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As-resistance in laboratory-reared F1, F2 and F3 generation offspring of the earthworm *Lumbricus rubellus* inhabiting an As-contaminated mine soil

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ABSTRACT

Previous studies provided no unequivocal evidence demonstrating that field populations of *Lumbricus rubellus* Hoffmeister (1843), exhibit genetically inherited resistance to As-toxicity. In this study F1, F2 and F3 generation offspring derived from adults inhabiting As-contaminated field soil were resistant when exposed to 2000 mg kg^{-1} sodium arsenate. The offspring of uncontaminated adults were not As-resistant. Cocoon viability was 80% for F1 and 82% for F2 offspring from As-contaminated adults and 59% in the F1 control population. High energy synchrotron analysis was used to determine whether ligand complexation of As differed in samples of: resistant mine-site adults, the resistant F1 and F2 offspring of the mine-site earthworms exposed to the LC_{25} sodium arsenate (700 mg kg^{-1}) of the F1 parental generation; and adult *L. rubellus* from an uncontaminated site exposed to LC_{25} concentrations of sodium arsenate (50 mg kg^{-1}). XANES and EXAFS indicated that As was present as a sulfur-coordinated species.

1. Introduction

Earthworms have particularly intimate contact with soil, consuming large quantities of it, and their highly respiratory gas-permeable body surface provides an ineffective physical barrier to most inorganic constituents of pore water (Peijnenburg, 2002). Earthworms are widely used as indicators of soil contamination (Spurgeon et al., 2003). This, together with their status as ecosystem engineers (Jouquet et al., 2005) exerting major roles in the development and maintenance of soil physical structure, in nutrient cycling, and promotion of the activities of soil microflora makes them valuable indicators of soil quality and health. Through their activities earthworms can influence the speciation mobilities of soil-borne inorganic contaminants (Wen et al., 2004).

Arsenic is a widely distributed metalloid in many soils, with a mean concentration of around 5 mg kg^{-1} (Alloway and Ayers, 1994). However, much higher concentrations can occur in mine spoils and areas contaminated by industrial wastes. Under aerobic conditions inorganic arsenic is present predominantly as the phosphate-analogue, arsenate; but in anoxic soils the more toxic, S-seeking, reduced form, arsenite, predominates. Populations of the epigeic earthworm species, *L. rubellus* and *Dendrodrilus rubidus*, have been found inhabiting arsenic-rich mine-associated soils in the UK (Langdon et al., 1999, 2001, 2003; Button et al., 2009; Watts et al., 2008), with *L. rubellus* from Devon Great Consols mine and an uncontaminated site found to have LC_{50} values of 1510 mg As kg^{-1} and 96 mg As kg^{-1}, respectively (Langdon et al., 2001).

Evidence exists demonstrating that certain aquatic and terrestrial invertebrate species, including freshwater (Martinez and Levinton, 1996) and terrestrial (Rożen, 2006) oligochaetes, with multi-generational exposure histories can evolve site-specific heritable resistance to elevated Cd, Cu, or Zn levels in their native environments (reviewed by: Janssens, 2008). The criterion making this handful of published observations persuasive is that they were made on laboratory bred offspring from field populations and cultured until experimentally challenged on media devoid of stress evoking levels of the subject metal. Confirming the heritability of resistance traits is crucial for distinguishing between adaptation (i.e. genetically based resistance) and acclimation (i.e. tolerance conferred by a broad physiological response amplitude) (Morgan

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et al., 2007). There is an indication that a population of *L. rubellus* resident on an As-contaminated soil has evolved metalloid resistance (Langdon et al., 2003), but the evidence should be considered inconclusive because the observations were made on F1 adult offspring where the possibility of maternal confounding effects cannot be discounted (Morgan et al., 2007).

The primary aim of the present study was to determine whether the F1, F2 and F3 adult offspring of *L. rubellus* colonizing a highly As-contaminated soil, associated with a mine whose activities have been discontinued for several decades (Hamilton, 2000), are significantly less susceptible to acute As exposure than the equivalent progeny of parental earthworms with no field history of As exposure. No previous study has examined second and third generation earthworms to establish whether they have unequivocally inherited metal- or As-resistance traits from their parents.

