

Research



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Radiocarbon signature reveals that most springtails depend on carbon from living plants

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Terrestrial carbon cycling is largely mediated by soil food webs. Identifying the carbon source for soil animals has been desired to distinguish their roles in carbon cycling, but it is challenging for small invertebrates at low trophic levels because of methodological limitations. Here, we combined radiocarbon (¹⁴C) analysis with stable isotope analyses (¹³C and ¹⁵N) to understand feeding habits of soil microarthropods, especially focusing on springtail (Collembola). Most Collembola species exhibited lower $\Delta^{14}\text{C}$ values than litter regardless of their $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures, indicating their dependence on young carbon. In contrast with general patterns across all taxonomic groups, we found a significant negative correlation between $\delta^{15}\text{N}$ and $\Delta^{14}\text{C}$ values among the edaphic Collembola. This means that the species with higher $\delta^{15}\text{N}$ values depend on C from more recent photosynthate, which suggests that soil-dwelling species generally feed on mycorrhizae to obtain root-derived C. Many predatory taxa exhibited higher $\Delta^{14}\text{C}$ values than Collembola but lower than litter, indicating non-negligible effects of collembolan feeding habits on the soil food web. Our study demonstrated the usefulness of radiocarbon analysis, which can untangle the confounding factors that change collembolan $\delta^{15}\text{N}$ values, clarify animal feeding habits and define the roles of organisms in soil food webs.

1. Introduction

In terrestrial ecosystems, soil provides key ecosystem functions and services related to litter decomposition [1]. Decomposition is mainly driven by soil microorganisms and invertebrate animals via their transfers of energy and materials through the food web [2,3]. Thus, how to identify the food sources of these organisms has been a central theme for soil ecologists to understand their roles in the ecosystem. Despite difficulties inherent in the complexity of the soil matrix and limitations of conventional methods (e.g. gut content analysis and laboratory food choice experiments), stable isotope signatures have shown significant progress as a powerful tool [4,5]. The use of stable isotopes has revealed hidden trophic structures and trophic links in the soil. These studies provided evidence that food sources vary widely among organisms and include living plants and photoautotrophic microorganisms (e.g. cyanobacteria and algae) as well as decayed materials and related saprotrophic microorganisms [6–8]. Especially, studies using isotope tracers have revealed the underestimated importance of carbon inputs from living roots into the soil food web [9–12] and have raised questions over whether soil food webs are really based primarily on detritus.

Recently, an isotopic map that summarizes food sources and their effects on the bulk stable isotope values ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) of soil animals was proposed

[13]. This map covers most of the variation in soil animal food sources and enables us to estimate the primary food source of each animal taxon based on natural variations in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values. However, the interpretation of isotope values partly remains ambiguous because multiple factors alter isotope values in the same directions [14–16]. ^{15}N enrichment is a particular problem, since we cannot accurately distinguish between mycorrhizal effects and isotope enrichment by microbial decay processes, even for non-predatory taxa [13]. Because feeding on mycorrhiza could be a highly significant pathway for carbon input from living roots [15,17], the dependence of soil organisms on mycorrhiza or microbially decayed materials can lead to quite different consequences for their ecosystem functioning. Furthermore, assessing the degree of dependence on living roots is difficult. Soil animals, including many omnivores, can use both living and dead materials [18–20]. Most studies that assessed carbon inputs from living roots have used isotope tracers [10–12], which usually cannot be done simultaneously with the measurement of natural variations of the same isotope (but see [9]). Thus, we still cannot specify animal feeding habits directly related to their ecosystem functioning only from the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ isotopic map.

