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Freezing of body fluids induces metallothionein gene expression in earthworms (*Dendrobaena octaedra*)

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The molecular mechanisms activated by environmental contaminants and natural stressors such as freezing need to be investigated in order to better understand the mechanisms of interaction and potential effects that combined stressors may have on organisms. Using the freeze-tolerant earthworm *Dendrobaena octaedra* as model species, we exposed worms to freezing and exposure to sublethal copper in a factorial design and investigated the transcription of candidate genes for metal and cold stress. We hypothesised that both freezing and copper would induce transcription of genes coding for heat shock proteins (*hsp10* and *hsp70*), metallothioneins (*mt1* and *mt2*), and glutathione-S-transferase (*gst*), and that the combined effects of these two stressors would be additive. The gene transcripts *hsp10*, *hsp70*, and *gst* were significantly upregulated by freezing, but only *hsp10* was upregulated by copper. We found that copper at the time of sampling had no effect on transcription of two metallothionein genes whereas transcription was strongly upregulated by freezing. Moreover, there was a significant interaction causing more than additive transcription rates of *mt1* in the copper/freezing treatment suggesting that freeze-induced cellular dehydration increases the concentration of free copper ions in the cytosol. This metallothionein response to freezing is likely adaptive and possibly provides protection against freeze-induced elevated metal concentrations in the cytosol and excess ROS levels due to hypoxia during freezing.
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1. Introduction

Soil invertebrates of the cold temperate and subarctic regions that inhabit the superficial litter layer must tolerate long periods of subzero temperatures and recurring periods of thawing and freezing.

One such species, the earthworm *Dendrobaena octaedra* Savigny, has developed the ability to survive freezing of its body fluids (Berman et al., 2002; Rasmussen and Holmstrup, 2002). The biochemical mechanisms that support freeze tolerance in this species are the same as described for numerous other invertebrates and include accumulation of large stores of glycogen that can be converted to glucose serving both as a cryoprotectant and as fuel for anaerobic metabolism when oxygen supply is hindered by freezing of body fluids (Storey and Storey, 1990; Holmstrup et al., 2007a; Calderon et al., 2009). Metabolic rate of *D. octaedra* decreases to about 10% of aerobic rate at the same temperature which extends the period that glycogen reserves can support anaerobic metabolism and ensure survival in a frozen state for almost 6 months (Calderon et al., 2009).

Recently, our laboratory has focussed on the interactions between environmental contaminants (e.g. heavy metals) and natural stressors such as freezing and drought (Holmstrup et al., 2010; Laskowski et al., 2010). Emissions of heavy metals from smelters and other industrial sources have locally contaminated soils of northern Europe with impacts on the soil fauna (Bengtsson et al., 1983; Bengtsson and Rundgren, 1988; Spurgeon and Hopkin, 1999; Tosza et al., 2010). In addition to toxic effects of heavy metals *per se*, our studies show that sublethal concentrations of some heavy metals can reduce the ability of *D. octaedra* to tolerate freezing which adds another dimension to the problems of pollution (Bindesbøl et al., 2005, 2009; Noyes et al., 2009; Hooper et al., 2013). The molecular mechanisms activated by environmental contaminants (e.g. heavy metals) and natural stressors such as freezing and drought need to be investigated in order to better understand the mechanisms of interaction and potential effects that combined stressors will have on animals (Hooper et al., 2013). Here, we choose to investigate a number of candidate genes, representing such mechanisms.

It is well-known that anoxic or hypoxic conditions followed by reperfusion and high oxygen consumption may lead to generation of reactive oxygen species (ROS) that can cause oxidative damages such as denaturation of proteins and enzyme malfunctioning, lipid peroxidation, and damages to DNA (Hermes-Lima et al., 1998; Hermes-Lima, 2004). Freezing of body fluids also lead to hypoxic conditions because oxygen transport is limited in the frozen animal. The recurrence of oxic conditions upon thawing will subsequently initiate the generation

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of ROS which is a major problem for freeze-tolerant animals (Joanisse and Storey, 1996; English and Storey, 2003; Storey et al., 2013).

Earthworms living in soils polluted with copper take up free copper ions (Cu^{2+}) primarily across their skin (Vijver et al., 2003) through membrane-bound copper pumps (Vulpe and Packman, 1995). Once in the cytoplasm, Cu^{2+} is delivered to various cell targets with the help of copper chaperones. These chaperones include glutathione and metallothionein (Dallinger et al., 2000). The MT pathway is one of the most studied metal detoxifying mechanisms and known to be important for metal homeostasis in earthworms (Stürzenbaum et al., 1998, 2004; Spurgeon et al., 2004). Metallothioneins (MT) are cysteine-rich, low-molecular-weight proteins, which are known to have a high binding capacity for cadmium, copper, and probably also zinc (Dallinger et al., 2000; Spurgeon et al., 2004). If not bound to MT or other molecular chaperones, free cellular Cu^{2+} can result in generation of ROS via the Fenton reaction (Stohs and Bagchi, 1995; Valko et al., 2005).

It seems therefore that survival of both freezing and copper toxicity depends on efficient enzyme systems that can prevent generation of, or remove and detoxify ROS. We are particularly interested in uncovering the mechanisms underlying the interactions between effects of freezing and contaminants, and therefore have studied the transcription of genes involved in detoxification of ROS and heavy metals using *D. octaedra* as a model. In a factorial test design, we tested the effects of freezing and exposure for sublethal copper concentrations singly and in combination. We hypothesised that both freezing and copper would induce transcription of genes coding for heat shock proteins, metallothioneins, cytochrome oxidase, and glutathione-S-transferase, and that the combined effects of these two stressors would be additive.

