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Substantial nutritional contribution of bacterial amino acids to earthworms and enchytraeids: A case study from organic grasslands

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ABSTRACT

Many aspects of the feeding ecology of terrestrial oligochaetes are poorly understood despite the essential role of these soil and detritus feeders for maintaining soil fertility. To investigate dietary contributions of various soil components to two ecological groups of worms, anecic and endogeic species, we employed $\delta^{13}C$ fingerprinting of essential amino acids (EAA) for distinguishing between bacterial, fungal, and plant derived food sources. We collected earthworms and enchytraeids from organic grasslands with grass, clover, and mixtures of these two plants. Our results showed that the worms either relied on plants or bacteria as their primary EAA source, but not on fungi, and that EAA targets were unaffected by crop type. Two anecic species received 60–75% of their EAA from plant sources with bacterial contributions ranging from 18 to 23%. In contrast, both enchytraeids and an endogeic worms relied equally on bacterial and plant derived EAA. Our study provides answers to some of the long-standing questions in regards to the role of bacteria for earthworm nutrition. While bacterial EAA contribution to anecic worms was relatively modest, less than one-quarter, bacterial contribution to endogeic and enchytraeid worms was substantial comprising almost half of their EAA. Our findings are important for understanding how different ecological groups of terrestrial oligochaetes meet nutritional needs and partition food resources.

1. Introduction

Earthworms and enchytraeids are dominant detritus feeders in many soils where they affect soil fertility by ingestion of litter, excretion of nutrient rich casts, and creation of pores (Curry and Schmidt, 2007; Didden, 1993). How these soil oligochaetes influence soil fertility is largely determined by their physiology and feeding preferences (Eisenhauer et al., 2009a; Griffith et al., 2013). Both earthworms and enchytraeids are bulk feeders with highly complex mechanisms for selecting and digesting their detrital diets (Dózsa-Farkas, 1982; Hendriksen, 1990; Liebeke et al., 2015). Enchytraeids differ from earthworms by their smaller size, lack of pigmentation and inability to masticate plant litter larger than their buccal cavity (Didden, 1993; Gelder, 1984). In spite of both groups of oligochaetes being well studied, many aspects of their feeding ecology are unknown (Vliet and Hendrix, 2011). It has long been documented that dietary preferences and ability to digest complex compounds differ greatly between annelid species; however, the direct dietary contributions of bacteria and fungi have remained elusive (Crotty et al., 2011; Didden, 1993; Edwards, 2004). Closing this gap can help to better understand the feeding ecology of earthworms, and how they affect the interplay between basal soil resources and microbiota.

Earthworms dwelling predominantly belowground can be divided into two ecological groups; endogeic worms live typically below the topsoil and ingest large quantities of nutrient-poor mineral soil, and anecic worms making vertical burrows presumably feed on a mixture of detritus and litter from different soil layers including surface litter (Bouché, 1977). These different food sources among worms are also reflected in their ^{15}N and ^{13}C values. Endogeic worms usually have the most enriched values, which has

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been ascribed to utilization of more humified organic matter, i.e. older carbon sources (Briones et al., 2005; Pollierer et al., 2009; Schmidt et al., 2004). Both endogeic earthworms and enchytraeids tend to have similar isotope values and ingest food sources of the same age, 5–10 years after photosynthetic fixation (Briones and Ineson, 2002; Scheu and Falca, 2000; Schmidt et al., 2004). It remains controversial whether the carbon sources assimilated by terrestrial oligochaetes derive directly from humified plant matter or indirectly from microbes using humified plant matter as a substrate and energy source (Curry and Schmidt, 2007; Marhan et al., 2007; Sampedro et al., 2006). For this reason, application of novel *in situ* approaches is required to distinguish between plant and microbial derived sources.

Protein amino acids are in many soils considered limiting nutrients for terrestrial oligochaetes (Pokarzhevskii et al., 1997; Pokarzhevskij et al., 1989). Like other metazoans, soil oligochaetes cannot synthesize the carbon skeletons of about half of the 20 protein amino acids *de novo* and therefore rely on dietary sources (Costa et al., 2015). For endogeic earthworms and enchytraeids, obtaining sufficient amino acids may be particularly challenging because the composition and concentration of amino acids is more imbalanced in old than fresh detritus (Moriarty and Pullin, 1987). For endogeic earthworms, it has been suggested that are able to mobilize old organic carbon pools in soils (Marhan et al., 2007). A considerable fraction of carbon bound in organo–mineral complexes is inaccessible to degradation without physical disruption (Six and Paustian, 2014). However, within the gizzards of earthworms these complexes are macerated with mucus and then physically disrupted by muscles and sand particles making them available for subsequent digestion. By comparing fatty acid ^{13}C values of earthworms and various particle size fractions, Ferlian et al. (2014) found evidence that endogeic earthworms utilize old carbon enclosed in organo–mineral complexes, which led the authors to conclude that stabilized fatty acids of bacterial origin can contribute to earthworm nutrition. For enchytraeids, it is widely assumed that bacteria constitute an important nutrient source (Didden, 1993) in part because their digestive system is better adapted at digesting bacterial cell walls than complex plant polymers (Dash et al., 1981; Mothes-Wagner et al., 1996; Reichert et al., 1996).