A survey of the invertebrate literature indicates that genetically differentiated populations express a number of physiological strategies for resisting inorganic contaminants in their native environments. These range from avoidance behaviour in the freshwater pulmonate snail *Physella columbiana* (Lefcort et al., 2004), to enhanced excretion or periodic-shedding of immobilized metal in the collembolan *Orchesella cincta* (Posthuma et al., 1992), and higher turnover of Cd-sequestering metallothionein (MT) in the marine polychaete *Hediste diversicolor* (Mouneyrac et al., 2003). However, increased bio-immobilization capacity may be the most common strategy, as exemplified by the enhanced ability of a resistant ecotype of the freshwater oligochaete *Lumbriculus hoffmeisteri* to reduce the solubility of Cd (Wallace et al., 1998), a mechanism that is probably supported by MT gene-amplification (Martinez and Levinton, 1996). Modifications of the promoter regions of the MT gene in Cd-resistant populations of *O. cincta* can also increase the efficiency of the expression of the protective metalloprotein (Roelofs et al., 2007).

Few biochemical or physiological studies have been made on As-resistance mechanisms in earthworms. Electron microprobe (Morgan et al., 1994), biochemical (Langdon et al., 2002), and immuno-localisation studies (Langdon et al., 2005) have demonstrated that As^{3+} thiol complexes are probably involved in resistance. It has been shown that cysteine-rich MT is a significant thiol donator. Two MT isoforms have been characterised in adult *L. rubellus* (Sturzenbaum et al., 2004), but it is not presently known whether As in any of its chemical forms is able directly to induce the expression of either gene. A recent study indicated that speciation of As in earthworm tissues may be multi-phasic (Watts et al., 2008). Langdon et al. (2005) in a preliminary synchrotron-based X-ray absorption spectroscopy (XAS) study observed that As is accumulated by earthworms in the form of arsenobetaine, but that –SH coordinated species corresponding with MT complexation were also present in certain tissues.

The second aim of this study was to deploy XAS to determine whether the ligand-binding speciation of As in laboratory-reared F1 and F2 generation offspring derived from *L. rubellus* adult worms sampled from an As-contaminated mine soil reflects the multi-generational exposure history of the parental population. This was achieved by comparing the XAS spectra of adult F1 and F2 earthworms from contaminated site and uncontaminated site parents after identical experimental exposures to an LC_{25} concentration of As. Our aim was to provide some insight into population-specific heritable differences, if they exist, in As metabolism and detoxification at the molecular level.

2. Materials and methods

2.1. Earthworm collection

Mature *L. rubellus* were collected by digging and hand sorting from an arsenic-contaminated location at Devon Great Consols, an abandoned copper and arsenic mine near Tavistock, Devon, UK (Ordnance Survey Grid Reference SX 423736) (Hamilton, 2000). The earthworms were transported to the laboratory in their native soil. Reference worms were purchased from an earthworm supplier (Ecology Earthworms, Hubbards Hall Farm, Bently, Ipswich, IP9 2LS, UK).

2.2. Laboratory culture of F1 and F2 generation *L. rubellus*

Field-collected (Devon Great Consols) clitellate non-moribund individuals were cultured ($n = 4$ per vessel) in twelve 600 ml vessels (diameter 125 mm, depth 50 mm) with sealable lids (pierced with a mounted needle to allow ventilation) in a temperature-controlled incubator (450l Series 3 Cooled Incubator, LMS Ltd, Kent, UK) at 15 °C in 24 h darkness. Culture vessels contained pre-sterilised Kettering loam soil (Broughton Loam, Kettering, UK) with a moisture content of 25–30%. Approximately 30–40 g of rewetted oven-dried (at 105 °C), urine-free horse manure (produced from a single known horse not subject to any medication) was applied to the soil surface. Individual biomass was recorded at the outset and at subsequent sampling when survival was also recorded.

