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No costs on freeze tolerance in genetically copper adapted earthworm populations (*Dendrobaena octaedra*)

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A B S T R A C T

For nearly three centuries the area around Gusum, in south-east Sweden, has been highly polluted with copper. An earlier study in this area showed that populations of the freeze-tolerant earthworm *Dendrobaena octaedra* were genetically adapted to copper. Apparently, no life-history costs to reproduction or growth were imposed by this adaptation. In the present paper we therefore investigated how laboratory raised F1-generations of these populations coped when exposed to increased copper concentrations in the soil and to sub-zero temperatures. We found that *D. octaedra* from polluted sites accumulated the same amount of copper as reference worms. Furthermore, earthworms from polluted sites survived equally to reference worms when exposed to freezing temperatures (−8 or −12 °C). However, when simultaneously exposed to the lowest temperature and copper, the worms from polluted sites survived significantly better than reference worms. The overall conclusion of this study is that worms from polluted sites seem to be better at handling copper and accrue no costs in terms of reduced cold tolerance in connection to genetic adaptation in these populations.

1. Introduction

Atmospheric emissions from metal industry and smelters have caused pollution of the environment over large areas with several heavy metals (Hopkin, 1989). In terrestrial habitats metals are accumulated in the top-soil and litter layers meaning that species living there are potentially more exposed to emitted metals compared to species living in deeper soil layers (Bengtsson et al., 1983; Bengtsson and Rundgren, 1988; Hopkin, 1989). In these areas top-soil living species such as surface-dwelling earthworms will assimilate metals when feeding on soil and litter containing metals. The surface-dwelling earthworm *Dendrobaena octaedra* both lives and deposits its cocoons under moss in the top-soil and litter layer (Stöp-Bowitz, 1969; Rundgren, 1975; Edwards and Bohlen, 1996). Thus, this species is highly exposed to metals in polluted areas. Despite this, *D. octaedra* has been found at several metal contaminated sites in Europe, including Gusum in Sweden and Olkusz in Poland (Bengtsson et al., 1983, 1992; Rozen, 2006; Tosza et al., 2010; Holmstrup et al., 2011). This shows the potential of *D. octaedra* to adapt to metal exposure. Fisker et al. (2011) have recently shown that *D. octaedra* populations originating from contaminated sites near Gusum and tested in both control soil and copper polluted soil, had higher population growth rates as compared to reference populations, and thus apparently suffer no cost of adaptation. However, it is unknown how these populations differ from reference populations in tolerating and handling copper.

Overall, adaptations can be categorised into two different types: 1) physiological adjustment of the exposed individual (phenotypic acclimation) and 2) adaptation of a population during generations through alterations in genes and/or expression of genes (genetic adaptation). One classic example of acclimation response to heavy metal exposure is the induction of metallothionein, which is a protein that binds, and thereby, detoxifies free metals e.g. cadmium and copper (Dallinger, 1996; Dallinger et al., 2000). The induction of metallothionein enables the organism to tolerate a higher concentration of metals. In contrast to this, genetic adaptation of a population is expressed constitutively and could occur if a population is exposed to heavy metals through generations. Studies by Maroni et al. (1987) and Sterenborg and Roelofs (2003) have shown some examples of how populations could be genetically adapted to metals. Maroni et al. (1987) showed that *Drosophila* populations adapted to metals have duplicated the number of metallothionein genes and Sterenborg and Roelofs (2003) showed that *Orchesella cincta* originating from polluted sites had a significant increase in expression relative to the increase in the control population when fed with cadmium-contaminated algae.

Both acclimation and genetic adaptation are usually expected to entail benefits and costs (Posthuma and Van Straalen, 1993; Posthuma, 1997). The costs of phenotypic acclimation and genetic adaptation are defined as "costs of exposure" and "costs of tolerance", respectively (Posthuma and Van Straalen, 1993). Bindesbøl et al. (2005) have shown that *D. octaedra* exposed to sub-lethal effects of

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copper entailed costs of exposure, which were manifested as a decrease in freeze tolerance. When considering genetic adaptation to metals, costs of tolerance have often been suggested to appear as reduced tolerance to other stress factors (Posthuma and Van Straalen, 1993; Posthuma, 1997). A major natural stress factor of importance in northern regions is low winter temperatures that cause freezing of soil water. In that case surface dwelling earthworms face freezing of body water because external ice will inoculate freezing of internal compartments (Rasmussen and Holmstrup, 2002). For most earthworm species, freezing of body water is lethal, but *D. octaedra* has evolved freeze tolerance and endures temperatures as low as −20 °C (Holmstrup et al., 2007; Holmstrup and Overgaard, 2007). Having shown that *D. octaedra* from copper polluted areas near Gusum have better population growth rates than reference populations (Fisker et al., 2011) it is interesting to investigate if this genetic adaptation has costs in terms of reduced freeze tolerance.

