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Fatty acid analyses to detect the larval feeding preferences of an omnivorous soil-dwelling insect, *Anomala cuprea* (Coleoptera: Scarabaeidae)

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ABSTRACT

Omnivorous soil-dwelling insects in soils profoundly affect plants, but their feeding preferences are largely unknown, in part because few methods allow tracking of trophic interactions in soils. Here, we propose a fatty acid (FA) analysis to examine the diet of an omnivorous soil-dwelling insect, *Anomala cuprea* (Coleoptera: Scarabaeidae). Larvae were grown for one month on single diets (soil organic matter (SOM), wood flakes, or the roots of *Lolium perenne* or *Plantago lanceolata* grown in sand) or a mixture of these diets. FA profiles of the single diets differed significantly, and FA profiles of the larvae depended on those in their diet. FA profiles of SOM exhibited some typical marker FAs known to be bacteria-specific. Some of the typical markers in SOM were also found in larvae, suggesting that the larvae fed on the SOM. FA analysis, therefore, revealed the feeding preferences of an omnivorous soil-dwelling insect.

1. Introduction

There is increasing awareness of the importance of the belowground components of terrestrial ecosystems, because the soil ecosystems exhibit high species diversity (Wardle et al., 2004; Bardgett, 2005; Dighton and Krumins, 2014). Until recently, it was quite difficult to study food web interactions in soils because direct observations of the animals are difficult due to the opacity of soils (Brose and Scheu, 2014) and also because many soil animals are omnivorous (Traugott et al., 2013; Brose and Scheu, 2014). However, state-of-the-art methods have begun to unravel some of the trophic interactions in soils: stable-isotope analyses can document broad patterns across entire food webs (Brose and Scheu, 2014), and DNA-based techniques can provide a detailed snapshot of a herbivore's diet (Staudacher et al., 2011; Pompanon et al., 2012; Schmidt et al., 2012; Traugott et al., 2013; Brose and Scheu, 2014). Food web studies in soils will be greatly advanced by the adoption of such methods and their use to create a more integrative understanding of the effects of food webs on carbon and nutrient cycling and primary productivity.

Omnivorous soil-dwelling insects, such as grubs and wireworms, have profound effects on plant growth and mortality (Brown and Gange, 1990; Blossey and Hunt-Joshi, 2003; Tsunoda et al., 2014a) as well as on primary production, species composition, and succession rates in plant communities (Brown and Gange, 1992; Gange and Brown, 2002; De Deyn et al., 2003; Schädler et al., 2004; Stein et al., 2010). They often dominate these communities in the field and have the strongest negative impact on plants (Whittaker, 2003; Seastedt and Murray, 2008; Zvereva and Kozlov, 2012; Johnson and Rasmann, 2015). Omnivorous soil-dwelling insects can feed on the living roots of various species, as well as on a wide range of soil organic matter (SOM), and this makes it difficult to identify their diet (Traugott et al., 2013, 2015; Brose and Scheu, 2014). Therefore, the mechanisms by which belowground herbivores affect plant communities have rarely been determined because of the difficulties in examining what they eat (Hunter, 2001; Rasmann et al., 2011; but see Schallhart et al., 2012 and Wallinger et al., 2013).

Fatty acid (FA) analysis, an effective approach to elucidate details of the soil food web (Haubert et al., 2011; Traugott et al., 2013; Brose and Scheu, 2014), can reveal the diet of omnivores based on the distinct signatures of the FAs in their food sources. FAs are major components of lipids, and widely distributed in all living cells (Ratledge and Wilkinson 1988). FAs consist of a carbon chain that is either fully saturated or unsaturated, and a carboxylic acid.

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Some FAs are specific for certain organisms: Branched chain and cyclic FAs are specific markers for bacteria (Welch, 1991; Zelles, 1999; Haubert et al., 2006): Linoleic acid (18:2ω6,9) is a marker of fungi but nor very specific (Frostegård and Bååth, 1996). Plants often have high proportions of saturated long-chain FAs (20:0 and 24:0) (Zelles, 1999; Ruess et al., 2007). By using the diverse array of specific FAs that originate in specific taxonomic groups, we can evaluate the consequence of the relatively long-term diets of target animals because most FAs are deposited without changes in the bodies of the consumers, thereby leaving signatures of their diet; this is called "dietary routing" (Ruess and Chamberlain, 2010; Traugott et al., 2013). However, to study the omnivores in natural systems, it is essential to consider their multi directional feeding preferences, because they may also consume SOM that have been incorporated into the soil. Therefore, it is necessary to confirm that FA analysis is an effective approach to reveal the actual diets of the omnivores.

In the present study, we tested whether FA analysis of an omnivorous soil-dwelling insect, *Anomala cuprea* (Coleoptera: Scarabaeidae) can indicate its diet. The insect was reared under the combination of roots and the cultivation media in a pot. We used three plant cultivation media, SOM, wood flakes, and sand. Therefore, the roots, the SOM or wood flakes are potential diets. After one month rearing, FAs of the insect and diets were analysed. We tested whether FA analysis of the insect can identify the diets of which the insect fed on SOM, wood flakes or living root. Actually, we tested the following three hypotheses: *Hypothesis 1*, the concentration of marker FAs of bacteria (*e.g.*, branched chain FAs such as i16:0 and 18:0 10-methyl, and 16:1ω5) and fungi (18:2ω6,9) in SOM are higher than those in roots and wood flakes, because SOM contains abundant microbial decomposers; *Hypothesis 2*, the concentration of plant marker FAs (*i.e.*, 20:0 and 24:0) are higher in roots than those in SOM and wood flakes; *Hypothesis 3*, marker FAs in a diet are only detectable in the insects that fed on the certain diet.

