lis* and *S. vulgaris*, statistical powers between foods containing 20 μL limonene *vs.* foods with lower limonene concentration were more than 0.80 (Table 4). In *C. erythraeus*, a statistical power exceeded 0.80 at a concentration of only 8 μL limonene. Therefore, the food consumption decreased significantly at 20 μL/g limonene concentration in two species of *Sciurus*. In contrast, the food consumption by *C. erythraeus* tended to decrease from 8 μL/g limonene concentration, reaching the lowest level at 16 μL limonene/g (Fig. 5, Table 4).

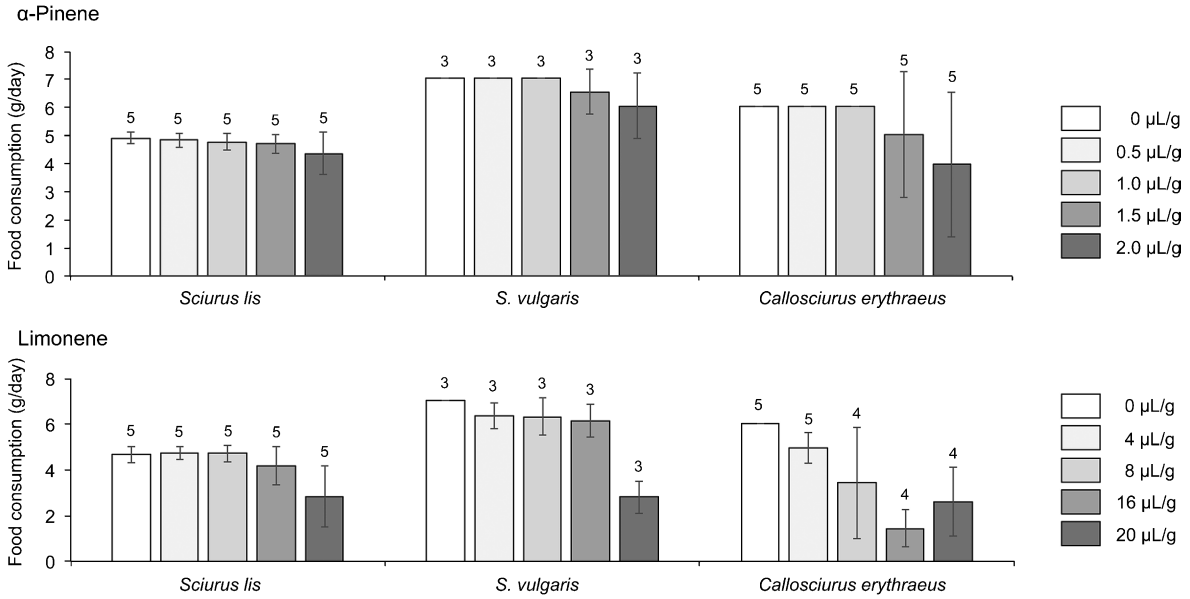

Fig. 5. Mean value (± *SD*) of consumption of 0–2.0 μL/g α-pinene-containing experimental foods (upper) and 0–20 μL/g limonene-containing experimental foods (lower) by three species of squirrels, *Sciurus lis* (*n* = 5 all pooled from Mt. Fuji and Mt. Takao), *S. vulgaris* (*n* = 3), and *Callosciurus erythraeus* (*n* = 5). The numbers above the bar indicate the number of individuals who participated in the feeding test for each type of food.

