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# Stable and radio-isotopic signatures reveal how the feeding habits of ants respond to natural secondary succession in a cool-temperate forest

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## Abstract

Ants are known to have various feeding habits and play important roles in many terrestrial ecosystems. However, little is known about how the feeding habits of each ant species are affected by the environmental changes associated with forest succession. In this study, we examined how feeding habits as well as the species composition and food preference of ants would respond to 105-year-old natural secondary succession of a cool-temperate forest. Stable carbon and nitrogen isotopes and radiocarbon were used to infer the feeding habits. The species richness was not affected by forest stand age. Redundancy analysis indicated that stand age, litter N concentration, and soil microbial respiration had significant relationships with the ant species composition. The bait experiments showed that honey bait became more attractive to ants relative to tuna bait in late succession. This was probably associated with changes in available food resources, such as nectar, during succession. Although the isotopic signatures differed significantly among the sample types (i.e., ant species, canopy leaves, and leaf litter), they were not significantly affected by forest stand age or by the interaction between sample types and stand age. This indicates that the ants showed consistent differences in feeding habits among the species during succession. Our results suggest that despite great changes in vegetation, each ant species would maintain functional roles in C and N flows during forest succession.

**Keywords** Ants · Natural secondary succession · Stable C and N isotopes · Radiocarbon · Diet ages

## Introduction

Knowledge on the feeding habits of animals is essential to understand not only their natural history, but also their functions in energy and nutrient flow through ecosystems. Ants (Hymenoptera: Formicidae) have diverse feeding habits, such as plant mutualists, nectar-feeders, and predators, and play important roles in many terrestrial ecosystems (Hölldobler and Wilson 1990; Folgarait 1998). Most ant species

need plant-based diets (e.g., extrafloral nectar and honeydew) as an energy source and animal-based diets as a protein source for the larvae and reproductive females; however, the relative importance of the diet sources differs among the ant taxa (Blüthgen et al. 2003; Davidson et al. 2003; Hyodo et al. 2011). Earlier studies have shown that ants adjust their diet preferences according to resource availability to maintain the nutritional balance at colony level (Kay et al. 2006; Wilder and Eubanks 2010; Pohl et al. 2016).

Recent forest development in temperate regions is expected to enhance biodiversity and ecological functions driven by a variety of organisms (Dunn 2004; Spake et al. 2015). Forest development often affects the ant species composition, but not their species richness (Maeto and Sato 2004; Ottonetti et al. 2006; Schmidt et al. 2013; Gallé et al. 2016). Forest succession can also affect the diet preferences of the ant community. A bait experiment showed that, in a tropical forest succession, animal-based diets (tuna) attracted more ants in young forest stands, whereas plant-based diets (honey) became more attractive to ants in old forest stands

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(Bihn et al. 2008). The preference shift of the ant community was considered to reflect that the ants foraged to compensate for nutritional deficiency: animal-based diets are less abundant and plant-based diets are more abundant in younger forest stands (Bihn et al. 2008). If ants are capable of maintaining the nutritional balance by compensatory feeding, the ant species should retain its own feeding habits during forest succession. However, little is known about whether the feeding habits of ants change in the face of environmental changes associated with temperate forest succession.

In the last two decades, stable carbon and nitrogen isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) have revealed the feeding habits of diverse consumer organisms and food web structures (Tayasu 1998; Feldhaar et al. 2010; Hyodo 2015). This approach is based on the finding that $\delta^{13}C$ values of animals are similar to those of the diets, whereas their $\delta^{15}N$ values increase by $3.4 \pm 1.1‰$ when compared with those of the diets (DeNiro and Epstein 1978; Minagawa and Wada 1984). This method has shown that most of tropical arboreal ants depend largely on plant-based diets and not on animal prey as thought previously (Blüthgen et al. 2003; Davidson et al. 2003). It has also been used to examine how the feeding habits of ants respond to different types of managements (pasture, revegetated pasture, and woodland) in south-eastern Australia (Gibb and Cunningham 2010) or logging in tropical forests (Woodcock et al. 2013). These studies have shown that the feeding habits of most ant species appear to be not affected markedly by these vegetation changes.

Besides stable isotopes, radiocarbon ($^{14}C$) has recently been used to estimate an aspect of feeding habit, diet age, which is the lag time between primary production and its use by animals (Hyodo et al. 2006; Hobbie et al. 2017). This approach is based on the fact that the $^{14}C$ content of atmospheric $CO_2$ reached a peak in 1963 because of nuclear bomb testing and gradually declined since then, and this $^{14}C$ trend is reflected in photosynthetic products (Beavan and Sparks 1998). The diet ages allow us to evaluate the feeding habits of terrestrial consumers in terms of the timeframe of C flow in ecosystems (Hyodo et al. 2012; Haraguchi et al. 2013). For example, the diet ages of tropical ants correspond well to their known feeding habits (e.g., 0 year for nectar- or honeydew-feeding species, such as *Tetraponera attenuata*, and 6 years for predacious species, such as *Leptogenys diminuta*) (Hyodo et al. 2015). Nonetheless, these isotopic methods have not been used to investigate the feeding habits of ants in temperate forest succession.

