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High-throughput gene expression in soil invertebrate embryos – Mechanisms of Cd toxicity in *Enchytraeus crypticus*

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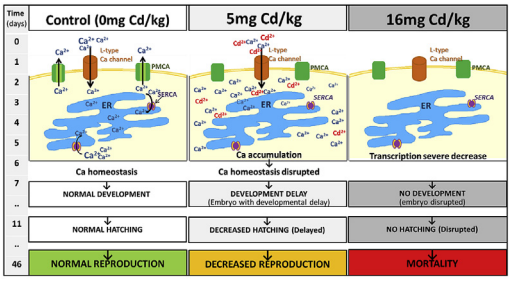
HIGHLIGHTS

- *E. crypticus* embryos are more sensitive to Cd than adults.
- Embryos' mechanisms were studied using high-throughput gene expression.
- Major modulators of Cd toxicity were identified in the embryos.
- Cd competes with Ca inside the cells (novel), besides extracellular (known).
- Adverse Outcome Pathway (AOP) was improved.

GRAPHICAL ABSTRACT



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ABSTRACT

Gene expression can vary with the organisms' life stage. It is known that embryos can be more sensitive to toxicant exposure, as previously demonstrated for *Enchytraeus crypticus* (Oligochaeta) exposed to cadmium (Cd), known to cause embryotoxicity and hatching delay. It was shown that Ca enters embryos via the L-type Ca channels in the cocoon membrane, this being affected in Cd exposed embryos (Cd-Ca competition is well-known). In the present study, the embryotoxic mechanisms of Cd were studied via high-throughput gene expression for *E. crypticus*. Cocoons (1–2 days old), instead of the adult organism, were exposed in Cd spiked LUFA 2.2 soil during 1 day. Results showed that Cd affected Ca homeostasis which is implicated in several other molecular processes. Several of the major modulators of Cd toxicity (e.g., impaired gene expression, cell cycle arrest, DNA and mitochondrial damage) were identified in the embryos showing its relevancy as a model in ecotoxicogenomics. The draft Adverse Outcome Pathway was improved. Previously was hypothesized that gene regulation mechanisms were activated to synthesize more Ca channel proteins – this was confirmed here. Further, novel evidences were that, besides the extracellular competition, Cd competes intracellularly which causes a reduction in Ca efflux, and potentiates Cd embryotoxicity.

1. Introduction

Gene regulation is known to differ with developmental stage, for instance in the fruit fly *Drosophila melanogaster*, methyltransferase expression occurs only in early embryo stages, restricting DNA methylation to the first developmental stages

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(Lyko, 2001). The developmental regulation of gene expression is also widely reported in organisms with distinct morphology along life cycle stages across taxonomical kingdoms, e.g., in African trypanosome species (Jackson et al., 2015), in the pathogenic fungus *Batrachochytrium dendrobatidis* (Rosenblum et al., 2008), or in the Atlantic salmon (*Salmo salar*) (Aubin-Horth et al., 2009).

In ecotoxicology, the standard guidelines focus mostly on the adult or juvenile life stage of organisms e.g. the enchytraeid reproduction test (ERT) (ISO, 2005; OECD, 2004a) or the acute toxicity test for earthworms (ISO, 1993). However, knowing that the life cycle of the organism is important, there have been, over the last decade, efforts to cover developmental stages in testing. For the aquatic environment, an example of this is the embryotoxicity test (Fish Embryo Toxicity, FET) that has emerged as an alternative approach to the classical standard fish acute testing (Lammer et al., 2009; OECD, 2013). For the terrestrial environment, examples are the embryotoxicity test for the snails, slugs and soil worms (Druart et al., 2010; Gonçalves et al., 2015). In addition, detailed full life cycle tests are also recently available, e.g. for the worm *E. crypticus* (Bicho et al., 2015) covering various developmental stages.

At the same time, there is increasing realization that the focus on apical endpoints cannot provide answers on the underlying biological responses. Hence, it is necessary to combine different lines of evidence to predict and understand hazardous properties. Since it is, as described above, well known that gene regulation differs with the developmental stage, it is logical to combine the approaches i.e. explore the omics at the different life stage while under toxicological pressure.

In the present study we use the soil worm *Enchytraeus crypticus* for which there are both tests for various developmental stages (see above) and well tested high throughput genomic tools (Gomes et al., 2018, 2017). This means that all necessary tools are available to perform highly integrative studies, allowing the linkage of the underlying biological responses to developmental stage specific apical effects, and building up towards a system toxicology approach (Sturla et al., 2014).

The test chemical used was Cadmium, both because the underlying biological mechanism is relatively well understood for adult organisms (e.g. (Cambier et al., 2010; Dabas et al., 2014; Fourie et al., 2007; Jia et al., 2011; Muangphra and Gooneratne, 2011; Novais et al., 2011)), but also because it was shown (Bicho et al., 2015; Gonçalves et al., 2015) that the embryos were the most sensitive developmental stage for *Enchytraeus crypticus*.