Table 4. Results of nonparametric test (Friedman tests, χ^2) for the effects of α-pinene and limonene concentrations on the food consumption of experimental foods (see Fig. 5)

Species	*Sciurus lis*	*S. vulgaris*	*Callosciurus erythraeus*
α-pinene	$\chi^2 = 9.33$, $df = 4$, $P = 0.05$	$\chi^2 = 8.00$, $df = 4$, $P = 0.09$	$\chi^2 = 10.67$, $df = 4$, $P = 0.03$
Significance			
Power			
Limonene	$\chi^2 = 12.46$, $df = 4$, $P = 0.01$	$\chi^2 = 9.96$, $df = 4$, $P = 0.04$	$\chi^2 = 16.30$, $df = 4$, $P < 0.01$
Significance		0–20 μL/g	0–16 μL/g
Power	0–20, 4–20, 8–20, 16–20 μL/g	0–20, 4–20, 8–20, 16–20 μL/g	0–20, 4–20, 0–16, 4–16, 0–8 μL/g

Siginificant effects ($P < 0.05$) are shaded. A combination of groups with a significant difference ($P < 0.05$) by the Dunn–Bonferroni *post hoc* test and a combination of groups with > 0.80 statistical power are also shown.

Chemical analyses of natural seeds

Total phenolic content in the edible parts of natural seeds, expressed as the gallic acid equivalent in mg/g dry weight, differed greatly among the species (Table 5). The *J. mandshurica* walnuts, *P. densiflora* seeds, and certain *Quercus* acorns such as *Q. gilva* and *Q. hondae* included little phenolics. The content of the two terpenes (α-pinene and limonene) in natural seeds, expressed by the dry weight base, differed greatly among coniferous species (Table 6). The foods with 2.0 μL/g α-pinene and 20 μL/g limonene, which were the maximum concentrations used in Experiment 2, corresponded to 2.8 and 25.8 mg/g in dry weight, respectively (Fig. 4).

Discussion

In the field studies by direct observation method, there were few reports that *S. lis* ate *Quercus* acorns (Kato 1985; Yatake et al. 1999). However, *Quercus* acorns are frequently used as food items by other species of squirrels, for example, *C. erythraeus* (Xiao et al. 2009), *S. vulgaris* (Krauze-Gryz and Gryz 2015), and *S. anomalus* (Malekian and Sadeghi 2020) in Eurasia, and *S. carolinensis* and *S. niger* (Smith and Follmer 1972) in North America. *Sciurus vulgaris* uses a wide range of seeds found in coniferous and deciduous forests and especially prefers *Q. robur* acorns (Krauze-Gryz and

Table 5. Mean and standard deviation (*SD*) of total phenolic content expressed in terms of gallic acid equivalent (mg/g dry weight) in the edible parts of seeds of *Juglans*, *Pinus*, and *Quercus* species

Family	Species	e/d[1)]	*n*	Mean	*SD*	Collecting sites
Juglandaceae	*Juglans mandshurica*	d	5[2)]	0.88	0.52	near Mt. Fuji, Yamanashi
Pinaceae	*Pinus densiflora*	e	3	0.47	0.16	near Mt. Takao, Tokyo
Fagaceae	*Quercus hondae*	e	3	18.56	5.54	Saiki, Oita
Fagaceae	*Quercus gilva*	e	1	25.78	–	Hachioji, Tokyo
Fagaceae	*Quercus variabilis*	d	3	43.47	11.96	Koyamadai and Hibiya, Tokyo
Fagaceae	*Quercus phillyraeoides*	e	3	47.07	14.45	Hachioji, Tokyo
Fagaceae	*Quercus dentata*	d	2	47.14	20.62[3)]	Matsumoto, Nagano; Fujiyoshida, Yamanashi
Fagaceae	*Quercus aliena*	d	3	54.85	11.09	Hachioji, Tokyo
Fagaceae	*Quercus serrata*	d	3	56.57	11.95	Hachioji, Tokyo
Fagaceae	*Quercus acutissima*	d	3	59.78	15.04	Hachioji, Tokyo
Fagaceae	*Quercus crispula*	d	3	68.77	20.44	near Mt. Hakkoda, Aomori
Fagaceae	*Quercus glauca*	e	3	95.25	30.74	Hachioji, Tokyo
Fagaceae	*Quercus sessilifolia*	e	3	127.76	18.21	Hachioji and Machida, Tokyo
Fagaceae	*Quercus salicina*	e	3	128.29	26.13	Hachioji, Tokyo
Fagaceae	*Quercus myrsinifolia*	e	3	129.84	51.42	Hachioji, Tokyo
Fagaceae	*Quercus acuta*	e	3	133.46	16.75	Izu Peninsula, Shizuoka

n, the number of trees examined.
1) evergreen/deciduous trees.
2) Number of seeds examined.
3) When $n = 2$, it means the range.