In this study, we used stable isotopes and radiocarbon to examine the feeding habits of six ant species that persisted during a 105-year-old natural secondary succession in a cool-temperate forest. We also explored how ant species composition is influenced by forest stand age, nitrogen availability, and soil microbial biomass and performed a bait experiment using honey and tuna baits to confirm that

the forest succession affected the environment and resource availability for ants found previously in other study systems (Andersen and Sparling 1997; Bihn et al. 2008). We hypothesized that, in contrast to the species composition and bait preference, the feeding habits evaluated using the isotopic signatures of each ant species would not be influenced significantly by the forest stand age. By measuring the isotopic signatures of ants during succession, we aimed to better understand how changing environments influence the functions of ants in temperate forests.

## Materials and methods

### Study sites

This study was conducted in a cool-temperate deciduous forest in Ogawa Forest Reserve and the surrounding secondary forests in northern Ibaraki, Japan (36°56′N, 140°35′E, 580–800 m a.s.l.). The mean annual temperature is 12.3 °C and mean annual precipitation is about 1,750 mm (Shibata et al. 2014). The study sites were under natural secondary succession created by clear-cutting for pulp production. The chronosequence consisted of six study sites that had stand ages of 1, 7, 11, 24, 51, and 105 years. The above- and below-ground properties of the study sites have already been characterized (Hyodo et al. 2016a) and are listed in Table 1. The locations of the forest stands were reported in a previous study (Haraguchi and Tayasu 2016), and the area of each forest stand was larger than 4 ha. At each stand, a 30 × 30-m plot, at least 20 m away from the stand edge, was established and the following experiment and samplings were performed.

### Ant sampling for species compositions and isotopic measurements

We sampled the ants to examine the species compositions and the isotopic signatures at the six forest stands in July and August 2011. We performed hand sampling of ants at both ground and arboreal levels to characterize the ant community at each stand. The sampling was conducted by a single person who searched for foraging ants during a time span of 90 min on the ground in the daytime (9 a.m. to 4 p.m.). Ants found on the litter and understory vegetation were caught using trays and forceps or aspirators and then placed in plastic bags and frozen within a few hours of collection. In addition, ants foraging on tree trunks and canopy layers of five-to-seven trees were observed for 90 min using the single-rope technique. This sampling allowed us to investigate approximately 70–90% of the canopy surface and all the tree trunks of the target trees. At the same time, we recorded whether there were ants feeding

**Table 1** Ant species richness and above- and below-ground properties in each forest stand

| Stand age (year) | Ant species richness[a] | Total basal area ($m^2\ ha^{-1}$) | Litter fall (t $ha^{-1}$) | Litter N concentration (%) | Soil pH | SIR[b] ($\mu g$ $CO_2$–C $h^{-1}$ soil $g^{-1}$) |
|---|---|---|---|---|---|---|
| 1 | 8 (3) | 3.5 | 2.66 | 1.08 | 5.59 | 6.0 |
| 7 | 5 (3) | 18.5 | 2.20 | 0.90 | 4.72 | 12.1 |
| 11 | 5 (1) | 12.7 | 3.89 | 1.31 | 4.87 | 11.8 |
| 24 | 6 (0) | 16.4 | 3.93 | 1.61 | 4.16 | 10.1 |
| 51 | 7 (0) | 35.9 | 5.70 | 1.14 | 4.2 | 20.5 |
| 105 | 10 (0) | 44.6 | 5.32 | 0.92 | 4.9 | 10.8 |

The above- and below-ground properties in these forest stands were obtained in the previous study (Hyodo et al. 2016a)

[a]The numbers in the parenthesis indicate species richness of ants observed to feed on extrafloral nectaries or honeydews in canopy layers

[b]SIR stands for substrate-induced respiration

on extrafloral nectaries or honeydew in the canopy layers. We regarded the ants with a swollen gaster as those feeding on carbohydrate sources, even when we were unable to observe their feeding behaviour directly. The samplings of both ground and canopy layers were performed twice on different days [i.e., 90 min × two times × two habitats (ground and canopy) = 360 min per site]. The observed ant species and the rarefaction curves for each forest stand are shown in Appendices S1 and S2, respectively. We did not sample more than 15 individuals of the same species of ants at each sampling occasion, except for *Pheidole fervida* (Fabricius) (maximum, 26 individuals), to not spend too much time for one sampling, and changed the sampling location at each stand. We assumed the number of sampling occasions for each species was proportional to their abundance in the plot and then used it to examine the relationship between the ant species composition and environmental variables. During the canopy sampling, several canopy leaves were sampled as representatives of the current-year products from three to five trees and placed in paper bags.

For isotopic measurements, the frozen ants and canopy leaves were dried in an oven at 60 °C for 24 h. We also used the ants collected similarly from the ground in the same sites in June 2009. We assumed that the 2-year difference would not affect the overall trend of isotopic signatures across the 105-year-old chronosequence. The dried ants were used for identification and isotopic measurements as detailed below. The identification of ant species was based on a guide and a key in Ants of Japan (Japanese Ant Database Group 2003). The voucher specimens are deposited at Research Core for Interdisciplinary Sciences, Okayama University, Okayama, Japan.

