Biochemical and Biophysical Research Communications 430 (2013) 1306–1311

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Biochemical diversity of betaines in earthworms

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ARTICLE INFO

Article history:
Received 30 November 2012
Available online 19 December 2012

Keywords:
Betaine
Nuclear magnetic resonance
HSQC
Untargetted profiling
Comparative biochemistry
Earthworm
Megascolecidae
Lumbricidae
Glossoscolecidae

ABSTRACT

The ability to accumulate osmoprotectant compounds, such as betaines, is an important evolutionary feature in many organisms. This is particularly the case for organisms that live in variable environments, which may have fluctuations in moisture and salinity levels. There is, surprisingly, very little known about betaines in soil invertebrates in general, and there is almost no information about earthworms – a group that are important 'ecosystem engineers' and key indicators of soil health. Here, we describe a fast and reliable $^{1}H–^{13}C$ heteronuclear single quantum coherence (HSQC) 2D NMR approach for the metabolic profiling of a series of betaines and related metabolites in tissue extracts, and list ^{1}H and ^{13}C chemical shifts for the trimethylammonium signal for 23 such compounds. The analysis of ten different species from three different families (Lumbricidae, Megascolecidae and Glossoscolecidae) showed an unexpected diversity of betaines present in earthworms. In total ten betaines were identified, including hydroxyproline-betaine, proline-betaine, taurine-betaine, GABA-betaine and histidine-betaine, and a further eleven as-yet unassigned putative betaine metabolites detected. The findings clearly indicate a hitherto-unappreciated important role for betaine metabolism in earthworms.

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1. Introduction

Betaines – trimethylammonium derivatives of amino acids and related compounds – are an important set of metabolites for many organisms, including microbes, algae, plants, and animals, including humans [1–4]. They play a pivotal role in osmoprotection of cells and tissues to maintain cellular homeostasis, and to protect them against environmental stresses like high salinity and extreme temperatures. Additionally, they serve as a catabolic resource of methyl groups in different biochemical pathways (transmethylation).

Betaines are well studied in bacteria [5], plants and algae [1,6], but less is known about their occurrence in many other species. This is true for invertebrates, and in particular terrestrial invertebrates. Earthworms are a classic example where fundamental knowledge about their occurrence and distribution is lacking. Earthworms can survive and flourish in soils with both high and low extremes of water stress [7]. Soil moisture is considered to be the primary factor limiting survival of different earthworm species [8,9], therefore they must preserve an efficient osmoregulatory system. Surprisingly little is known about betaines and other trimethylammonium compounds in earthworms. Our knowledge of which compounds may be present and their natural distribution is far from complete, but for instance metabolic profiling approaches by ^{1}H NMR have detected both glycine-betaine and choline in *Lumbricus rubellus* [10] and *Eisenia fetida* [11]. This lack of knowledge motivated us to study the landscape of betaine compounds in earthworms. Additionally, we aimed to develop a fast and reliable method for the detection of these compounds, which can be easily applied to other sample types.

Betaines are zwitterionic quaternary ammonium compounds and so are not trivial to analyze by common separation methods such as reversed phase HPLC, although methods have been developed using hydrophilic interaction liquid chromatography [12] or a pentafluorophenylpropyl stationary phase [13] to separate betaines. More time-consuming protocols, including previous derivatization of betaines, can also be applied to improve HPLC retention characteristics [14]. In contrast, nuclear magnetic resonance (NMR) spectroscopy can also be used for analysis of betaines, and does not require physical separation of metabolites [1]. Betaine metabolites give rise to singlet resonances from the trimethylammonium group; because these are based on nine protons, and there is no resonance splitting, these compounds have relatively low detection levels by ^{1}H NMR. However, it is difficult to assign betaines in 1D spectra based on the trimethylammonium peak alone; unfortunately, these are often the only peaks easily detected in crude cell/tissue extracts (especially for low-concentration compounds) because the other compound resonances are obscured by overlapping signals from other metabolites. Either homonuclear or heteronuclear two-dimensional NMR experiments are required for confident assignment of betaine metabolites in a given sample; in particular, heteronuclear $^{1}H–^{13}C$ methods make advantage of the inherently wide chemical shift distribution of the ^{13}C nucleus.