Table 6. α-Pinene and limonene content expressed as mg/g dry weight in a mass of each species of coniferous seeds

Family	Species	Locality	α-Pinene	Limonene
Cupressaceae	*Cryptomeria japonica*	Chiyoda, Ibaraki	0.025	0.002
Pinaceae	*Pinus densiflora*	Tsukuba, Ibaraki	0.034	0.007
Pinaceae	*Pinus parviflora*	Fujiyoshida, Yamanashi	0.041	0.003
Pinaceae	*Picea jezoensis*	Furano, Hokkaido	0.120	0.063
Pinaceae	*Abies sachalinensis*	Horonobe, Hokkaido	16.000	8.100
Pinaceae	*Abies mariesii*	Kamihozawa, Yamagata	20.000	5.900
Pinaceae	*Abies firma*	Hachioji, Tokyo	54.000	59.000

Gryz 2015), which have a total phenolic content of 52 mg/g (Shimada and Saitoh 2006). *Callosciurus erythraeus* is known to eat *Q. variabilis* acorns (49.4 mg/g total phenolic content, Table 5), with the embryos removed to prevent the acorns from sprouting before feeding (Xiao et al. 2009).

In the cafeteria-style experiment, *S. lis* at Mt. Fuji removed more acorns compared to those at Mt. Takao. This result indicates that the dependence on acorns may differ between populations. In other species of rodents, regional differences in tannin resistance within the same species have also been reported (Atsatt and Ingram 1983; Briggs and Smith 1989; McEachern et al. 2006). In the cafeteria experiment, however, it was unclear whether or not the removed food was actually eaten. It is possible that squirrels may remove food, but not eat it. Under the captive conditions, no significant difference was observed in the effect of tannin concentration between the squirrels captured at Mt. Fuji and Mt. Takao. In addition, the two populations are connected via forests, only 60 km apart from each other. Therefore, it is unlikely that only the squirrels at Mt. Fuji acquired the physiological adaptation to tannins.

The response of squirrels to tannins under the experi-

mental conditions was significantly different between *S. lis* and the other two species. By increasing the lipid content in this experiment, *S. lis* ate a food containing a maximum of 4% tannic acid, whereas *S. vulgaris* and *C. erythraeus* were able to eat a food containing a maximum of 8% tannic acid. In laboratory experiments, *S. carolinensis* is known to eat foods containing 9% tannins (Barthelmess 2001) and 8% tannins (Chung-MacCoubrey et al. 1997). Even *Tamiasciurus hudsonicus*, which prefers conifer seeds, ate foods containing 5% tannins under experimental conditions (Eberly et al. 1982). Apparently, *S. lis* tended to rarely eat foods containing tannins compared to such tree squirrels. Irrespective of the amount of tannins, the consumption of Food C by *S. lis* was significantly lower, suggesting that the low lipid content may be one of the reasons for not using *Quercus* acorn as food. Interspeciefic differences in tannin detoxification mechanisms and the lipid content effects on food consumption are still unknown, and further physiological studies are needed.

Unlike tannins, the consumption of foods with terpenes by *S. lis* was not lower compared to that of other species of squirrels. The content of α-pinene had no significant effect on food consumption in the range of concentration used in the present study. The response patterns of *S. vulgaris* and *S. lis* to terpenes were similar, but those of *C. erythraeus* were not. There were significant individual differences in the food consumption, and foods containing a higher concentration of terpenes tended to be radically avoided.