## A bait experiment

In 2011, we performed a bait experiment to examine whether the diet preference would shift during the temperate forest succession, as observed in tropical forest succession (Bihn et al. 2008). The bait experiment was performed according to Bihn et al. (2008). A 30-m transect was randomly set in each study plot, and 10 bait stations were installed at 3-m intervals. Each bait station consisted of two white acrylic plates (10 cm × 10 cm). Two types of baits (3 g of tuna or a cotton soaked with 20% honey solution) were placed on the plates that were 20-cm apart from each other. In total, 120 acrylic plates were set in the field sites. The number of ants foraging on the plate was counted at 60 min after bait placement. A few individuals of the ants were preserved in ethanol for identification at the end of the investigation.

## Stable and radio-isotopic measurements

For stable carbon and nitrogen analysis, we used six ant species collected from the ground in at least four sites during the survey in 2009 and 2011. The ant species were *Aphaenogaster japonica* Forel, *Camponotus obscuripes* Mayr, *Formica hayashi* Terayama & Hashimoto, *Lasius japonicus* Santschi, *Paratrechina flavipes* (F. Smith), and *Pheidole fervida* (Fabricius). A few individuals of each species, which were found at the same sampling location in each plot, were ground using a mortar and pestle. Although gut content (or gaster) could have lower $\delta^{15}N$ values than the rest of body tissues (Blüthgen et al. 2003), we did not remove it, because gut contents are considered to contribute little to the N content of worker ants (Davidson 2005). We treated the ants found at different locations more than 5-m apart as replicates in each forest stand and thus separately measured the isotopic signatures. The number of replicates of each ant species (*n* = 1–9) in each forest stand is listed in Appendix S3. The canopy leaf samples collected in 2011 were ground using a ball mill. In this study, we also used litter samples, which were collected from 10 subplots (20 × 20 cm) at each site just after litter fall in November 2009 (Hyodo et al.

2016a), to correct variations in the baseline $^{15}$N of food chains.

The powdered samples were placed in tin capsules. The C and N isotope ratios of these samples were measured using an isotope mass ratio spectrometer (Delta V Advantage, Thermo Fisher Scientific Inc., Waltham, MA, USA) connected to an elemental analyser. The natural abundances of $^{13}$C or $^{15}$N were expressed in per mil (‰) deviation from the international standards using the following equation: $\delta^{13}$C or $\delta^{15}$N $= (R_{sample}/R_{standard} - 1)$, where $R_{sample}$ and $R_{standard}$ are the isotopic ratios ($^{13}$C/$^{12}$C or $^{15}$N/$^{14}$N) of the sample and standard, respectively. Vienna Pee Dee belemnite and atmospheric nitrogen were used as the international standards for carbon and nitrogen, respectively. The standard deviation of the working standards (DL-Alanine) measured for every six samples was better than $\pm 0.2$‰ for both isotope ratios.

For the radiocarbon analysis, we used three ant species (*A. japonica*, *C. obscuripes*, and *F. hayashi*) that were collected from at least three stands during our survey in 2009 and 2011 and had enough amount of body tissues. Several individuals of *A. japonica* and *F. hayashi* were pooled to obtain the amount (1 mg C) required for the radiocarbon measurements. The gaster of the ants was removed before the weighing, because the gut contents, especially carbohydrates, could affect the radiocarbon contents. For most of the ant samples, two replicates were used (Appendix S4). Three replicates were used for the canopy leaf samples that were collected, as mentioned above. The samples were combusted in evacuated and sealed quartz tubes with CuO and Ag wire at 850 °C for 2 h. After cooling, the quartz tubes were cracked on a vacuum line, and $CO_2$ was cryogenically purified. The purified $CO_2$ was graphitized under Fe catalysis at 650 °C for 4.5 h (Kitagawa et al. 1993). The graphite samples were sent to Rafter Radiocarbon Laboratory, Institute of Geology and Nuclear Sciences, New Zealand, for accelerator mass spectrometry measurements of the radiocarbon. Radiocarbon values were reported as $\Delta^{14}$C (‰), which is the part per 1000 deviation from the activity of nineteenth century wood, and were corrected for fractionation using stable C isotope ratios of the samples (Stuiver and Polach 1977). The average analytical error during the sample measurements was $\pm 3.7$‰.

We estimated the diet age by calculating the difference between the sample collection year and the year ($t$) when the $\Delta^{14}$C value of a sample matched that of atmospheric $CO_2$. In this study, the year ($t$) was calculated using the regression curve, year ($t$) $= 2060.5 - 13.43 \ln(\Delta^{14}C)$ ($R^2 = 0.997$, $P < 0.0001$), which is based on $^{14}CO_2$ data for the northern hemisphere from 2000 to 2009 (Hua et al. 2013). The analytical error and yearly change in $\Delta^{14}$C value of atmospheric $CO_2$ (ca. 4.7‰.) indicated that the diet age could be estimated with a precision of 1–2 years. The time elapsed, since the production of canopy leaves was also estimated as

diet age for comparison. The average $\Delta^{14}$C values (27.9‰) of the canopy leaves across the forest stands ($n = 18$) were lower than those (39.9‰) of atmospheric $CO_2$ expected by the regression curve. Earlier studies have shown that local variation in atmospheric $^{14}CO_2$ is likely due to the effect of $CO_2$ derived from the burning of fossil fuel, of which the $\Delta^{14}$C value is $-1000$‰ (Takahashi et al. 2002; Pataki et al. 2010). Assuming that the depletion in $^{14}$C was constant from 2000 to 2011, we have added the difference (12.0‰) to the $\Delta^{14}$C values of ants and canopy leaves. This correction produced the 0-year-old diet age of the canopy leaves of the deciduous trees.