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0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.12.049

However, these can be time consuming, especially if aiming to give high-resolution spectra across the full range of proton and carbon shifts found in tissue extracts. Fortunately, as already described, betaines possess a characteristic trimethylammonium resonance, and spectral acquisition times can be drastically reduced (while maintaining resolution in both dimensions) by concentrating on this spectral region.

We present here an analytical approach that provides both qualitative and quantitative data, by combining short 1D and 2D HSQC NMR acquisitions of spectra from tissue extracts. We also provide a reference database for a range of amino acid derived betaines to consistently identify as many as possible compounds in various samples, and apply this to characterize the betaine profiles in 10 different earthworm species.

2. Materials and methods

2.1. Earthworm species

Lumbricus rubellus (Lumbricidae) worms were a gift from David Spurgeon (CEH Wallingford, UK). *Aporrectodea chlorotica* (Lumbricidae) worms were collected from field populations within the UK. *Lumbricus terrestris*, *E. fetida*, and *Dendrobaena veneta* (all Lumbricidae), were purchased from Blades Biological Ltd. (Edenbridge, UK). Four additional species from two families were collected in glasshouses with tropical climate and flora based in the Royal Botanic Gardens, Kew, London (UK). These were *Amynthas rodericensis, Amynthas corticis* (putative assignment – identification was to a lower level of confidence than for the other species), *Pithemera bicincta* (all Megascolecidae), and *Pontoscolex corethrurus* (Glossoscolecidae). *Amynthas gracilis* (Megascolecidae) worms were a gift from Peter Kille (University of Cardiff, UK) and originally collected from the island of Furnas (Portugal).

2.2. Metabolite extraction

The worms were frozen in liquid N_2 and the frozen tissue cryogenically ground using a FreezerMill 6870 (Spex SamplePrep, Stanmore, UK). The milled tissue was extracted with a method described in detail elsewhere [15]. Briefly, 20 mL ice cold acetonitrile:methanol:water 2:2:1 (vol./vol.) were added to 1–3 g tissue powder and mixed in a vortexer for 1 min, freeze–thawed, and mixed again and centrifuged (4000*g*, 5 min). The supernatant was transferred into glass tubes and dried down in a rotary vacuum concentrator (Eppendorf, Cambridge, UK).

2.3. NMR analysis

The dried extracts were resuspended in 650 μL D_2O with 0.1 M phosphate buffer, pH 7.0, containing DSS as internal standard (5 mM). Samples were centrifuged at 10,000 rpm for 2 min and the supernatant was transferred into 5 mm NMR glass tubes. Samples were analyzed with a 800 MHz Bruker Avance spectrometer equipped with a triple resonance cryoprobe. The sample temperature was set to 300 K; the temperature was not calibrated directly for this experiment, but is calibrated for this probe at regular intervals. For all samples a one-dimensional ^{1}H NMR spectrum with solvent suppression on the residual water peak was acquired