To resolve that difficulty, we used radiocarbon (^{14}C) analysis to add information on the carbon age in animals into the biplot of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$. The radiocarbon technique provides *in situ* information about the age (i.e. the time elapsed after photosynthesis) of assimilated carbon, which can distinguish taxa dominated by carbon that is younger than litter from taxa that depend more on carbon from the litter and humus. This technique is based on the atmospheric $^{14}\text{CO}_2$ peak in the early 1960s (the ‘bomb peak’) that resulted from nuclear bomb testing and the 1963 Nuclear Test Ban treaty. The ^{14}C content of atmospheric CO_2 has continued to decline since this peak, and this decay curve is reflected in the ^{14}C content of photosynthate carbon. ^{14}C analysis has been used to estimate the feeding habits of some soil animals, such as termites [21–24], ants [23,25], earthworms [24,26,27] and enchytraeids [28]. We can estimate the diet ages from the ^{14}C contents in most soil animals, with exceptions for several wood-feeding termites, by using the decay curve since the peak in atmospheric $^{14}\text{CO}_2$ [22]. In this study, we conducted the first trial that has used ^{14}C to assess the feeding habits of soil microarthropods, especially focusing on Collembola. Given that Collembola are the primary prey of many soil animals at higher trophic positions, their carbon sources will clearly affect the whole soil food web [2,29]. We assessed animal feeding habits by revealing the relationships between the age of assimilated carbon and the stable isotope values for multiple collembolan species and other mesofaunal taxa. We then assessed the utility of the multi-dimensional isotopic map that includes ^{13}C , ^{15}N and ^{14}C signatures.

2. Material and methods

The research site was a warm temperate natural forest of Japanese cypress (*Chamaecyparis obtusa*), one of the major species for timber production in Japan, at the Kamigamo Experimental Forest Station of Kyoto University, Japan (35°04' N, 135°43' E) [30]. The vegetation consisted of a canopy layer of *C. obtusa* and an understory of shrubs, such as *Cleyera japonica*, *Eurya japonica* and *C. obtusa* saplings. The soil has a moder humus with an organic layer (A_0) 3 to 5 cm thick above a poorly developed A horizon

that was 1 to 2 cm thick and BC horizons. In the A_0 layer, densely distributed fine roots of *C. obtusa* formed a root mat. More than 30 papers have been published about the ecology of the soil microarthropod community, including its interactions with *C. obtusa* roots, at this research site (e.g. [31,32]).

We collected samples of the soil organic layer, including soil animals and *C. obtusa* fine roots, at monthly intervals from November 2018 to March 2019. Soil animals were extracted alive into deionized water using Tullgren funnels at 35°C. The extracted animals were stored in a fridge at 4°C and immediately identified and divided into taxonomic groups under a stereomicroscope to prevent decay. Most Collembola were grouped at the species level, whereas mites were grouped at the sub-order level and other animals were combined at the order or class level (table 1). These samples were stored frozen at –20°C and then freeze-dried for isotope analyses. Fine roots that were 1 mm in diameter or less and the first-order roots (i.e. root tips) were removed from the soil samples and ground into powder using an agate mortar. The soil organic layer was separated into the litter (L) layer and the fragmented litter and humus layer (i.e. the FH layer), and each layer (excluding roots) was ground into a powder using a ball mill. We obtained three samples of the dominant collembolan species (*Folsomia octoculata*, *Tetracanthella sylvatica* and *Tomocerus varius*): sample 1 in November and December 2018, sample 2 in January and February 2019, and sample 3 in March 2019. We combined the samples of the other taxa to obtain enough material (more than 2 mg) to permit the three isotopic analyses (i.e. $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\Delta^{14}\text{C}$). For the substrates (i.e. roots and litter), we took small random samples from each harvesting date and combined them, then dried them at 70°C for 48 h. Details of isotopic analyses are in the electronic supplementary material, S1. We used Pearson’s correlation coefficient (*r*) to analyse the relationships among pairs of the three isotopic signatures (i.e. $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\Delta^{14}\text{C}$).

3. Results

The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures of soil animal taxa spanned 6.1 and 9.6‰, respectively (figure 1a; electronic supplementary material, S2). The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures of the L layer were lower than those of most soil animals, except for the $\delta^{15}\text{N}$ of *Tomocerus ocreatus*. The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures of soil organic matter in the FH layer were higher than those in the L layer but lower than those of most FH layer inhabitants. The $\delta^{13}\text{C}$ signatures of the root substrates were higher than litter, whereas the $\delta^{15}\text{N}$ signatures were between those in the L and FH layers. The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures were slightly lower in the first-order roots than in the fine roots.

