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Natural variability and correlations in the metabolic profile of healthy *Eisenia fetida* earthworms observed using ^{1}H NMR metabolomics

Melissa Whitfield Åslund, Magda Celejewski, Brian P. Lankadurai, André J. Simpson, Myrna J. Simpson *

Environmental NMR Centre and Department of Physical and Environmental Sciences, University of Toronto, 1265 Military Trail Toronto, Ontario, Canada M1C 1A4

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ABSTRACT

^{1}H NMR metabolomics can be used to assess the sub-lethal toxicity of contaminants to earthworms by identifying alterations in the metabolic profiles of contaminant- exposed earthworms in contrast to those of healthy (control) individuals. In support of this method this study sought to better characterize the baseline metabolic profile of healthy, mature earthworms of the species, *Eisenia fetida,* which is recommended for both acute and sub-lethal toxicity testing for soil contaminants. Profiles of D_2O-buffer extracted metabolites were determined using ^{1}H NMR spectroscopy and both inter-individual metabolic variability and pair-wise metabolic correlations were assessed. The control earthworm extracts exhibited low overall inter-individual metabolic variability, with a spectrum-wide median relative standard deviation (%RSD = standard deviation/mean × 100) of 14%, which suggests that the metabolic profile of *E. fetida* earthworms is well controlled in laboratory conditions and supports further use of this organism in environmental metabolomics research. In addition, strong positive correlations were detected between the levels of maltose, betaine, glycine, and glutamate as well as between the levels of lactate, valine, leucine, alanine, lysine, tyrosine, and phenylalanine which had not previously been reported. Since comparison of pair-wise metabolic correlations between control and treated organisms can reveal changes in the underlying pattern of biochemical relationships between the metabolites, identification of these significant metabolic correlations in control earthworms provides an additional characteristic that may be applied to delineate between control and treated earthworms in future NMR-based metabolomic studies.

1. Introduction

In environmental metabolomics, analytical techniques such as gas chromatography/mass spectrometry (GC/MS) or nuclear magnetic resonance (NMR) spectroscopy are used to detect and quantify endogenous metabolites in tissues or biofluids collected from study organisms under varying environmental conditions (Viant, 2008). Changes observed in the metabolic profile can reveal modifications of the energetic, reproductive, or oxidative status of the organism in response to the experimental treatment (Malmendal et al., 2006; Overgaard et al., 2007; Turner et al., 2007; Viant, 2008; Reinke et al., 2010; Schock et al., 2010). This technique has identified changes in earthworm metabolic profiles following exposure to metals and/or organic contaminants in both soil exposures and contact tests, suggesting that metabolomics is a powerful tool for evaluating the sub-lethal toxicity of environmental contaminants to earthworms (Bundy et al., 2004, 2008; Jones et al., 2008; Brown et al., 2009, 2010; Guo et al., 2009; McKelvie et al., 2009, 2010). This method can complement traditional ecotoxicological techniques since it is sensitive to subtle biochemical responses and may provide new information about the mechanism of action (MOA) of adverse environmental stressors (Guo et al., 2009; Vulimiri et al., 2010).

Detection of metabolomic differences between the treated and control (untreated) organisms can be obscured if the natural metabolic variation is too high (e.g. due to genetic or environmental variation (Viant, 2008)). Therefore it is important to understand the baseline metabolic variability of the study organism (Lay et al., 2006). The analysis and reporting of metabolic variability for groups of healthy human subjects in metabolomics has been studied in detail (Lenz et al., 2003; Saude et al., 2007; Slupsky et al., 2007; Psihogios et al., 2008; Crews et al., 2009; Mi Park et al., 2009). These studies showed that human metabolic profiles respond to factors such as genetics, age, diet, mass, and time of day, but that overall inter-individual metabolic variability is still low enough to detect treatment-related metabolic responses. To date, the inter-individual metabolic variability of study organisms has not been explicitly addressed in many environmental metabolomics studies. In light of the ongoing development of earthworm metabolomics, examination of the biological variability of the earthworm metabolome is warranted. Therefore, this study was designed to quantify the degree of inter-individual metabolite variability in tissue extracts from a sample of healthy mature *Eisenia fetida* earthworms,

* Corresponding author. Tel.: +1 416 287 7234.
E-mail address: myrna.simpson@utoronto.ca (M.J. Simpson).

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which are recommended for both acute and sub-lethal soil contaminant toxicity testing (OECD 1984, 2004).

In addition, due to the relationships between metabolites in major metabolic pathways, pair-wise metabolite correlations may exist which are considerably less variable than individual metabolite concentrations (Camacho et al., 2005; Steuer, 2006). Experimental treatments that disrupt aspects of normal metabolic function can alter the pattern of these metabolic correlations (Steuer, 2006; Southam et al., 2008). Therefore, even for organisms which exhibit high levels of inter-individual metabolic variability, comparison of pair-wise metabolic correlations may improve discrimination between treatment and control groups and may also help identify which metabolic processes were altered by the study treatment (Roessner et al., 2001; Southam et al., 2008; Zhang et al., 2008). To date, metabolic correlation analysis has been applied to discriminate between plant phenotypes (Roessner et al., 2001), control and diabetic rats (Zhang et al., 2008) and healthy and tumor hepatic tissue collected from dab flatfish (Southam et al., 2008). In the studies by Roessner et al. (2001) and Zhang et al. (2008), comparisons of pair-wise metabolic correlations provided supplementary information which assisted in the interpretation of the metabolomic differences observed between the treatment groups using a traditional metabolomic statistical approach based on metabolite concentrations (i.e., PCA). In addition, this approach offers a means to detect metabolomic responses between treatment groups that are not detected by traditional metabolomic statistical methods (Southam et al., 2008). For instance, Southam et al. (2008) was unable to distinguish between the metabolic profiles of tumor and healthy liver samples collected from dab flatfish using PCA due to high levels of biological variability in the metabolite concentrations within each treatment group. However, pair-wise correlation analysis of the same dataset successfully identified more than 30 pair-wise metabolic correlations that differed significantly between the two tissue types (Southam et al., 2008). This demonstrates that comparative analysis of pair-wise metabolic correlations offers significant potential as a complementary method to traditional multivariate approaches for interpreting metabolomic data. However, this approach has not yet been considered in the field of earthworm metabolomics. Therefore, the current study sought to assess and quantify the baseline pattern of pair-wise metabolic correlations within a sample of healthy control *E. fetida* earthworms.