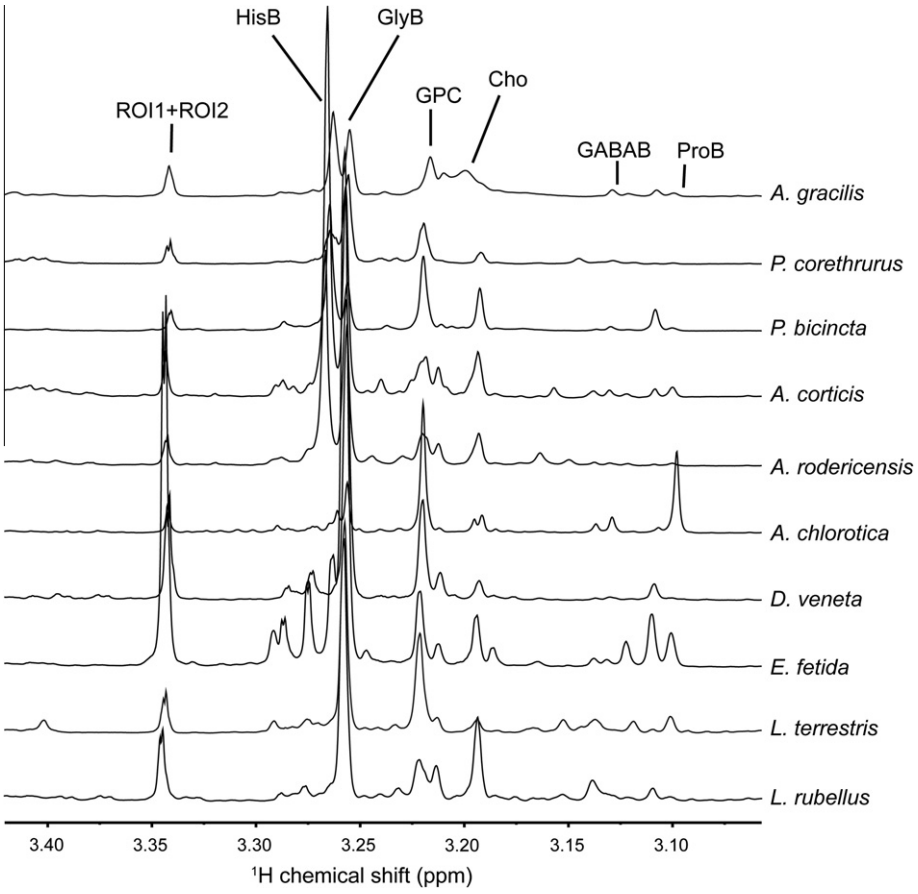

Fig. 1. One-dimensional ^{1}H NMR spectra of all earthworm species, showing a region for potential betaine signals. Selected signals are indicated, proline-betaine (ProB), GABA-betaine (GABAB), choline (Cho), glycerophosphocholine (GPC), glycine-betaine (GlyB), histidine-betaine (HisB), unidentified metabolites ROI1 and ROI2.

over a spectral width of 20 ppm sampled into 64k data points, as previously described [16]. Two dimensional NMR spectra were recorded with standard sequences, $^{1}H–^{13}C$ HSQC (Gradient enhanced phase-sensitive HSQC spectra [17] were collected with 4096 points in t_2 and 512 points in t_1 over a bandwidth of 12.5 ppm in ^{1}H and 200 ppm in ^{13}C with 8 scans per t_1 value (giving a total acquisition time of 2 h). The resulting HSQC NMR spectra were processed in Topspin® 3.0 (Bruker, Biospin) using standard methods, with 90° shifted sine-squared apodization and phase correction in both dimensions and zero filling in t_1 to yield a transformed 2D dataset. Additional $^{1}H–^{13}C$ HSQC spectra were recorded using a focused ^{13}C bandwidth of 30, 15 and 8 ppm with a midpoint (frequency offset) at 52 ppm. Those spectra were acquired using a decreased number of increments (16–64 points in t_1) to gain short acquisition time. All full range spectra were referenced to DSS ($\delta = 0$ for both ^{1}H and ^{13}C). These spectra were used to determine the chemical shift of peaks from the endogenous earthworm metabolites glycine-betaine and lombricine (both found in all species), which were then used as internal ^{13}C reference peaks for the narrow-range HSQC spectra ($^{1}H/^{13}C$ shifts for glycine-betaine and lombricine were 3.256/55.9 ppm and 3.46/44.34 ppm, respectively).