## Statistical analysis

We tested the relationship between the ant species richness and the forest stand age using single regression analysis. In addition, we used redundancy analysis (RDA) to explore the effect of a set of environmental variables on the ant species composition (i.e., the number of sampling occasions for each species) (ter Braak 1994; Borcard et al. 2011). For this analysis, the ant species composition data were Hellinger-transformed to not give high weights to the rare species (Legendre and Gallagher 2001). The environmental variables used in this study were forest stand age (year), substrate-induced soil microbial respiration (SIR), and litter N concentration (litterN), which were examined in an earlier study (Table 1). A permutation test of all the combinations (719) was conducted to test the significance of the set of environmental variables under a reduced model.

We examined the effects of forest stand age on the bait preference of the ants using a generalized linear mixed model (GLMM) with a binomial distribution and a logit link function. The bait preference was evaluated as the honey preference ratio, which was the ratio of abundance of all the ant species in the honey bait to the total abundance of all the ant species in the honey and tuna baits at each bait station. In the GLMM, the honey preference ratio was the response variable, the effect of forest stand age was the explanatory variable, and each forest stand and each bait station were treated as random effects. We also used the GLMMs to evaluate the preference ratio of each of the three ant species (*A. japonica*, *Ph. fervida*, and *Pa. flavipes*), which were frequently observed in the bait stations.

We tested whether forest stand age and sample types (ant species, litter, or canopy leaves) would affect the $\delta^{13}$C and $\delta^{15}$N values and diet ages of the ants using GLMM. Because $\delta^{15}$N of consumer organisms could be affected by variation in $\delta^{15}$N of plants across study sites, we corrected $\delta^{15}$N of ants ($\delta^{15}N_{corrected}$) using fresh litter collected in November 2009 as follows: $\delta^{15}N_{corrected}$ of ants $= \delta^{15}$N of ants $- \delta^{15}$N of litter for each stand. In the GLMM, $\delta^{13}$C, $\delta^{15}$N, $\delta^{15}N_{corrected}$, or diet ages were treated as the response variables; forest

stand age, sample types, and the interaction term (forest stand age × sample type) were the explanatory variables; and each forest stand was a random effect. For the response variable $\delta^{15}N_{corrected}$, the sample type consisted of ant species or canopy leaves. Tukey–Kramer post-hoc tests were performed to investigate the significant difference among the sample types. R (ver.3.3.3, R core team) and package vegan (Oksanen et al. 2017) were used for RDA and GLMM for the bait preference test, and a statistical software JMP (ver.9.02 for Mac, SAS Institute, CA), for the rest of the statistical analyses.

## Results

The number of ant species observed in our study sites ranged from five to ten species with the highest in the oldest stand (Table 1 and Appendices S1 and S2); however, the relationship between ant species richness and forest stand age was not significant ($r^2 = 0.538$, $P = 0.097$). *Crematogaster teranishii* was the species found only in the canopy layers (Appendix S1). During our sampling of the canopy layers, three ant species (*F. hayashi, C. obscuripes*, and *Camponotus kiusiuensis* Santschi) in the 1-year-old stand, three ant species (*F. hayashi, C. obscuripes*, and *Pa. flavipes)* in the

7-year-old stand, and one ant species (*F. hayashi*) in the 11-year-old stand were observed to have a swollen gaster, indicating their feeding on carbohydrate sources (e.g., nectar and honeydew; Table 1). RDA showed that the first two axes explained 68% of the variation in species composition (43% by Axis 1 and 26% by Axis 2). The RDA results show that the ant species composition was significantly affected by the set of the environmental variables ($F = 2.9$, $P = 0.038$; Fig. 1) and that each environmental variable, the forest stand age ($F = 3.9$, $P = 0.022$), litterN ($F = 3.0$, $P = 0.04$), and SIR ($F = 2.9$, $P = 0.05$), had significant influences on the ant species composition.

Six ant species (*A. japonica, C. obscuripes, F. hayashi, L. japonicus, Pa. flavipes*, and *Ph. fervida*) were observed on the bait stations (Appendix S5). The forest stand age had significant effects on the honey preference ratio of all the ant species ($\chi^2 = 20.4$, $P < 0.0001$) (Fig. 2). This indicates that honey baits became more attractive relative to tuna baits as forest succession proceeded. When the honey preference ratio was considered for each species, there were no significant effects of the forest stand age on the honey preference ratios of *A. japonica* ($\chi^2 = 0.105$, $P = 0.746$), *Pa. flavipes* ($\chi^2 = 1.46$, $P = 0.226$), or *Ph. fervida* ($\chi^2 = 2.10$, $P = 0.148$).