2. Materials and methods

2.1. Earthworms and soil

Dendrobaena octaedra were collected near Valdemarsvik, south-east Sweden, in autumn 2009. About 15 adult worms were collected from each of three locations (about 1 km apart from each other) where the vegetation was dominated by mosses in mixed pine and birch forest. The area was not contaminated by human activities. The earthworms were transported to the laboratory in Silkeborg, Denmark, and were used for establishment of laboratory cultures.

The adult worms from the collection sites were cultured as three separate populations in uncontaminated agricultural soil at 15 °C and fed with cow dung as described by Fisker et al. (2011a). Cocoons were harvested every month and hatched in Petri dishes, of which the bottom was layered with wet filter paper. The soil used in this experiment originated from the agricultural research facilities at Askov, Denmark (see Fisker et al. (2011a) for further details on soil properties). The soil was dried for 24 h at 80 °C before it was re-watered to 18% of dry weight. Cow dung was dried and finely ground, and worm food was prepared by mixing moist soil, dried cow dung, and water in the ratio: 42:21:37 w/w/w. Soil for the copper treatment was spiked with anhydrous $CuCl_2$ (Cu^{2+}) by dissolving it in water before mixing it into the dry soil. The food for the copper-exposed worms was made on basis of the copper-spiked soil mixed with dried cow dung in the same ratio as for the control food. The soil for the experiment was made 1 day before use to equilibrate and was kept at 15 ± 1 °C.

2.2. Treatments

The present study used 3rd generation worms with an age of approximately 4 months to conduct a combined freeze and copper tolerance study. Worms were placed in either uncontaminated control soil or soil spiked with copper to a nominal concentration of 160 mg kg^{-1} dry soil. A previous study showed that this concentration of copper is sublethal and that worms exposed to soil spiked with 160 mg Cu kg^{-1} had an increased internal copper concentration (between 110 and 150 μg g^{-1} dry tissue) compared to worms from control soil (13–16 μg g^{-1} dry tissue)(Fisker et al., 2011b). The two groups were slowly acclimated to low temperatures and thereafter exposed to sub-zero temperatures. The worms were placed separately in 200 mL plastic beakers containing 70–75 g soil and roughly 4 g food (n = 10–15). Worms were acclimated at 10 °C for 1 week followed by 1 week at 5 °C, and finally 4 weeks at 2 °C. After the cold acclimation period, the worms were moved to 50 mL plastic beakers containing the same mixed moist soil and food (about 30 g fresh weight) and kept at 2 °C for two more weeks to ensure that the worms were cold hardy and would survive the freezing treatment (Holmstrup et al., 2007b). Half of the worms were kept at +2 °C as a control group, and the rest of the worms were placed in a walk-in freezer cabinet at −1 ± 0.2 °C for 16 h. A small piece of ice (50–100 mg) was added to the soil surface to seed the freezing of soil and worm. The worms were checked by prodding to confirm that their body fluids were frozen. Temperature was then lowered to −2 ± 0.2 °C and kept here for 14 h until they were sampled for analysis. At the end of the treatments, worms were quickly rinsed in demineralised water, placed in 2 mL centrifuge tubes, and snap frozen in liquid nitrogen. All thawed worms looked healthy and were responding to handling upon thawing. The samples were kept at −80 °C until further analysis.

2.3. RNA extraction and cDNA synthesis

Four replicate worms from each population and treatment were used for testing the relative gene expression. The samples were homogenised in 1 ml 50 mM phosphate buffer (pH 7.4) using a Tissue-lyser II with a steel bead at 30 Hz for 20 s (Qiagen, Copenhagen, Denmark). RNA extraction was done by using the RNeasy Mini kit with on-column DNAse treatment (Qiagen, Copenhagen, Denmark) according to the instructions provided by the manufacturer. The success of the DNAse treatment was later verified by the absence of amplifiable DNA in a qPCR assay on RNA samples. The concentration of RNA was determined by using an Implen NanoPhotometer spectrophotometer (AH Diagnostics, Aarhus, Denmark). By following the manufacturer's instructions, cDNA was synthesised from 1.2 μg total RNA using the Omniscript Reverse Transcriptase kit (Qiagen) and Anchored $Oligo(dT)_{20}$ primers (Invitrogen A/S, Taastrup, Denmark). Furthermore, cDNA was diluted 14-fold, to a concentration equivalent to 4 ng total RNA μL^{-1}, and stored at −20 °C until further use.

2.4. Relative quantification of messenger RNA (qPCR)

The sequences of the analysed genes were obtained from a transcriptome of *D. octaedra* (M. Holmstrup et al., unpublished). Preliminary annotated isotigs were identified and further verified by blastx analyses against the non-redundant protein database in GenBank (http://www.ncbi.nlm.nih.gov/genbank/). E-values for these blastx analyses are listed in Table 1. Primers were designed by using Primer3 (Rozen and Skaletsky, 2000) and were synthesised by MWG (Ebersberg, Germany). Primer sequences for the target genes are listed in Table 1. Details of these analyses are presented in Fisker et al. (2013).

Stratagene Brilliant® II SYBR® Green qPCR Mastermix (AH Diagnostics, Aarhus, Denmark) was used for real-time quantitative polymerase chain reaction (qPCR) conducted on a Stratagene MX3005P (AH Diagnostics, Aarhus, Denmark). Each reaction contained 5 μL of cDNA template (equivalent to 20 ng total RNA) along with 900 nM primers in a final volume of 15 μL and these reactions were run in duplicate. The amplification was performed under the following conditions: 95 °C for 10 min to activate the DNA polymerase, then 40 cycles of 95 °C for 10 s and 60 °C for 60 s. Melting curves of the raw qPCR data were inspected to confirm the presence of a single amplification product with no primer-dimers. Furthermore, all products were run on a 1% agarose gel to verify that only one PCR product was present. No major

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