For selected samples Heteronuclear Multiple Bond Correlation (HMBC) $^{1}H–^{13}C$ spectra were acquired using standard Bruker pulse sequence (4096 points in t_2 and 512 points in t_1 over a bandwidth of 10.5 ppm in ^{1}H and 205 ppm in t_2) and processed as described above. These spectra were used to help metabolite assignment from the complex mixtures, and a typical example is shown as Fig. S1, online supplementary information.

For quantification of signal areas in the HSQC spectra the open source software rNMR Version 1.1.7 was used [18].

2.4. Betaine standards

Glycine-betaine, choline and carnitine were purchased from Sigma Aldrich, UK. Non-commercially available betaines were synthesized after standard procedures described earlier [19]. Briefly, respective amines were dissolved in 2 mL methanol:water (1:1, vol./vol.) with 0.1 g sodium hydrogen carbonate, and methyl iodide was added (final concentration amine to CH_3I 0.1:0.16 mmol). This mixture was shaken at room temperature for 24 h in darkness. The mixture was dried under reduced pressure at 45 °C. Afterwards betaines were extracted by addition of chloroform and water to the residue, and the aqueous phase was stored at −20 °C until analysis.

3. Results

To assess the occurrence of betaines in earthworms we screened 10 different earthworm species by a 1D ^{1}H NMR metabolic profiling approach and refined a common 2D HSQC NMR approach into a rapid and sensitive method for betaine detection. The species were taken from three earthworm families, Lumbricidae, Megascolecidae and Glossoscolecidae. The Lumbricidae is the only earthworm family indigenous to the UK, and so, in order to increase the phylogenetic diversity of our study, we collected tropical megascolecid and glossoscolecid worms from a botanical garden [20], and also obtained the megascolecid *Amynthas gracilis* from a population originally collected from an Atlantic island. We analyzed all samples first by 1D ^{1}H NMR, which is a rapid means to screen for putative betaine metabolites, as they give singlet resonances between δ 3.0 and δ 3.4 ppm.

Examining the 1D spectra from all 10 species analyzed shows a large number of peaks in this region, including many which appear to be singlets and hence are potential trimethylammonium compounds (Fig. 1). Not all of the peaks are resolved, and it is not possible to confidently decide in all cases if a peak is derived from the same metabolite or not across multiple samples, especially for the lowest-concentration metabolites. Nonetheless it does already indicate that there is a large amount of cross-species biochemical diversity: clearly, not all peaks are present in all samples. It is impossible to confidently identify all the peaks from 1D ^{1}H–NMR spectra, so the acquisition of heteronuclear 2D NMR spectra is essential. A $^{1}H–^{13}C$ HSQC spectrum of an earthworm tissue extract is still quite complex (Fig. 2A), and entails relatively long acquisition times if it is to be acquired with high resolution in the F1 dimension. However, in the current case, it is possible to restrict the HSQC sweep width to cover a much smaller ^{13}C chemical shift range and decrease the number of data points needed for a good signal-to-noise ratio. Different ranges and respective number of data points were evaluated for earthworm tissue extracts to yield comparable results to the standard full range HSQC (data not shown) with special focus on betaine cross peaks. A final method was set up with a total acquisition time of 25 min, targeting ^{13}C signals from 37–67 ppm (Fig. 2A). As little as 0.5 g wet weight of tissue was needed, and the acquisition time was shortened 5-fold. Example narrow-range HSQC spectra are shown for *Pontoscolex corethrurus* and *L. terrestris* (Fig. 2B). The spectra for all species are given as Fig. S2, online supplementary information. In all cases the NMR spectra show several N-trimethyl peaks; the *L. terrestris* sample had the greatest number, with 13 putative compounds.