The lipid content of acorns distributed in Japan ranges from 1.5% to 4.6% (Shimada and Saitoh 2006), which corresponds to the lipid content of Food C in this experiment. When limited to Food C, *S. lis* can eat only 1% tannic acid (Fig. 3), corresponds to a total phenol content of natural seeds of 2.5 mg/g (Fig. 2). All species of *Quercus* acorns showed higher values than this tolerable limit (Table 5), although it is known that the tannin content varies among individual trees and even among acorns within the same tree (Steele et al. 1993; Shimada and Saitoh 2006; Shimada et al. 2015). Further, we have to pay attention that all of total phenolics in plants cannot be eluted through chewing and digestion, and so acceptance limits of squirrels may be higher in natural food than those of the purified tannic acid. However, it was at least suggested that *S. vulgaris* and *C. erythraeus* were more acceptable to the food containing more tannin than *S. lis*. These two species will be able to consume some species of deciduous *Quercus* acorns (Table 5), because they can eat Food C with up to 4% or 5% tannic acid and Food A with up to 8% (Fig. 3) which corresponds natural seeds with a total phenolic content of 20.5 or 26.5 mg/g and 51.4 mg/g, respectively (Fig. 2).

The concentrations of terpenes that three species of squirrels were able to eat under experimental conditions exceeded those contained in *Cryptomeria*, *Pinus*, and *Picea* seeds (Table 6). The genus *Abies* tended to have a particularly high content of terpenes, but *S. lis* and *S. vulgaris* were able to eat experimental foods containing similar concentrations of limonene. However, in *C. erythraeus*, the consumption of experimental foods containing > 2.2 mg/g of α-pinene and > 5.4 mg /g of limonene were drastically decreased (Fig. 5), suggesting that they are unlikely to eat *Abies* seeds.

We speculated from these results that *S. lis* originally adapted to the environment of coniferous forests. From the early to middle Pleistocene (~0.4–1.2 Mya), fossils of plants and pollens reveal that coniferous trees, *P. koraiensis*, *Picea jezoensis*, and others that are currently distributed in the subalpine zone, were dominant in the lowlands of Honshu and Shikoku (Momohara et al. 2017). These plants are consistent with the foods preferred by *S. lis*, and a suitable habitat for *S. lis* is suspected to have widespread during the middle Pleistocene. After the late Pleistocene, the vegetation changed drastically including the distribution of broad-leaved trees such as *Quercus* (Sohma and Tsuji 1988). In the current Japanese archipelago, the once widely distributed *P. koraiensis* is limited to the northern part of Honshu or a few subalpine places in central Honshu and central Shikoku (Okitsu and Momohara 1997). Based on fossil records, an ancestral *S. vulgaris* immigrated from Hokkaido to Honshu and Shikoku through the land bridge formed during the middle Pleistocene, and then *S. lis* speciated from the ancestral *S. vulgaris* due to the isolation of Honshu and Shikoku from Hokkaido (Kawamura 1988; Kawamura et al. 1989). The divergence time between the two species estimated based on the cytochrome *b* gene was earlier than that estimated by the fossil records, approximately 4.0–5.2 Mya (Oshida and Masuda 2000). Although there is a variation in the estimated divergence time for the two species, it is considered that at least *S. lis* evolved under the environment of Honshu and Shikoku during the early to middle Pleistocene. Since *S. lis* is a relict species, it is important to preserve such a limited suitable habitat.

Eastern grey squirrels (*S. carolinensis*), originally distributed in deciduous forests in eastern North America and introduced to Europe, have a mechanism for tannin

detoxification and are even more resistant to tannins than native *S. vulgaris* (Chung-MacCoubrey et al. 1997). In the United Kingdom, they predominantly consume acorns that should originally be stored by *S. vulgaris* (Wauters et al. 2002). Since *S. vulgaris* is endangered in the UK, forest management towards conifer-dominated forests that encourage *S. vulgaris* and prevent alien *S. carolinensis* is considered an effective conservation strategy (Lurz et al. 1995; Gurnell et al. 2002; Andrew et al. 2020). Similarly, *C. erythraeus* and *S. vulgaris* are resistant to tannins, and some common *Quercus* acorns currently distributed in Japan can be used and are more likely to spread in Japan. Early control measures of alien squirrels and forest management based on the knowledge of habitat requirements are essential for conservation of the endemic *S. lis*.

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