The actual nutritional contribution of bacteria is difficult to quantify with prevailing methods (Crotty et al., 2011; Didden, 1993; Waldrop et al., 2012). A newly developed approach based on naturally occurring stable isotope patterns, $\delta^{13}C$ fingerprinting of amino acids ($\delta^{13}C_{AA}$) (Larsen et al., 2009, 2013) may be able to circumvent some of the issues of undetermined sources and isotope fractionation that can be problematic with bulk and phospholipid fatty acid based approaches. The $\delta^{13}C$ fingerprints represent the sum of the isotopic fractionations, associated with the individual biosynthetic pathways and the specific set of precursors used for synthesizing each amino acid (Hayes, 2001). Owing to lineage specific pathways for synthesizing amino acids, $\delta^{13}C_{AA}$ fingerprinting can distinguish between bacterial, fungal, and plant origins of amino acids (Larsen et al., 2009) regardless of variability in isotope baseline values (Larsen et al., 2013, 2015). The $\delta^{13}C$ values of essential amino acids (EAA) are particularly diagnostic for lineage-specific isotope effects because have more complex and conserved biosynthetic pathways than the non-essential amino acids (Larsen et al., 2009). The fingerprinting approach has the advantage that biosynthetic origins of EAA can be inferred indirectly from "training data", i.e. potential food sources cultured in the laboratory, saving the complicated process of isolating microorganisms from the soil matrix or gut content of the study animals.

In the present study, we collected earthworms and enchytraeids from agricultural plots with grass, clover, and a mixture of clover and grass. The mixed treatment was included to test whether clover or grass resources would support oligochaetes. Grasslands are mainly root-driven food webs (Crotty et al., 2014), and earthworms may therefore utilize different basal resources under different plant functional group regimes. To investigate dietary patterns and basal resources of earthworms and enchytraeids, we relied on $\delta^{13}C_{EAA}$ fingerprints (Larsen et al., 2009, 2013). We hypothesized that anecic earthworms would depend on plant litter rather than bacterial inputs. For the endogeic earthworms and enchytraeids, we expected that they would rely more on bacterial amino acids than their anecic counterparts. We also expected that the anecic species would track bulk $\delta^{15}N$ values of clover more closely than grass because earthworms have been shown to perform better in the presence of legumes than grasses, presumably due to N-rich legume litter and root exudates (Eisenhauer et al., 2009a; Milcu et al., 2008).

2. Methods and materials

2.1. Field site and climate data

Plant and fauna samples were collected on September 12–13 2011 from an organic dairy crop rotation (ley-arable) experiment established in 1987 at Foulumgaard Experimental Station (09°34′ E, 56°29' N; mean annual precipitation 770 mm; mean annual temperature 7.7 °C (Eriksen et al., 2014)). An additional sampling for enchytraeids was performed on the September 26 to get more specimens for species identification. The grass-clover leys comprised of plots with perennial ryegrass (Grass: *Lolium perenne* L.) only, clover (Clover: white clover; *Trifolium repens* L., red clover; *Trifolium pratense* L) only, and a mixture of grass and clover (Mixed). The grass-clover leys were established in a randomised block design with four replicates (henceforth referred to as plots) of each plant cover. See Supplementary figures S1-S3 for climate data, soil water content and aboveground plant biomass.

Within each plot, six subsamples of enchytraeids were collected randomly with a soil corer (inner diameter 5.5 cm; depth 9 cm). The samples were kept at 5 °C until extraction, which was initiated within two weeks. The extraction procedure was a modified version of O'Connor's wet funnel extraction with a stepwise increase in temperature at the sample surface from 25 °C to 50 °C in 5 h (O'Connor, 1962). The enchytraeids were collected in tap water and stored for 24–48 h at 5 °C. For species identification, three subsamples from each plot were first pooled and then identified in vivo to species or genus level at the University of A Coruña by Dr. Schmelz using the taxonomic keys of Schmelz and Collado (2010). Each of the remaining subsamples were counted and weighed (Sartorius Micro SC 2 balance – Göttingen, Germany) before drying the worms at 60 °C for 24 h. The subsamples were weighed again after drying, and stored for isotope analyses. Earthworms were collected from two subsamples of (20 cm × 20 cm x 20 cm) in each plot. The blocks were transported to the laboratory where the soil was broken up and the earthworms hand-sorted, counted and left overnight in Petri dishes with wet filter paper to empty their gut. The following day all individuals were identified according to species, weighed, freeze-dried, and crunched. The gut were removed from the earthworms, but not from the enchytraeids prior to freeze-drying. All samples were stored at −18 °C until further analyses. Clover and grass leaves and roots were collected from the earthworms blocks (20 cm*20 cm*20 cm) in all the plots. The plants were immediately sorted into leaves and roots, and stored at 5 °C for one day. The leaves and roots were then washed with ELGA-water, frozen, freeze-dried and crunched. For soil analysis, we collected three subsamples from each plot. These subsamples were subsequently pooled and freeze-dried before analysis. Samples