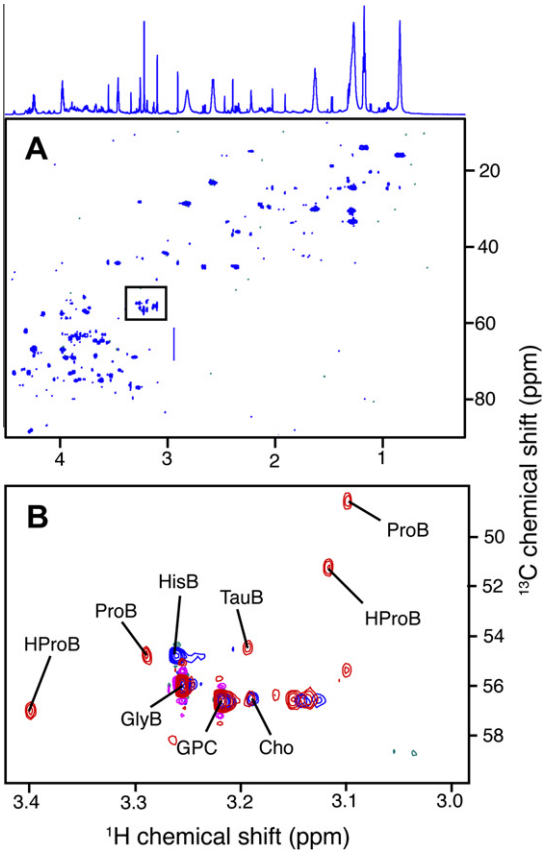

Fig. 2. (A) $^{1}H–^{13}C$ HSQC spectrum of an earthworm tissue extract (aliphatic region only shown here), showing the complexity of the earthworm metabolome. The inset rectangle marks the chemical shift region chosen for a restricted-range HSQC for betaine analysis. (**B**) Overlay of restricted-range spectra for *L. terrestris* (Lumbricidae) (red) and *Pontoscolex corethrurus* (Glossoscolecidae) (blue). Individual assigned metabolites are indicated directly on the plot: proline-betaine (ProB), hydroxyproline-betaine (HProB), choline (Cho), glycerophosphocholine (GPC), taurine-betaine (TauB), glycine-betaine (GlyB), and histidine-betaine (HisB). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