The $\delta^{13}C$ and $\delta^{15}N$ values and diet ages of the ants and plant materials did not show significant responses to forest



**Fig. 1** Bi-plots of the ant community obtained from the redundancy analysis. The bottom and left scales are the site scores for the forest stands and species scores for the ant species, and the top and right scales are the bi-plot scores for the environmental variables (i.e., forest stand age, year; litter N concentration, litterN; and substrate-induced soil microbial respiration, SIR). The blue and red arrows indicate the directions of increases for the environmental variable and ant abundance, respectively. The angles between the arrows reflect their correlations. Projecting a forest stand at right angle to the direction of an arrow for an environmental variable or ant abundance approximates the value of the stand along that variable
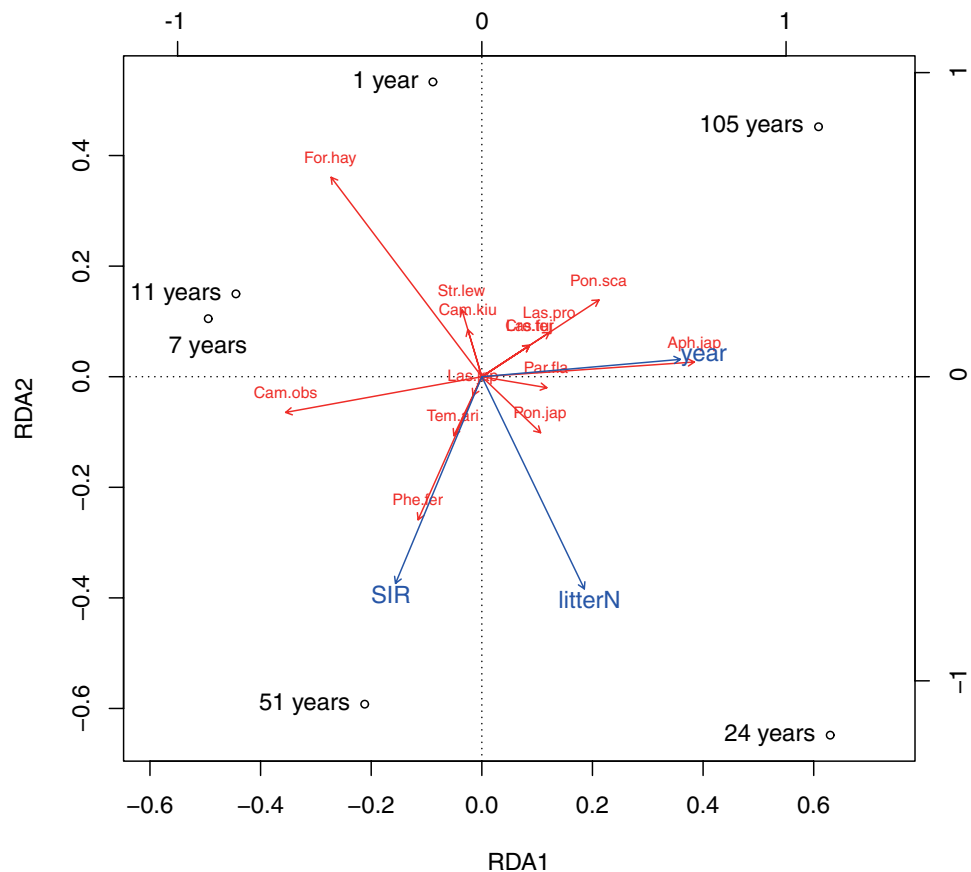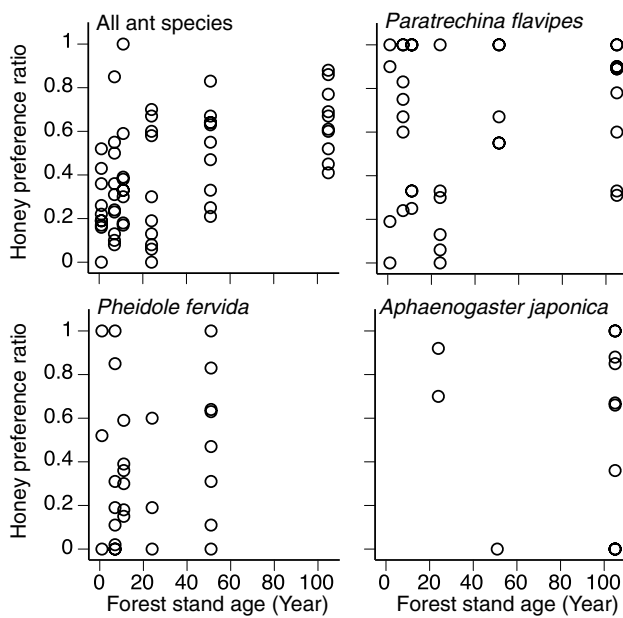
**Fig. 2** Changes in the honey preference ratios of the ant community at each bait station during forest succession. The honey preference ratios were calculated as the ratio of ant abundance in honey bait to the total abundance in honey and tuna baits at each bait station. The ratios of all the ant species, *Ph. fervida*, *Pa. flavipes*, and *A. japonica* are presented