The $\Delta^{14}\text{C}$ signatures of the soil animal taxa spanned 64.5‰, with values ranging from –34.8‰ for Entomobryidae to 29.7‰ for Pseudoscorpiones (figure 1a,b; electronic supplementary material, S2). The $\Delta^{14}\text{C}$ signatures of the substrates showed a broad range from 13.0‰ for the first-order roots to 56.2‰ for soil organic matter in the FH layer. However, most Collembola (except for Neanuroidea) had a lower $\Delta^{14}\text{C}$ value than the substrates. Based on the simulation calculated by Graven *et al.* [35], the atmospheric $\Delta^{14}\text{C}$ value was declining and estimated to be about 5–10‰ at the global scale in 2019. Lower $\Delta^{14}\text{C}$ values in soil microarthropods than the globally estimated values indicate that $\Delta^{14}\text{CO}_2$ levels at our site were affected by local CO_2 emissions derived from fossil fuels. Local variation in atmospheric $^{14}\text{CO}_2$ has been widely reported and ascribed to local CO_2 emissions derived from fossil fuel ($\Delta^{14}\text{C} = -1000$ ‰), which decreases the $\Delta^{14}\text{C}$ values in the curve [36,37]. If we assume the lowest

Table 1. Soil animal taxa identified in this study. The vertical habitats of the collembolan species are based on studies by Takeda [33,34]. FH, fragmented litter and humus; L, litter.

| sample | ID | notes on family, order and class | habitat |
|---------------------------------|---------------------|---|--|
| Collembola | | | |
| <i>Folsomia octoculata</i> | <i>F_octoculata</i> | Isotomidae | FH layer |
| <i>Tetracanthella sylvatica</i> | <i>T_sylvatica</i> | Isotomidae | L layer, but shows vertical migration |
| <i>Isotoma carpenteri</i> | <i>I_carpenteri</i> | Isotomidae | FH layer |
| <i>Onychiurus flavescens</i> | <i>O_flavescens</i> | Onychiuridae | FH layer |
| <i>Onychiurus sibiricus</i> | <i>O_sibiricus</i> | Onychiuridae | FH layer, but shows vertical migration |
| <i>Oncopodura yosiiiana</i> | <i>O_yosiiiana</i> | Oncopoduridae | FH layer |
| <i>Tomocerus varius</i> | <i>T_varius</i> | Tomoceridae | L layer |
| <i>Tomocerus ocreatus</i> | <i>T_ocreatus</i> | Tomoceridae | L layer |
| Entomobryidae | Entomobryidae | Entomobryidae: mix of <i>Entomobrya</i> spp., <i>Lepidocyrtus</i> spp., <i>Homidia</i> spp. and <i>Sinella</i> spp. | L layer |
| Neanurinae | Neanurinae | Neanuridae: mix of <i>Neanura kitayamana</i> , <i>Vitronura pygmaea</i> and <i>Vitronura mandarina</i> | L layer |
| Neanuroidea | Neanuroidea | Neanuridae, Odontellidae: mix of <i>Friesia japonica</i> , <i>Pseudachorutes</i> spp. and <i>Superodontella</i> sp.1 | L layer |
| trophic position | | | |
| others | | | |
| Prostigmata | Prostigmata | Acari | predator |
| Mesostigmata | Mesostigmata | Acari | predator |
| Oribatida | Oribatida | Acari | decomposer |
| Araneae | Araneae | Araneae | predator |
| Geophilomorpha | Geophilomorpha | Chilopoda | predator |
| Lithobiomorpha | Lithobiomorpha | Chilopoda | predator |
| Diplopoda_adult | Diplopoda_ad | Diplopoda | decomposer |
| Diplopoda_juvenile | Diplopoda_ju | Diplopoda | decomposer |
| Pseudoscorpiones | Pseudoscorpiones | Pseudoscorpiones | predator |
| Symphyla | Symphyla | Symphyla | decomposer |

$\Delta^{14}\text{C}$ signature of -34.8% for Entomobryidae to be the current photosynthate C at our site in 2019, 17.2‰ of $\Delta^{14}\text{C}$ value in the tree fine roots is equivalent to the value of 12–13 year-aged C on the simulation curve [35]. The results are consistent with the findings that C in fine roots was aged 10 ± 1 years on average because fine roots are produced from stored non-structural carbohydrates [38].