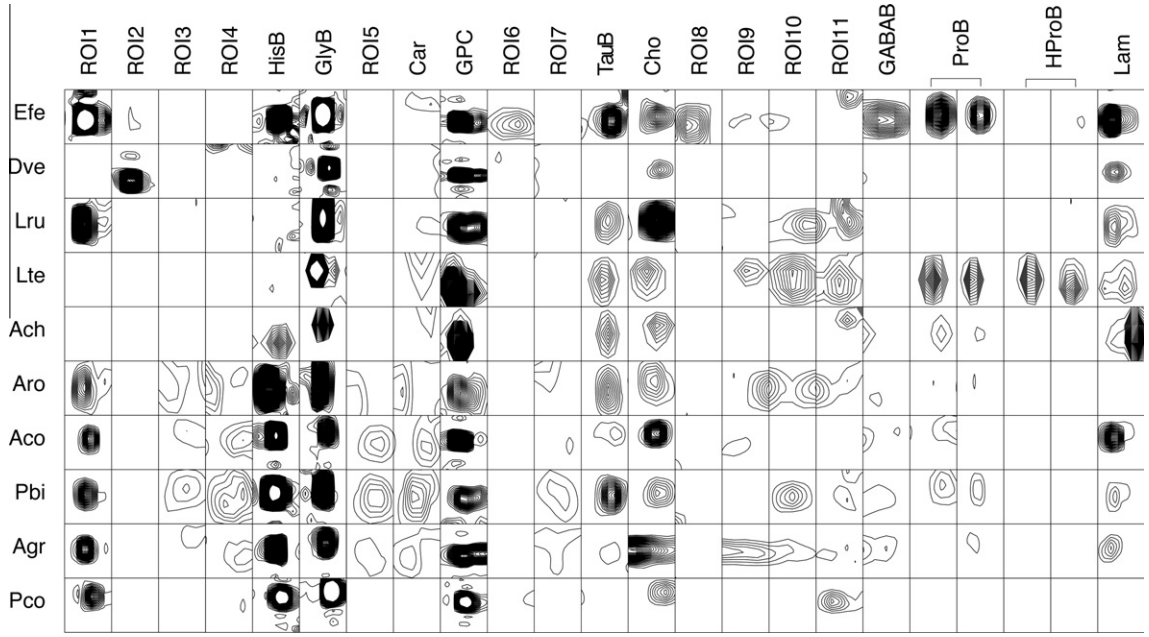

Fig. 3. Betaine metabolites are highly diverse considered across multiple earthworm taxa. Individual spectral trimethylammonium peaks from betaine metabolites (columns) observed in 10 different earthworm tissue extracts (rows). Chemical shift values for individual regions of interest (ROI) are given in Table 2. Metabolites labeled on plot: HisB – histidine-betaine, GlyB – glycine-betaine, Car – carnitine, GPC – glycerophosphocholine, TauB – taurine-betaine, Cho – choline, GABAB – GABA betaine, ProB – proline-betaine, HProB – hydroxyproline-betaine. Earthworm species are listed by the first letter of the genus and the first two letters of the species name.

Table 1
Assigned betaine metabolites and signals from putative unassigned betaine compounds found in earthworm species. Relative concentrations with respect to glycine-betaine; dash indicates no peak observed. Chemical shift values for individual regions of interest (ROIs) are given in Table 2.

	Trimethyl-N metabolite	ROI1	ROI2	ROI3	ROI4	HisB	GlyB	ROI5	Car	GPC	ROI6	ROI7	TauB	Cho	ROI8	ROI9	ROI10	ROI11	GABAB	ProB	HProB	Lam
L	*E. fetida*	1.02	0.00	–	–	0.22	1	–	0.00	0.26	0.02	–	0.12	0.04	0.04	–	–	0.02	0.05	0.07	–	0.17
	D. veneta	–	0.48	–	0.04	0.04	1	–	0.02	0.63	–	–	–	0.05	–	–	–	–	–	–	–	0.08
	L. rubellus	0.40	–	–	–	0.02	1	–	0.00	0.47	–	–	0.06	0.30	–	–	0.06	0.13	–	–	–	0.08
	L. terrestris	–	–	–	–	–	1	–	0.02	0.63	–	–	0.04	0.04	–	0.01	0.09	0.09	–	0.05	0.06	0.04
	A. chlorotica	–	–	–	–	0.68	1	–	0.11	3.19	–	–	0.32	0.33	–	–	–	0.35	0.06	0.12	–	2.22
M	*A. rodericensis*	0.28	–	0.08	0.08	2.02	1	0.04	0.07	0.54	–	0.02	0.18	0.13	–	0.09	0.15	0.11	–	0.00	–	–
	P. bicincta	0.55	–	0.08	0.22	4.20	1	0.12	0.14	2.38	–	0.01	0.11	0.75	0.01	0.04	–	0.00	0.04	0.11	–	0.68
	A. corticis	0.28	–	0.07	0.19	2.70	1	0.09	0.12	0.57	–	0.03	0.31	0.10	0.01	–	0.08	0.04	0.02	0.06	–	0.06
	A. gracilis	0.68	–	0.03	0.10	1.85	1	0.04	0.08	1.89	–	0.05	0.05	1.47	–	0.30	0.14	0.03	0.08	–	–	0.12
G	*P. corethrurus*	0.31	–	0.00	0.00	0.69	1	–	–	0.68	0.01	–	0.01	0.13	–	–	–	0.15	–	–	–	–

All spectra contained signals from the trimethylammonium metabolites choline, glycerophosphocholine, and glycine-betaine. However, the diversity of betaine compounds was, like in the 1D spectra, very obvious (Fig. 3).