**Table 2** Results of GLMM for the effects of forest stand age, sample type, and the interaction term (forest stand age × sample type) on $\delta^{13}C$, $\delta^{15}N$, $\delta^{15}N_{corrected}$, and diet ages

| Response variables | Explanatory variable and covariate | df | F | P |
|---|---|---|---|---|
| $\delta^{15}N$ | Stand age | 1 | 0.004 | 0.952 |
| | Sample type | 7 | **272.5** | **< 0.0001** |
| | Interaction | 7 | 0.546 | 0.798 |
| $\delta^{15}N_{corrected}$ | Stand age | 1 | 0.509 | 0.515 |
| | Sample type | 5 | **84.25** | **< 0.0001** |
| | Interaction | 5 | 0.891 | 0.489 |
| $\delta^{13}C$ | Stand age | 1 | 0.008 | 0.933 |
| | Sample type | 7 | **82.62** | **< 0.0001** |
| | Interaction | 7 | 0.615 | 0.743 |
| Diet age | Stand age | 1 | 1.088 | 0.533 |
| | Sample type | 3 | **52.46** | **< 0.0001** |
| | Interaction | 3 | 0.762 | 0.523 |

Significant effects at $P = 0.05$ are indicated in bold

stand age, but significantly differed among the sample types (ant species, canopy leaves, or litter; Table 2; Figs. 3, 4). Because significant interaction (forest stand age × sample type) with $\delta^{13}C$, $\delta^{15}N$, and diet ages was not found, this result shows that the difference in $\delta^{13}C$, $\delta^{15}N$, and diet ages among the sample types was consistent during the succession. *A. japonica* and *C. obscuripes* were most enriched and

depleted in $^{15}N$ among the ant species, respectively. The canopy leaves and litter had the lowest $\delta^{15}N$ values among the samples. The correction of ant $\delta^{15}N$ by litter ($\delta^{15}N_{corrected}$) at each stand did not affect the statistical results (Table 2). The $\delta^{13}C$ values of *C. obscuripes* and *F. hayashi* were significantly higher than those of *Pa. flavipes*, and the rest of the ant species showed intermediate values. The canopy leaves and litter were most depleted in $^{13}C$ among the sample types. The diet ages of *A. japonica* and *F. hayashi* (least square means, 5.7 years and 4.3 years, respectively) were significantly older than that of *C. obscuripes* (1.7 years), and canopy leaves had the youngest diet age (0 year) among the samples (Fig. 4).

## Discussion

As we hypothesized, the present results demonstrated that, in contrast to ant species composition and diet preference, the isotopic signatures of ants were not significantly influenced by the forest stand age. In the studied region, Makino et al. (2006) had already shown that ant species richness, which was estimated using litter extraction and pitfall traps, varied from six to ten species and exhibited no significant relationship with forest stand age. In other regions, ant species richness also did not respond to the forest successional stages, whereas the compositions did (Maeto and Sato 2004; Ottonetti et al. 2006; Gallé et al. 2016). The RDA results showed that the forest stand age in our study sites had significant effects on the ant species composition. As shown in the bi-plot of RDA, *A. japonicus* tended to be more abundant in the old stands, whereas *C. obscuripes* was less abundant in the young stands. The RDA also indicated that belowground properties such as soil microbial biomass and N availability were important factors that influenced the ant species composition (Andersen and Sparling 1997; Mezger and Pfeiffer 2011): *F. hayashi* and *Pa. flavipes* tended to be more abundant in forest stands with low N availability and low soil microbial biomass, respectively. The effect of the forest stand age on the ant species composition might be partly explained by changes associated with the availability of food resources during the succession as discussed below.