Hence in the present study the mechanisms of gene regulation to Cd were studied in *E. crypticus* embryos. The cocoons (containing the embryos) were exposed to Cd for 1 day (5 and 16 mg Cd/kg soil, concentrations known to affect hatching at 80 and 100%, via delay and disruption of embryo development, respectively (Gonçalves et al., 2015)). The high-throughput gene expression microarray (Gomes et al., 2018, 2017) was used. This is the first time this is done for a soil invertebrate to the best of our knowledge.

In summary, we aimed to answer the following: 1) Is it feasible to use the method in embryos? 2) What is the gene-expression pattern in embryo life stage of *E. crypticus*? and 3) What is the Cd-embryo gene profile.

2. Materials and methods

2.1. Test organism

Enchytraeus crypticus (Enchytraeidae, Oligochaeta) was used. The cultures were kept in agar, consisting of Bacti-Agar medium (Oxoid, Agar No. 1) and a sterilized mixture of four different salt solutions at the final concentrations of 2 mM $CaCl_2 \cdot 2H_2O$, 1 mM $MgSO_4$, 0.08 mM KCl, and 0.75 mM $NaHCO_3$, at the temperature of 19 ± 1 °C with photoperiod of 16:8 h light:dark. Cultures were fed on ground autoclaved oats twice per week.

2.2. Test soil and spiking procedure

The natural standard LUFA 2.2 soil (Speyer, Germany) was used for the toxicity test, and had the following main characteristics: pH (0.01 M $CaCl_2$) = 5.4; organic carbon = 1.61%, cation exchange capacity (CEC) = 9.7 meq/100 g, maximum water holding capacity (maxWHC) = 44.8%, and grain size distribution of 8% clay (<0.002 mm), 15.8% silt (0.002–0.05 mm), and 76.2% sand (0.05–2.0 mm).

Cadmium chloride hemi-pentahydrate ($CdCl_2 \cdot 2^{1/2}H_2O$) was purchased from Sigma-Aldrich (Fluka, 98% purity) and tested at 0, 5, and 16 mg Cd/kg soil dry weight (mg/kg soil DW). The concentrations were selected based on known embryo-toxicity effects, i.e. hatching delay at 5 mg Cd/kg and total embryo disruption at 16 mg Cd/kg (Bicho et al., 2015; Gonçalves et al., 2015), to include two different effects. Cd was added to pre-moistened soil as aqueous solutions. Stock solution was prepared with deionized water and serially diluted. Soil batches per concentration were homogeneously mixed and allowed to equilibrate for 3 days after which water was added up to 50% of the soil maxWHC.

2.3. Exposure details

Ten cocoons of 1–2 days old (for details on culture synchronization see (Bicho et al., 2015)) were introduced in each well (6-well plates, 35 mm ø) containing 5 g of moistened soil, in a random design. Four biological replicates were performed per test condition, each consisting of a pool of 300 cocoons. This corresponds to a total of 120 wells (1200 cocoons) per test condition, i.e. 3600 cocoons in total. Test performance followed the procedures described in Gonçalves et al. (2015). In short, the cocoons were evenly distributed (in groups of 2) and covered with soil to avoid dehydration and ensure exposure. Exposure lasted 1 day (corresponding to days 2–3 of embryonic development) under controlled conditions of photoperiod (16:8 h light:dark) and temperature (20 ± 1 °C). After that, the cocoons were carefully removed from the soil to a Petri dish with reconstituted water (ISO water (OECD, 2004b)) to remove soil particles, snap frozen and stored at −80 °C until further analysis.

2.4. Microarrays analysis

2.4.1. RNA extraction, labelling and hybridizations

RNA was extracted from each replicate (300 cocoons, to ensure enough material). Three biological replicates per test treatment (from the 4 performed) were used. Total RNA was extracted using SV Total RNA Isolation System (Promega). The quantity and purity of the isolated RNA were measured spectrophotometrically with a nanodrop (NanoDrop ND-1000 Spectrophotometer) and its quality was checked on a denaturing formaldehyde agarose gel electrophoresis.

A single-colour design was used. In brief, 500 ng of total RNA was amplified and labelled with Agilent Low Input Quick Amp Labelling Kit (Agilent Technologies, Palo Alto, CA, USA). Positive controls were added with the Agilent one-colour RNA Spike-In Kit (Agilent Technologies, Palo Alto, CA, USA). Purification of the amplified and labelled cRNA was performed with the RNeasy columns (Qiagen, Valencia, CA, USA).

The cRNA samples were hybridized on the Custom Gene Expression Agilent Microarrays (4 × 44k format) developed for this species (Castro-Ferreira et al., 2014). Hybridizations were performed using the Agilent Gene Expression Hybridization Kit