We aimed to establish a fast pipeline from sample to relative quantities of betaines. After the HSQC acquisition we used rNMR, a freely available software package for 2D NMR processing. By the use of user-defined regions of interests (ROIs) it is possible to compare many peaks across multiple spectra (Fig. 3) and extract peak integrals for relative quantification (Table 1). A comprehensive ROI list in the rNMR environment was established for earthworm extracts based on cross-peak comparisons derived from betaine standards. The quantification within 2D NMR spectra overcomes deconvolution problems in 1D ^{1}H NMR spectra and makes it possible to describe in detail the betaine composition of the different species. As well as the compounds already described, we detected signals from GABA-betaine, histidine-betaine, taurine-betaine, proline-betaine, hydroxyproline-betaine, and Nε,-Nε,Nε-trimethyllysine (laminine). Chemical shift values for these metabolites, the unassigned ROI peaks, and the additional betaine chemical standards are given in Table 2.

Glycine-betaine is the only betaine compound that was detected in all of the species with relatively high abundance. A remarkable difference at the family level is the presence of a second major betaine metabolite in megascolecid and glossoscolecid earthworms, immediately downfield of the glycine-betaine peak. We identified this metabolite as histidine-betaine (Nα,Nα,Nα-trimethylhistidine, or hercynine). It is absent or appears only at low levels in lumbricid worms (Table 1). Conversely, Nα,Nα-dimethylhistidine (DMH) was present at high concentration in all lumbricid earthworms tested, but nearly absent in megascolecid and glossoscolecid earthworms (singlet peak at 2.94 ppm; data not shown). DMH was previously tentatively assigned in *L. rubellus* [10]. We were unable to obtain a pure commercial standard of DMH, but confirmed the assignment here by, firstly, some of the dimethyl product being formed in the incomplete synthesis reaction; and, secondly, matching mass spectra obtained from GC–MS of tissue extracts to standards in the NIST database (data not shown). Some betaine metabolites were only detected in a single species, e.g. hydroxyproline-betaine in *L. terrestris*, or an unknown compound in *D. veneta* (ROI2). Others, like proline-betaine, taurine-betaine and laminine, were present in multiple species.

Table 2
List of trimethylammonium ^{1}H and ^{13}C chemical shifts for a range of betaine metabolites: data acquired using authentic standards (^{13}C data not available for spermine-betaine and putrescine-betaine). Chemical shifts are also listed for putative unassigned betaine metabolites from earthworm extracts, identified here as individual regions of interest (ROI). Selected metabolites also have ^{13}C chemical shifts listed for the $RCH_2N(CH_3)_3$ methylene carbon.

	^{1}H (–NMe_3)	^{13}C (–NMe_3)	^{13}C (–CH_2–N)
Hydroxyproline-betaine	3.116	51.2	75.2
	3.401	57.1	77.4
Proline-betaine	3.099	48.1	77.7
	3.287	54.8	75.3
Histidine-betaine	3.265	55.2	
Glycine-betaine	3.256	55.9	68.8
Carnitine	3.220	56.6	72.8
Glycerophosphocholine	3.211	56.6	68.6
Phosphocholine	3.207	57.2	68.9
Taurine-betaine	3.193	54.7	69.2
Choline	3.190	56.1	70.1
GABA-betaine	3.121	55.2	68.5
Laminine	3.108	55.5	68.2
Lysine-betaine	3.121	54.9	
	3.165	55.7	
Beta-Alanine-betaine	3.115	56.1	
Alanine-betaine	3.180	55.3	
Aspartate-betaine	3.189	54.9	
Citrulline-betaine	3.173	54.6	
3-Aminobutyric acid-betaine	3.080	56.1	
Beta-Aminoisobutyric acid-betaine	3.107	56.5	73.0
6-Aminohexanoic acid-betaine	3.098	56.2	70.1
Gamma-Aminovaleric acid-betaine	3.100	56.4	
Ornithine-betaine	3.170	55.3	
	3.129	56.2	
Spermine-betaine	3.210		
	3.250		
Putrescine-betaine	3.231		
	3.269		
ROI1	3.340	46.1	
ROI2	3.340	56.3	74.9
ROI3	3.290	55.5	
ROI4	3.270	57.7	
ROI5	3.240	57.7	
ROI6	3.210	54.7	
ROI7	3.210	56.8	
ROI8	3.138	56.6	
ROI9	3.170	56.7	67.0
ROI10	3.160	56.8	68.0
ROI11	3.140	56.7	67.9