As observed in tropical forest successions, the bait preference shift occurred during the temperate forest succession: the ant community preferred honey bait over tuna bait to a greater extent in late succession. The greater preference for honey baits of the ant community was not caused by the dominance of nectar-feeding ant species in the old forest stands. For example, *A. japonica*, which has a predatory feeding habits as demonstrated by the high $\delta^{15}N$ values, became abundant in the 105-year-old stand (Appendix S5). Although the preference shift of each ant species may have contributed to the observed preference shift of all the
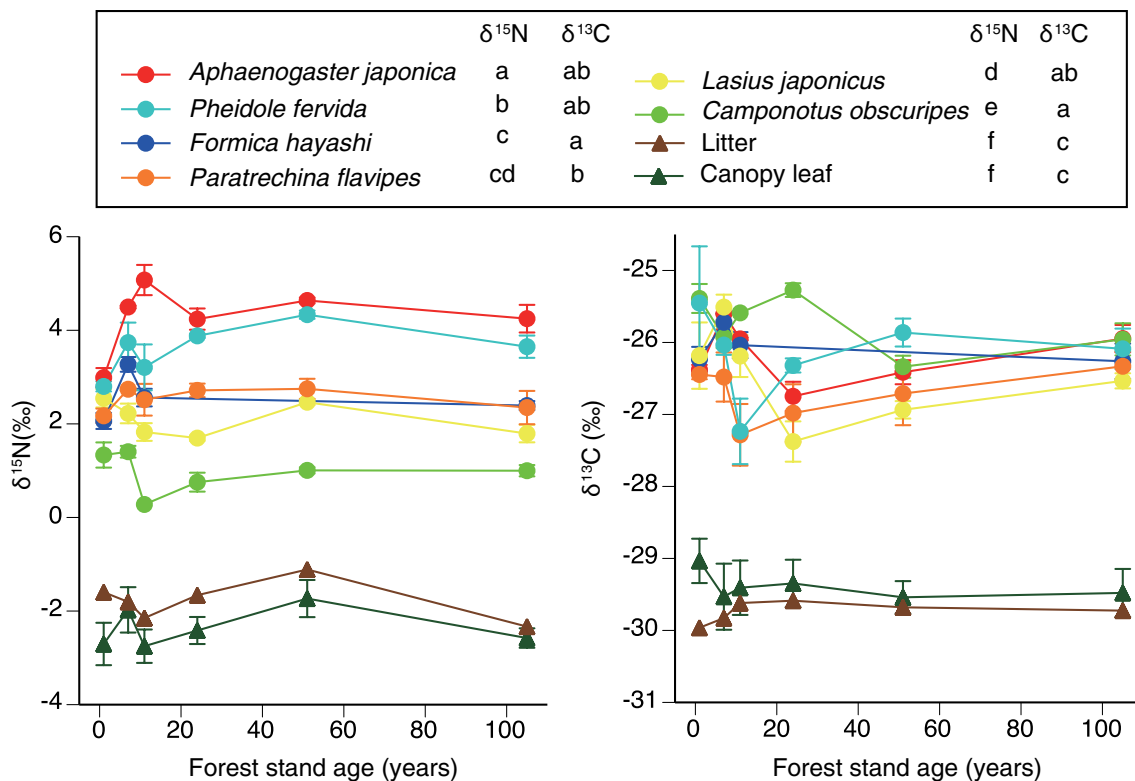
**Fig. 3** $\delta^{13}C$ and $\delta^{15}N$ values (mean ± SE) of six ant species, canopy leaves, and leaf litter in natural secondary succession of a cool-temperate forest. The post-hoc results are shown next to the symbols for each sample type. The values with the same letters indicate that they do not differ significantly across the sample types at $P = 0.05$

ant species, this was not statistically supported. Bihn et al. (2008) considered that the preference shift in tropical forest succession was due to changes in the relative availability of food resources rich in carbohydrates (e.g., extrafloral nectar) and proteins (e.g., litter arthropods): plants with extrafloral nectar dominated in early succession and litter accumulated in late succession. The explanation about the changes in vegetation could be applied to our sites, because tree species with extrafloral nectaries mostly occurred in the young sites [e.g., *Viburnum wrightii* Miq. and *Cerasus maximowiczii* (Rupr.) Kom.], whereas two *Quercus* species (*Q. crispula* Blume and *Q. serrata* Murray) that are not known to produce extrafloral nectar (Weber et al. 2015) became dominant in the 51- and 105-year-old stands (Hyodo et al. 2016a). Indeed, ants that use nectar or honeydew on tree trunks were not observed during our survey in forest stands older than 24 years. Meanwhile, greater mass accumulation of forest floor litter occurred in old succession in our study sites (Table 1). However, it seems unlikely that the increased amount of litter in the old stands directly enhanced prey availability for these ants, because the ants had diet ages (2–6 years) older than the litter (generally 0–2 years). In addition, the previous studies have shown that despite the increase in litter amount, the abundance of collembolans,

soil microbial biomass, and soil organic matter did not increase along the succession in the study area (Hasegawa et al. 2006; Hyodo et al. 2016a).