For all collembolan samples combined, $\Delta^{14}\text{C}$ values were significantly positively correlated with $\delta^{13}\text{C}$ values, but not with $\delta^{15}\text{N}$ values ($\delta^{13}\text{C}$: $r = 0.55$, $p < 0.05$; $\delta^{15}\text{N}$: $r = -0.09$, $p = 0.72$, $n = 17$; figure 2a,b). We obtained similar results for the epigeic collembolan species (i.e. L-layer inhabitants), except for Neanurinae and Neanuroidea (which have blade-like mandibles); that is, $\Delta^{14}\text{C}$ values were significantly positively correlated with $\delta^{13}\text{C}$ values, but not with $\delta^{15}\text{N}$ values ($\delta^{13}\text{C}$: $r = 0.77$, $p < 0.05$; $\delta^{15}\text{N}$: $r = 0.33$, $p = 0.42$, $n = 8$; figure 2c,d). By contrast, among the edaphic collembolan species (i.e. FH-layer inhabitants), the positive correlation between $\Delta^{14}\text{C}$ and $\delta^{13}\text{C}$ values was only marginally significant, but the $\Delta^{14}\text{C}$ values were significantly negatively correlated with $\delta^{15}\text{N}$

($\delta^{13}\text{C}$: $r = 0.73$, $p = 0.06$; $\delta^{15}\text{N}$: $r = -0.89$, $p < 0.01$, $n = 7$; figure 2e,f). For all taxa combined, $\Delta^{14}\text{C}$ values were significantly positively correlated with $\delta^{13}\text{C}$ values and $\delta^{15}\text{N}$ values ($\delta^{13}\text{C}$: $r = 0.49$, $p < 0.01$; $\delta^{15}\text{N}$: $r = 0.39$, $p < 0.05$, $n = 27$; figure 2g,h).

4. Discussion

Most Collembola showed lower $\Delta^{14}\text{C}$ values than the litter, regardless of their $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures. Only the Neanuroidea sample (which included *Friesia japonica*, which is known to be a carnivore [33]) had a higher $\Delta^{14}\text{C}$ value than litter in the L layer. These results indicate that Collembola generally depend more on carbon younger than litter, such as carbon from living roots and algae. *T. ocreatus*, which is a litter surface dweller and exhibited lower $\delta^{15}\text{N}$ than the litter, likely depends on algae as its main food source [6,39]. On the other hand, *T. varius*, *F. octoculata* and *Onychiurus flavescens* could depend on root-derived C as a main pathway