4. Discussion

Our final analytical method was fast, relatively sensitive, and can detect a whole range of betaines, including novel or unassigned compounds. The final acquisition time was reduced to 25 min, but still gave comparable signal-to-noise ratio to a two-hour HSQC experiment that covered the full chemical shift range. In fact, by reducing the chemical shift range and number of increments still further (e.g. to 8 ppm and 16 increments), we could reduce the total acquisition time to around 3 min per sample. The signal:noise ratio was slightly worse in this case, and so because we were only analyzing a small number of samples, we chose to use the 25-min experiment. However, this could be very useful in the future if higher sample-throughput were required. We did not attempt to measure absolute concentrations in our current study – however, it would be relatively straightforward to use an internal standard to obtain molar concentrations for future work. Ideally, this should be calibrated against each analyte of interest, or could be used in combination with a standard addition procedure [21].

Previous studies have given lists of NMR chemical shifts for betaine compounds – e.g. Blunden et al. [1] had extensive data on a range of metabolites. However, these often give only proton chemical shifts – and as we have shown, this is often not sufficient for confident identification. What is more, we observed slight differences in the chemical shifts that we measured compared to previous studies. This is not uncommon for NMR of biological extracts, and therefore we decided to present both ^{1}H and ^{13}C chemical shifts for 20 metabolites (16 chemicals that we synthesized, plus four compounds that we obtained from commercial suppliers). This list should be of value for future NMR-based studies.

Screening 10 different earthworm species showed surprisingly high biochemical diversity in the number of betaines found as well as their quantitative distribution. The detection of multiple abundant betaines per species indicates the presence of a specialized and regulated system, possibly related to osmoregulatory function. Earthworms can undergo considerable changes in their tissue moisture levels depending on the environment [22], so it would be expected that they would possess biochemical adaptations to deal with this.

There is very little known about betaine metabolites (other than glycine-betaine) in animals. Most work has been performed on plants, especially marine algae, and such animal studies as there are tend to focus on marine organisms [23,24]. Other betaines such as proline-betaine in mammals have been ascribed to diet [25]. We are unable to say if the betaines are produced by earthworm enzymes, or if they are acquired from the diet, or from associated gut microbes. However, we think that it is probable that they are the direct result of earthworm metabolism. This is because the earthworms were from very different sources, and did not all have identical diets. The presence of a wide range of betaine compounds argues for the existence of methyltransferase enzymes with low specificity. Alternatively, gene duplication could lead to a paralogous series of enzymes with related function. Our current study is essentially qualitative, and does not attempt to define robust species differences in betaine composition that are maintained across different conditions. However, earthworms are an excellent biological group in which to study evolutionary adaptations, as they have wide dispersion across diverse environments with restricted population migration. There is wide genetic diversity even within defined species [26]. We speculate that the diversity of betaines could provide an interesting biochemical phenotype for studying earthworm adaptation and speciation. For instance, we observed histidine-betaine in non-lumbricid species, and the related metabolite DMH in the lumbricid species. Histidine-betaine is found in fungi [27], as is the related metabolite ergothioneine, but it has not been reported as an animal metabolite, that we are aware of. This family-level difference could well be the result of a slight alteration in enzyme function.

Acknowledgments

We thank Emma Sherlock (Natural History Museum, London, UK) for her invaluable help in identifying worm species; the Director and Trustees of the Royal Botanic Gardens, Kew, London, UK, for access for worm collection; David Spurgeon (CEH Wallingford, UK) for provision of *L. rubellus* worms; and Peter Kille (University of Cardiff, UK) for provision of *A. gracilis*. This study was supported by the Natural Environment Research Council (NERC), under grant NE/H009973/1.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.12.049.

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