Cocoons were collected after 21 and 50 days by wet sieving the culture substrate through a series of graded sieves (6.7, 3.35 and 2.00 mm). Cocoons were placed in Petri dishes on filter paper (Whatman no. 1), provided with excess water to prevent dehydration and maintained at 15 °C in 24 h darkness. The dishes were checked regularly for hatchlings, allowing calculations to be made on viability and length of incubation. The latter was calculated as time to hatch plus half the time between sampling periods.

On emergence F1 generation hatchlings were transferred to 250 ml plastic vessels with sealable lids (ventilated as above) containing a soil and horse manure mixture. Hatchlings were kept at 4 °C to impede development until all viable cocoons from a sampling period were spent at which time hatchlings were transferred to 15 °C ($n = 4$ per vessel) in 600 ml vessels (as for field-collected adults). Earthworm condition and sexual development were monitored every 12–14 weeks, when soil and feed were replaced with fresh material. On maturation (determined by the presence of a swollen clitellum) the process of cocoon collection, incubation and growth of F2 generation hatchlings was repeated following the procedure described for F1 generation. A control group of F1 laboratory-reared *L. rubellus* (produced by uncontaminated *L. rubellus* from a commercial supplier) were also cultured following the same protocols.

The F3 Devon earthworms were cultured as above. There were no F3 *L. rubellus* originally from Devon in the Daresbury EXAFS (extended X-ray absorption fine structure) and XANES (X-ray absorption near edge structure) as funding for this aspect of the research terminated before the F3 generation *L. rubellus* were produced.

2.3. Toxicity tests

Soil purchased from Broughton Loam, Huntingdon, Cambridgeshire, UK was partially air-dried, sieved through a 2.8 mm mesh, and rewetted to a moisture content of 53% (dry weight equiv.) using a solution of sodium arsenate ($Na_2HAsO_4{\cdot}7H_2O$) to give a concentration of 2000 mg As kg^{-1} dry weight of soil. Mean soil pH (aqueous suspension) ± standard error (SE) was 5.43 ± 0.05. Moistened soil (69 g) was weighed into each of a series of 20 × 25 cm polythene bags. The bags were placed into plastic flower pots to give some rigidity.

One *L. rubellus* was introduced into each bag, the earthworms being assigned to treatments at random (treated and uncontaminated soil). Mean body weights ± SE ($n = 10$) were: uncontaminated culture 0.844 ± 0.044 and As-treated 0.779 ± 0.049 mg, Devon Great Consols F1 control 0.822 ± 0.043 Arsenic treated 0.899 ± 0.059, F2 control 0.817 ± 0.020 As-treated 0.766 ± 0.035 and F3 control 0.662 ± 0.035 As-treated 0.597 ± 0.033 mg, with 10 earthworms used per treatment. The bags were kept for 28 d at 12 °C with a 53% dry weight equivalent moisture content. Specimens were examined at weekly intervals and assigned one of the condition index scores, 0 = dead, 1 = moribund (flaccid, unresponsive to tactile stimulation) and 2 = turgid (responsive to tactile stimulation) as detailed in Langdon et al., 2001. Assessment was carried out "blind", with an assistant presenting the specimens for assessment to the recorder.

2.4. EXAFS and XANES

L. rubellus adults collected from Devon Great Consols were cultured in uncontaminated soil to produce F1 and F2 generation offspring. The F1 and F2 generation adults were placed in soils treated with 2000 mg kg^{-1} (see Methods above) or the LC_{25} concentration of sodium arsenate calculated from Devon Great Consols (700 mg kg^{-1}) (Langdon et al., 2002). Earthworms were extracted from soil and kept on moist tissue paper for 24 h to evacuate their guts prior to sample preparation. Whole earthworms, body wall sections and anterior and posterior gut dissections from the adult earthworms were frozen in liquid nitrogen prior to experimental analysis. F1 and F2 generation adults from an uncontaminated population were cultured and prepared as above with the equivalent LC_{25} concentration of sodium arsenate (50 mg kg^{-1}) (Langdon et al., 2002).

X-ray absorption spectra at the As K-edge were collected on Station 16.5 at the CLRC Daresbury SRS operating at 2 GeV with an average current of 140 mA, using