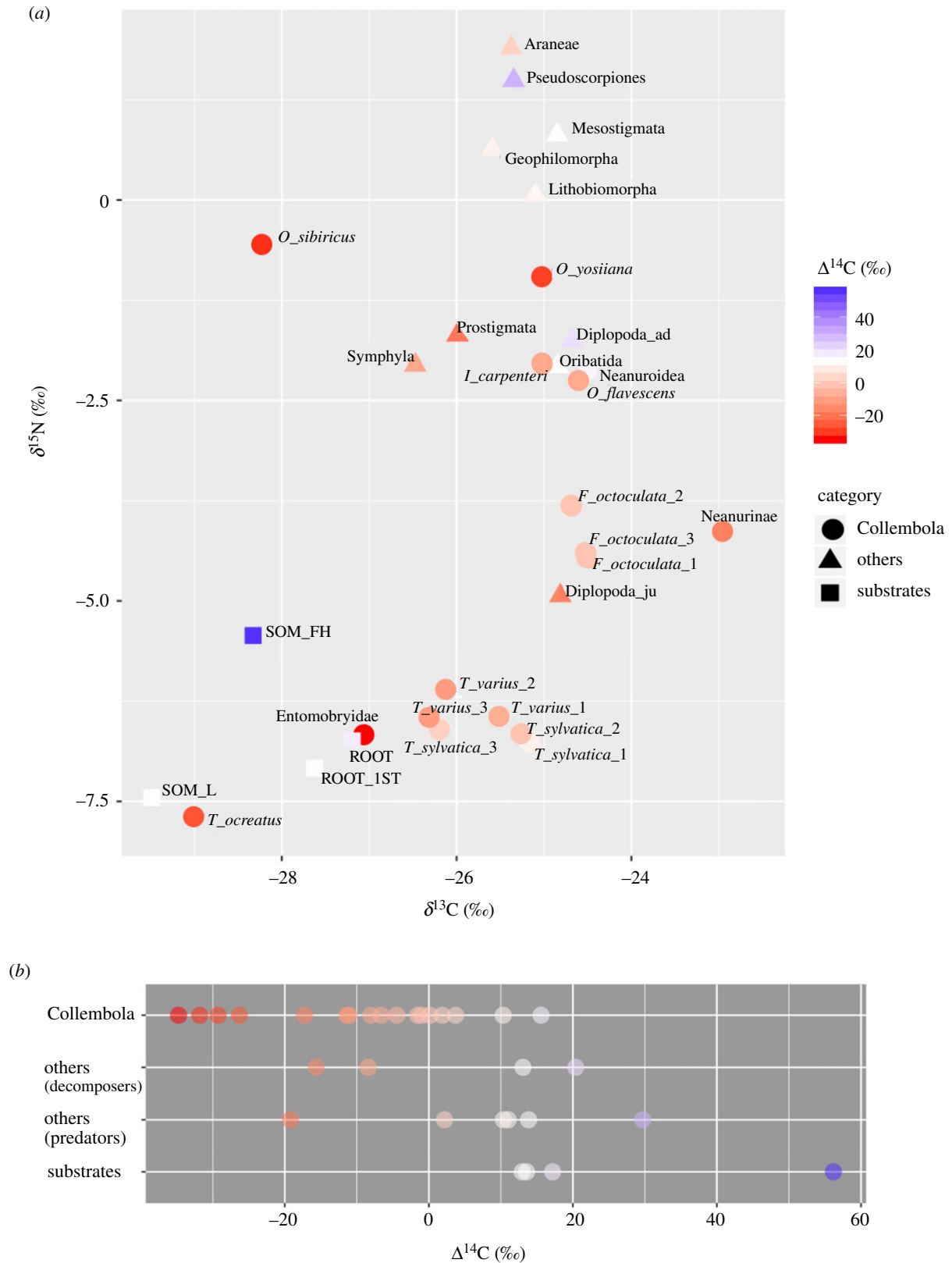


Figure 1. (a) Isotope values (i.e. $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, $\Delta^{14}\text{C}$) of the soil fauna and of the litter and root substrates. Colours represent the $\Delta^{14}\text{C}$ values: white indicates the $\Delta^{14}\text{C}$ value for the litter layer (i.e. 13.6‰), with higher values as blue gradation and lower values as red gradation. The shape key is as follows: circles, Collembola; triangles, other fauna; squares, substrates. Abbreviations for soil fauna are shown in table 1; abbreviations for substrates are SOM_L for the litter (L) layer soil organic matter; SOM_FH for the FH layer; ROOT for the fine roots and ROOT_1ST for the first-order roots. (b) The ranges of $\Delta^{14}\text{C}$ value for Collembola, other fauna and substrates. For other fauna, different trophic positions (i.e. decomposers and predators, table 1) are displayed separately. Each dot shows each sample. Colour representation is the same as figure 1a.

for the younger carbon input, although each species showed different combinations of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values. These species all showed clear responses to recent photosynthate C in a $^{13}\text{CO}_2$ pulse-labelling experiment using greenhouse pots

with *C. obtusa* seedlings and soil collected from the same research site [11]. Differences in stable isotope signatures among these species could reflect differences in their habitat [14] (table 1), leading to differences in the foods they use in