Despite changes in species compositions and diet preferences, the $\delta^{13}C$ and $\delta^{15}N$ values and diet ages of each ant species did not show significant responses to the forest stand age, but showed a consistently significant difference among the taxa across different aged successional stands. The differences in $\delta^{13}C$ and $\delta^{15}N$ values of the ant taxa seemed to be in agreement with those of congeners previously reported (Tillberg et al. 2007; Caut et al. 2014). Hyodo et al. (2015) showed that predatory ant species with high $\delta^{15}N$ values tends to exhibit old diet ages (ca. 6 years), while nectar-feeding ants with low $\delta^{15}N$ values tend to have young diet ages (0–2 years). The diet ages of the ants examined in this study also showed a similar pattern. *A. japonica*, which had the highest $\delta^{15}N$ values among the ants examined, exhibited the oldest diet ages. This implies that the predatory ant was largely dependent on detritivores, even though they were attracted to the honey bait as well as the tuna bait. In accordance with this, detritivorous dipterans had relatively high $\delta^{15}N$ values (4‰) as well as old diet ages (ca. 6 years) in the present study sites (Haraguchi et al. 2013), and dipterans are known to be an important animal-based diet for the
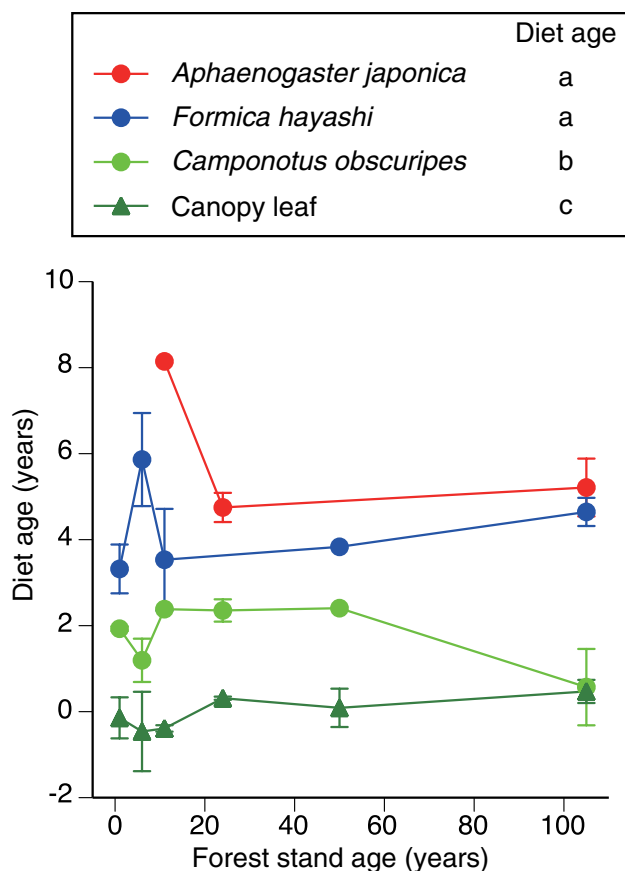
**Fig. 4** Diet ages (year; mean ± SE) of three ant species and canopy leaves in natural secondary succession of a cool-temperate forest. The post-hoc results are shown next to the symbol for each sample type. The values with the same letters indicate that they do not differ significantly across the sample types at $P = 0.05$

congener species *Aphaenogaster senilis* (Caut et al. 2013). The range of diet ages of the temperate forest ants examined (0–8 years) was similar to that of tropical rain forest ants, in spite of large differences in litter and labile soil C decomposition rates between the two climate regions (Fissore et al. 2009; Prescott 2010). This result supports the finding of a previous study that the time frame of C flows in many terrestrial food webs would mostly be within 10 years (Hyodo et al. 2012).

The rather constant isotopic signatures of ants across different vegetation or landscapes have already been reported in some earlier studies. For example, Gibb and Cunningham (2010) compared the $\delta^{15}N$ values of several ant species among pastures, regeneration sites, and remnant woodlands in south-eastern Australia. They found that the $\delta^{15}N$ values of ants differed consistently among taxa across the study sites. Caut et al. (2014) found that fire largely changed vegetation and arthropod community composition, but the $\delta^{13}C$ and $\delta^{15}N$ values of ant species were not altered. Furthermore, although ant species

richness in tropical secondary forests was positively influenced by the proportions of surrounding remnant primary forests, the surrounding environment did not significantly affect the diet ages as well as $\delta^{13}C$ and $\delta^{15}N$ values of the ant species (Hyodo et al. 2016b). The $\delta^{15}N_{corrected}$ values of some tropical ant species differ significantly between logged and unlogged forests (Woodcock et al. 2013) and among different forest types (Pfeiffer et al. 2014). However, the mean differences were generally small (1‰ or less) and no significant difference in $\delta^{15}N_{corrected}$ values was observed across the study sites for 10 out of 14 species in the former study and for four out of nine species in the latter study. These isotopic results suggest that the nutrient balance between plant- and animal-based diets is a conservative trait for each ant species.

Note that the relatively constant isotopic signatures of each ant species across environmental changes contrasts with the previous findings on three invasive ant species that showed dietary shifts in the introduced ranges. The Argentine ant (*Linepithema humile*) and fire ant (*Solenopsis invicta*) are known to shift their diet towards plant-based diets (extrafloral nectary or honeydew) in the introduced ranges, and the diet shift is actually reflected in the $\delta^{15}N$ values of the ants (Tillberg et al. 2007; Wilder et al. 2011). A termite-hunter needle ant (*Brachyponera chinensis*) exhibited younger diet age in the introduced ranges, probably by feeding more on herbivores and litter-dwelling invertebrates than on wood-feeding termites that have old diet ages (25–30 years) (Suehiro et al. 2017). It may be necessary to explore the capability of the dietary shift of more ant species in various habitats to elucidate whether the dietary shift is a unique trait of invasive ant species.

In conclusion, our results demonstrated that secondary natural forest succession affects the species composition and diet preferences of the ant community, whereas the feeding habits of each species are relatively constant likely due to compensatory feeding. Ants have been used as bioindicators of their habitats, because they respond rapidly to different land uses and disturbances (Andersen et al. 2002; Underwood and Fisher 2006). Our results suggest that their rapid responses to environmental changes could be associated with changes in the relative availability of plant- and animal-based diets in altered environments. Furthermore, we propose that, in addition to the species richness and composition, the bait experiment using ants has a potential to be an indicator of the relative availability of plant- and animal-based diets in temperate and tropical forests. Further studies would be required to confirm whether the patterns in this study could be observed in other forests and ecosystems. Nevertheless, our results suggest that each ant species would maintain the functional roles in energy and nutrient flow in changing environments, and therefore, the loss or gain of ant species could affect the ecosystem function.

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