2. Materials and methods

2.1. Study species

We used the larvae of *Anomala cuprea* Hope (Coleoptera: Scarabaeidae; hereafter, "grubs") as an omnivorous soil-dwelling insect. The grubs feed on roots of various herbaceous species (Okuno et al., 1978; Sakai and Fujioka, 2007) as well as on SOM (personal observation by TT). Grubs were grown from eggs laid in litters in the laboratory by adult *A. cuprea* that had been collected from a floodplain of the Tama River (35°38′N, 139°23′E) in July 2014.

Lolium perenne (Poaceae) and *Plantago lanceolata* (Plantaginaceae) were used as the host plants because their roots are known to be consumed by the grubs (Tsunoda et al., 2014a, 2014b, 2014c). Although there are no reports on FA profiles of these species, *L. perenne* and *P. lanceolata* are likely to exhibit different FA profiles. The two species commonly co-occur in semi-natural temperate grasslands and pastures (Grime et al., 1988; Joshi et al., 2000). Seeds of *L. perenne* cv. 'amazing' were obtained from a commercial supplier (Snow Brand Seed Co., Ltd, Sapporo, Japan). Seeds of *P. lanceolata* were collected from a floodplain of the Tama River (35°38′N, 139°23′E) in June 2010.

2.2. Sample preparation

The first-instar larvae were grown in a growth chamber (Koitotron, Koito Industries, Ltd., Kanagawa, Japan) with a 14-h day/10-h night photoperiod at 25 °C, using a two-way randomized full factorial design. The two factors were the plant species and the pot medium. The plant species had three levels: *L. perenne*, *P. lanceolata*, and a control without plants. The plants were grown in pots that contained one of the following three media: SOM, which was mainly composed of the leaves of broadleaved trees that were sieved to pass through a 6-mm mesh; wood flakes, "Kunugi-daioh" that contained mainly wood of *Quercus acutissima* (Mitani Co., Ltd, Ibaraki, Japan); and river sand. The treatment combination without plant in sand was not used because that treatment provided no food for the grubs. Each treatment had 20 replicates. We added five replicates that did not include grubs to evaluate the FAs in the plant roots and media.

We added 200 mL of each medium to 200 mL plastic pots. We then sowed ca. 100 seeds of each plant in early September 2014. We added 30 mL of nutrient solution (250 × Hyponex, 6:10:5 [N-P-K], Hyponex Japan, Osaka, Japan) once per week. In early October 2014, when roots had reached the bottom of the pot and would therefore provide enough food for the first-instar larvae, we placed one in each pot. One month after the addition, we placed the grubs in individual plastic bags that contained wet paper, and kept them there for three days to provide enough time to excrete their gut contents before the FA analysis. The grubs in some pots were not collected probably because they escaped from pots. The grubs were then freeze-dried at −20 °C. Plants from the additional five replicates were also harvested, and their roots were extracted from each medium, carefully washed, and then freeze-dried. The collected SOM and wood were also freeze-dried.

2.3. FA analysis

2.3.1. Extraction of FAs from SOM, wood, and roots

To detect the FA profiles of the dietary sources, we extracted FAs from the SOM, wood, and roots of *L. perenne* and *P. lanceolata* following the procedures of Frostegård et al. (1991) and Niwa et al. (2008). FAs were extracted from the finely ground freeze-dried samples (ca. 50 mg) by shaking the material in 135 mL of a single-phase extraction solvent composed of chloroform, methanol, and 0.05 M phosphate buffer (pH 7.4) at 1:2:0.8 v/v/v for 2 h. The chloroform fraction (which contained the FAs) was then separated from the solvent fraction by leaving the solution upright for 20 h. We tried to extract five samples of each diet but obtained only the results of four replicates.

2.3.2. Extraction of FAs from grubs

To detect the FA profiles of the grubs, we randomly chose five grubs from each combination of experimental treatment and analysed them using a modified version of the method of Ruess et al. (2004). FAs were extracted from the freeze-dried whole grubs by shaking the samples overnight in 5 mL of the single-phase extraction solvent. The solvent was then transferred to a new tube, and the grub was re-extracted by shaking for 3 h with an additional 2.5 mL of the single-phase solvent. The solvents were then combined and mixed with 0.8 mL of distilled water and 0.8 mL of chloroform. The solution was then centrifuged at 1500 rpm for 10 min to separate the solvent phases from the chloroform fraction.

2.3.3. Separating each FA fraction

The chloroform fraction from each sample was transferred to a silica acid column (BOND ELUT LRC-SI; Varian, Palo Alto, CA, USA). The FAs adsorbed in the column were then eluted with 5 mL of chloroform to obtain the neutral lipid FAs (NLFAs), with 10 mL of acetone to obtain the glycolipids, and 5 mL of methanol to obtain the phospholipid FAs (PLFAs). The NLFA and PLFA fractions were then reduced in volume under an N_2 flow on a 40 °C block heater. For the total FAs of the diets, the sum of the NLFAs and PLFAs was used in the statistical analysis. Because dietary routing of FAs