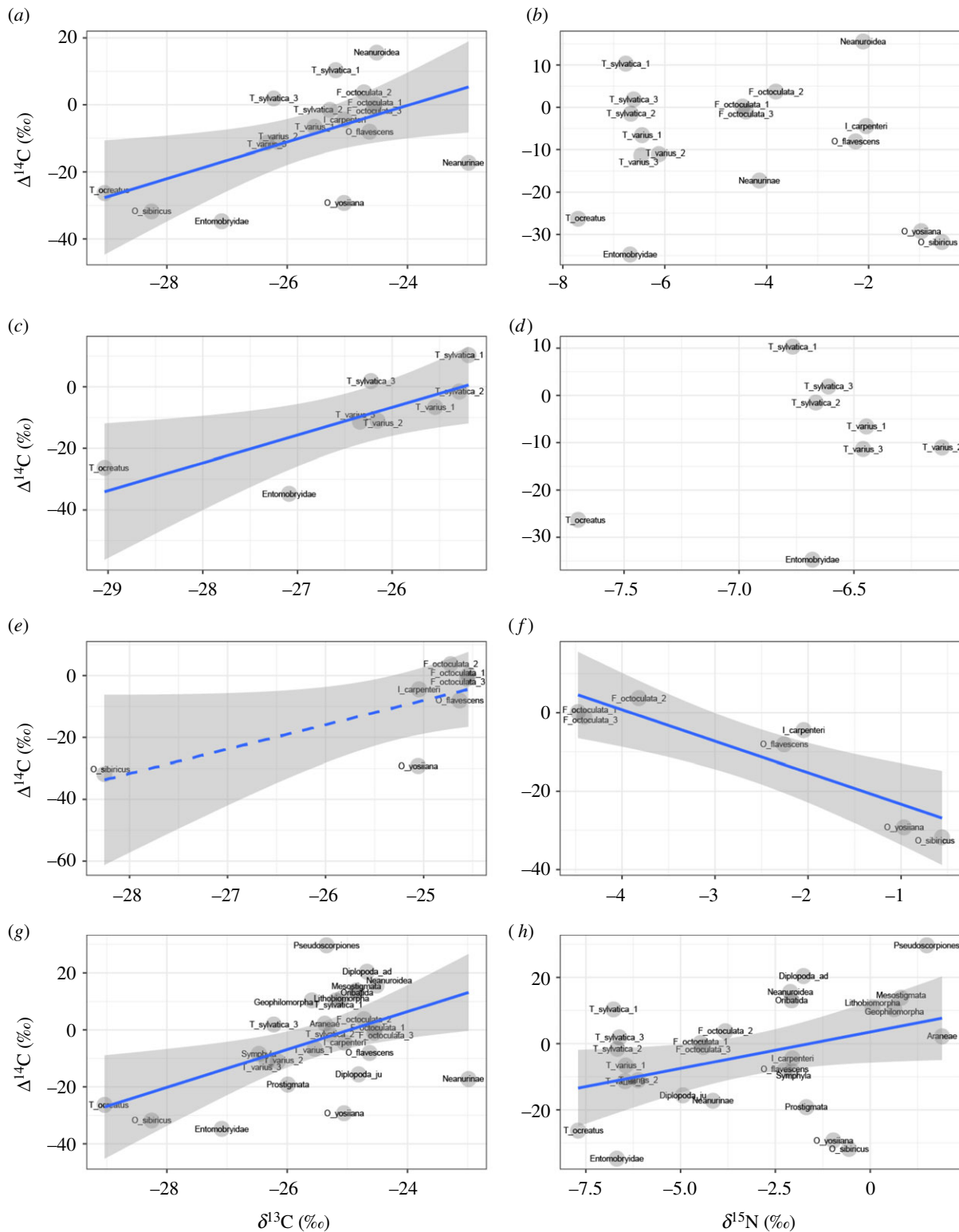


Figure 2. Correlations between (a) $\delta^{13}\text{C}$ and $\Delta^{14}\text{C}$ and (b) $\delta^{15}\text{N}$ and $\Delta^{14}\text{C}$ for all collembolan species combined, between (c) $\delta^{13}\text{C}$ and $\Delta^{14}\text{C}$ and (d) $\delta^{15}\text{N}$ and $\Delta^{14}\text{C}$ for the epigeic collembolan species (i.e. L-layer inhabitants), between (e) $\delta^{13}\text{C}$ and $\Delta^{14}\text{C}$ and (f) $\delta^{15}\text{N}$ and $\Delta^{14}\text{C}$ for the edaphic collembolan species (i.e. FH-layer inhabitants), and between (g) $\delta^{13}\text{C}$ and $\Delta^{14}\text{C}$ and (h) $\delta^{15}\text{N}$ and $\Delta^{14}\text{C}$ for all taxa combined. Abbreviations for collembolan species are shown in table 1. Solid lines show significant relationships ($p < 0.05$), while a dashed line shows a marginally significant relationship ($p < 0.1$) between variables. Shaded areas represent 95% confidence intervals.

addition to root-derived C and the relative proportions of these multiple foods. The stable isotope signatures could also reflect which materials they feed on directly to obtain root-derived C; these include rhizodeposits such as mucilage, mycorrhizal fungi, microorganisms that propagated on the rhizodeposits, and the living root itself, although these sources are still controversial [8,17,40].