2. Materials and methods

2.1. Earthworm maintenance

Twenty-four mature earthworms with a visible clitellum and an average mass of 0.50 g (range 0.28–0.71 g wet weight, standard error 0.03 g, $n = 24$) were sampled from a healthy population of *E. fetida* which has been maintained consistently since 2006, thereby minimizing inter-individual metabolic variations related to temperature, moisture, pH, diet, and other environmental factors (Brown et al., 2008). Progenitor earthworms were purchased from The Worm Factory (ON, Canada) and the population has been cultivated since then at a temperature of approximately 24 °C in earthworm bins containing sphagnum peat bedding (Magic Worm bedding; Magic Products; WI, USA) with a water content of approximately 67% water by weight (Brown et al., 2008) and a standardized diet of Magic Worm Food composed of 12% crude protein, 1% crude fat, and 6% crude fiber (Magic Products; WI, USA).

2.2. Earthworm tissue extraction

Earthworms were depurated (in groups of 10) in the dark for 96 h on Whatman 4 Qualitative filter paper in 500 mL jars (Brown et al., 2008). Earthworms were then flash–frozen in liquid nitrogen, lyophilized, reweighed and stored frozen until extraction. Prior to extraction, lyophilized earthworms were homogenized in a 1.5 mL centrifuge tube using a 5 mm wide stainless steel spatula. The homogenized tissue was extracted using 1.20 mL of a 0.2 M monobasic sodium phosphate buffer solution ($NaH_2PO_4{\cdot}2H_2O$; 99.3%; Fisher Chemicals) containing 0.1% (w/v) sodium azide (99.5% purity; Sigma Aldrich) as a biocide and 10 mg L^{-1} of 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS; 97%, Sigma Aldrich) as an internal standard (Brown et al., 2008). Buffer solution was made with D_2O (99.9% purity, Cambridge Isotope Laboratories) and adjusted to a pD of 7.4 using NaOD (30% w/w in 99.5% D2O, Cambridge Isotope Laboratories Inc.). This aqueous buffer was previously demonstrated to extract the largest quantity of the widest variety of metabolites from *E. fetida* tissue samples (Brown et al., 2008) and similar aqueous extractions have been applied in several other earthworm metabolomic studies (Bundy et al., 2001, 2004; Brown et al., 2010; McKelvie et al., 2009, 2010). Samples were vortexed for 30 s using a VX 100 vortexer (Labnet, NJ, USA) and sonicated for 15 min using a FS60 sonicator (Fisher Scientific) to aid with the extraction. Subsequently, samples were centrifuged at 14 000 rpm (~15 000*g*) for 20 min using an International Equipment Company 21 000 centrifuge (Fisher Scientific) and the supernatant was transferred into a new 1.5 mL centrifuge tube. The centrifugation procedure was repeated twice more to remove suspended particles. Samples were then transferred into 5 mm High Throughput^{plus} NMR tubes (Norell Inc.; NJ, USA) for ^{1}H NMR analysis.

2.3. ^{1}H NMR spectroscopy

^{1}H NMR spectra of the earthworm extracts were acquired with a Bruker Avance 500 MHz spectrometer using a ^{1}H-^{19}F-^{15}N-^{13}C 5 mm Quadruple Resonance Inverse (QXI) probe fitted with an actively shielded *Z* gradient. ^{1}H NMR experiments were performed using Presaturation Using Relaxation Gradients and Echoes (PURGE) water suppression and 128 scans, a recycle delay of 3 s, and 16 K time domain points (Simpson and Brown, 2005). Spectra were apodized through multiplication with an exponential decay corresponding to 0.3 Hz line broadening in the transformed spectrum, and a zero filling factor of 2. All spectra were manually phased and calibrated to the DSS internal reference methyl singlet, set to a chemical shift (δ) of 0.00 ppm. As many metabolite peaks as possible were identified in each spectra by comparison to previously published chemical shifts and peak multiplicities (Lenz et al., 2005; Brown et al., 2008; Cui et al., 2008).

2.4. Data analysis

Prior to statistical analyses, ^{1}H NMR spectra were analyzed between δ of 0.5 and 10 ppm and divided into bucket tables using a combination of equidistant and non-equidistant binning (Ross et al., 2007). Initially, spectra were divided into 0.01 ppm wide buckets and the area under each segment was integrated in AMIX 3.7.10 (Bruker BioSpin) with the integration mode of sum of intensities. However, this resulted in peak splitting for some of the identified metabolites, so bucket widths were adjusted for these compounds (Table 1, Bundy et al., 2007; Guo et al., 2009). With the remainder of each spectrum divided into 0.01 ppm wide buckets and the area between $\delta = 4.70$–4.85 ppm excluded to eliminate small residual H_2O/HOD signals, this resulted in a total of 896 buckets for each earthworm sample.

Initially, an integral normalization (IN), which divides the measured signal intensity for each bucket by the integral of the complete spectrum (i.e. 'scale to total intensity'), was applied to the data to correct for differences in the total NMR signal measured