In the present study we investigated laboratory raised F1-generations of *D. octaedra* populations from the copper polluted Gusum area in south-east Sweden. These populations were exposed to combinations of low temperatures (2, −8 and −12 °C) and copper (control and 160 mg Cu/kg dry soil) and their survival was compared to that of reference populations treated the same way. Furthermore, internal copper concentration of worms exposed to control temperature and copper was compared to study differences in accumulation of copper between populations. Overall, we test the hypothesis that populations of *D. octaedra* genetically adapted to copper accumulate copper differently and have a reduced tolerance of low temperatures compared to reference populations.

2. Materials and methods

2.1. Study area and field collected adults

The study was carried out using earthworms from south-east Sweden, near the village of Gusum. Since 1661 a brass mill has been operating in Gusum, which has resulted in pollution of the area with zinc, lead and especially copper. The old mill (OM) was replaced by a new mill (NM) in the 1960s, placed north-west of the old mill. The prevailing wind direction in the area is south-east, thus, the most polluted area is placed north-west of the two mills (Bengtsson and Rundgren, 1988).

D. octaedra were collected in October, 2009, from six sites in the area around Gusum (Table 1). Three of the sites were uncontaminated reference sites and the rest were contaminated with low, medium and high copper contamination. A detailed description of the six sites can be found in Fisker et al. (2011). From each site 13–20 adult earthworms were hand collected and used to culture an F1-generation of *D. octaedra* in the laboratory under standard conditions as described by Fisker et al. (2011).

2.2. Freeze and copper tolerance study

Newly hatched F1-worms were cultured in uncontaminated agricultural soil and fed with cow dung (Fisker et al., 2011). When the F1 generation had an age of approximately four months worms were used to make a combined freeze and copper tolerance study. This was done by placing the worms in either uncontaminated control soil or soil spiked with copper to a nominal concentration of 160 mg/kg dry soil. Subsequently, the two groups were slowly acclimated to low temperatures and thereafter exposed to sub-zero temperatures.

The soil used in this experiment originated from Askov, Denmark (see Fisker et al. (2011) for further details). 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 the $CuCl_2$ 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. This was done by using the copper-spiked soil and mixing it with clean water and dried cow dung in the same ratio as for the control food. The soil for the experiment was made one day before use to equilibrate and was kept at 15 ± 1 °C.

The F1-generation worms were placed separately in a 200 mL plastic beaker 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 mixed moist soil and food (around 30 g wet weight) and kept at 2 °C for two more weeks. Afterwards, the worms were exposed to three different temperatures: control temperature (+2 °C) and two exposure temperatures: −8 °C and −12 °C. Worms exposed to sub-zero temperatures were placed in a freezer cabinet at −2 °C for 24 h and a small ice crystal was added to initiate ice formation. After this the temperature was gradually lowered by 0.042 °C/h until target temperatures were achieved (Bindesbøl et al., 2005). The animals remained at the target experimental temperature for 24 h and were then transferred to 2 °C for recovery. Survival was checked within 1–4 days after thawing. The earthworms were considered to have survived if there was a reaction to tactile stimuli, normal locomotor activity, and no visible signs of freezing damage.

Table 1
Earthworm collection sites in Gusum, south-east Sweden. Three sites were contaminated with different levels of copper (High, Medium and Low) and three were reference sites (R1-3). The level of contamination is listed together with the site ID used throughout the paper. Furthermore, coordinates of the six collection sites are listed together with vegetation, number of collected adult earthworms and their internal copper concentrations (data from Fisker et al. (2011)). The distance to the old mill (OM) and new mill (NM) is also shown.

	ID	Coordinates	Number of collected earthworms	Internal copper concentration (μg/g dw)	Distance to point sources
High contamination	High	N 58° 16.667′ E 16° 28.550′	13	173.1	NM: 580 m OM: 1850 m
Medium contamination	Medium	N 58° 16.777′ E 16° 27.961′	20	33.9	NM: 1050 m OM: 2400 m
Low contamination	Low	N 58° 17.002′ E 16° 27.882′	13	20.6	NM: 1400 m OM: 2750 m
Reference 1	R 1	N 58° 11.030′ E 16° 44.356′	19	2.0	NM: 18 km OM: 16.5 km
Reference 2	R 2	N 58° 11.331′ E 16° 45.181′	15	1.4	NM: 19 km OM: 17 km
Reference 3	R 3	N 58° 12.055′ E 16° 48.141′	15	1.1	NM: 21 km OM: 19.5 km