Positive correlations between $\delta^{15}\text{N}$ and $\Delta^{14}\text{C}$ values have often been reported for earthworms and termites [24,26,41]. This pattern has supported enrichment of their diet (i.e. soil organic matter) in ^{15}N with humification by saprotrophic microorganisms, and this was often observed as a vertical isotopic gradient in the soil [4,14]. However, we did not find a similar relationship between the $\delta^{15}\text{N}$ value and the carbon

age for Collembola, likely because they feed more on microorganisms than on detritus. The significant positive correlation between $\delta^{13}\text{C}$ and $\Delta^{14}\text{C}$ values could be attributed to the same mechanism that is responsible for the soil ^{15}N enrichment [21], since soil enrichment in heavier isotopes during the humification process has been reported for both ^{15}N and ^{13}C [13,23]. This supports the hypothesis that collembolan species with higher $\delta^{13}\text{C}$ values could generally use more carbon derived from the litter and humus. By contrast, ^{15}N enrichment by a mycorrhizal pathway [42–44] may obscure the relationship between $\delta^{15}\text{N}$ and $\Delta^{14}\text{C}$ values in Collembola. Both factors (i.e. microbial humification and mycorrhizae) can increase $\delta^{15}\text{N}$ values with increasing soil depth [13,43], but have the opposite effect on $\Delta^{14}\text{C}$; that is, microbially humified soil exhibits a higher $\Delta^{14}\text{C}$ value, whereas mycorrhizae have a lower value [45]. The significant negative correlation between $\delta^{15}\text{N}$ and $\Delta^{14}\text{C}$ values in the edaphic Collembola (i.e. FH-layer inhabitants; figure 2f) indicates that collembolan species with higher $\delta^{15}\text{N}$ values depend more on recent photosynthate C, which means that the edaphic species generally feed on mycorrhizae to obtain root-derived C. For epigeic species, we cannot assess whether they feed on mycorrhiza or other materials such as microorganisms that are associated with rhizodeposits. Epigeic species may not feed on mycorrhiza from the point of their low $\delta^{15}\text{N}$ values compared to mycorrhizal $\delta^{15}\text{N}$ [40]. Whether Collembola have enough opportunities to encounter mycorrhizal mycelium in their living space could be critical in the first place, although it is likely influenced by the site-specific environment. Here, we can conclude that Collembola seem not to feed directly on fine roots at our coniferous forest site, because even the first-order roots had a much older carbon age than Collembola.

Predatory taxa with high $\delta^{15}\text{N}$ values [13,46] mostly showed higher $\Delta^{14}\text{C}$ values than Collembola, leading to the positive correlation between $\delta^{15}\text{N}$ and $\Delta^{14}\text{C}$ values across all taxa. Mesostigmata, Chilopoda and Pseudoscorpiones with high $\Delta^{14}\text{C}$ values may also feed on other animals with

higher $\Delta^{14}\text{C}$ values than Collembola, such as Oribatida and adult Diplopoda. In addition to Oribatida and Diplopoda, which have been reported to feed mainly on litter or humus-derived C [11,17], animals we did not sample, such as Diptera larvae, could contribute to increasing the carbon age of predators [47]. Isotopic signatures of other animals should also be assessed at the species level in future studies. However, our findings that many predators, including one of the top predators, Araneae, exhibited a younger carbon age than the litter, indicate non-negligible effects of the feeding habits of Collembola on the whole soil food web. These results emphasize that the soil food web does not necessarily function exclusively as a brown food web derived from detritus [9].

In this study, we showed the utility of radiocarbon analysis, which can compensate for the effects of confounding factors that alter $\delta^{15}\text{N}$ of Collembola (e.g. microbial humification and mycorrhizae). Although we should investigate the $\Delta^{14}\text{C}$ values for potential food sources themselves (especially the sources made out of the current carbon) in the future, our findings provide an important step to improve the assessment of the feeding habits of soil animals and ecosystem functioning through their impacts on the food web.

Data accessibility. All data are provided in the electronic supplementary material [48].

Authors' contributions. S.F. conceived the ideas and collected samples for isotope analyses. S.F. and T.F.H. did isotope analyses under the supervision of I.T. S.F. analysed the data and wrote the manuscript with critical inputs from T.F.H. and I.T. All authors were involved in revising the manuscript. All authors agree to be held accountable for the content therein and approve the final version of the manuscript.

Competing interests. We declare we have no competing interests

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