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Impact of Age of Immature *Dendrobaena octaedra* (Sav.), (Lumbricidae: Oligochaeta) at Cadmium Application on Life History Response

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Earthworms have been used as test organisms in numerous studies on the effects of heavy metals, pesticides or other chemicals on the environment (Greig-Smith et al. 1992), because of their importance in soil processes, ability to accumulate pollutants, and worldwide distribution. Short-term tests carried out on homogenous individuals are the standard in ecotoxicological studies (Reinecke 1992). In most tests the objects are adult or new born individuals exposed to high doses of pollutants. In the field, however, pollutants influence populations consisting of a mixture of animals of different developmental stages and ages. The initial age structure of a population does influence the impact of pesticides on populations (Stark and Banken 1999). Heavy metals negatively influence different parameters of an animal's life history, such as growth, reproduction and survival (Laskowski and Hopkin 1996; Rożen 2006; Sibly and Calow 1989).

Earthworms are characterized by a three-stage life cycle: nonreproductive (immature), reproductive (adults producing offspring) and postreproductive (senescent animals) (Edwards and Bohlen 1996). Sensitivity to heavy metals differs between life stages (Klok et al. 1996; Stark and Banken 1999). Juveniles are more sensitive, but there is still the question of whether within that stage there are differences in sensitivity connected with the animal's age at exposure to heavy metals. The answer can be of importance in ecotoxicological tests. In the present study, two series were established: animals cultured in cadmium-spiked soil for the whole life cycle (from the cocoon stage), and animals (immature individuals) transferred to cadmium-spiked soil after one year of culture in clean soil. Life history parameters (growth, reproduction, survival) were checked in both groups. The aim was to determine whether heavy-metal sensitivity within the juvenile life stage is age-dependent, and which life history parameters changes are connected with the age of the exposed animals.

MATERIAL AND METHODS

Adult individuals of *Dendrobaena octaedra* were collected by hand-sorting in June 1996 from three mixed oak-pine forests (*Pino-Quercetum*): the most polluted forest, near Olkusz (50°17'N, 19°04'E), affected by a zinc smelter (4 km from the smelter) with soil containing metal ore; in the Niepołomice Forest (50°05'N, 20°21'E), moderately polluted by industry (20 km from a steel mill); and the relatively

unpolluted Kampinoska Forest near Warsaw (52°21'N, 20°51'E) (for more details, see: Rożen 2006).

To establish the F1 generations, cocoons laid in laboratory culture by the parent generation were collected in July 1996 and September 1996, and transferred to containers (25 per container). Cocoons laid by individuals cultured in clean soil were placed in clean soil, and cocoons laid by individuals cultured in cadmium-spiked soil were placed in cadmium-spiked soil. The hatching rates were checked in January 1997 (cocoons collected July 1996) and May 1997 (cocoons collected September 1996). Hatched animals and cocoons were collected by rinsing the soil on a sieve. Hatched individuals were taken to culture the F1 generation. The medium for earthworm culture was artificial soil (OECD 207) (sand, sphagnum peat, kaolinite clay and calcium carbonate mixture; Reinecke 1992). Soil moisture was adjusted to 40% of the substrate's dry weight by the addition of deionized water. Two variants of culture were established. In the first variant, the animals were kept in clean artificial soil. In the second variant, cadmium chloride was added to soil and dung to establish the cadmium concentration at 20 mg kg^{-1} dry mass. The earthworms were transferred to new soil every month. The soil was prepared three days before transfer, in order to establish constant soil conditions. Individuals were introduced to separate plastic containers filled with 50 g wet artificial soil. Their food source was 0.3 g dry cattle dung placed on the soil surface every two weeks. The containers were kept at 15 ± 0.5°C (Ma 1984) and 80% humidity, under constant artificial light. Every month during 23 months of culture the animals and cocoons were removed from the soil by rinsing on a sieve, weighed, and transferred to new soil.

Table 1. Experimental design. Letters denote the experimental series.

Exposition time	Forest of origin		
	Heavily polluted	Moderately polluted	Unpolluted
Early exposed	**HE** (40)[a]	**ME** (20)	**UE** (22)
Late exposed	**HL** (31)	**ML** (44)	**UL** (25)

()[a] – denote number of *Dendrobaena octaedra* individuals in the treatments

Animals taken for culture from populations inhabiting heavily polluted (H), moderately polluted (M) and unpolluted (U) environments were divided into two groups: animals exposed to cadmium early (cultured in cadmium soil from the cocoon stage) (E), and animals exposed to cadmium late (transferred to cadmium-spiked soil after one year culture in clean soil) – L (Tab. 1).

For tissue cadmium concentrations analyses animals were depurated for 48 h in wet blotting paper, dried at 55°C in a vacuum drier for 24 h and weighed. Dried earthworms were digested in 0.5 ml 65% nitric acid (Suprapur, Merck) at boiling. When the fumes were white and the solution was completely clear it was cooled down to room temperature and filled to 5 ml with deionized water. Cadmium concentrations were measured by graphite furnace AAS (AAnalyst 800, PERKIN-

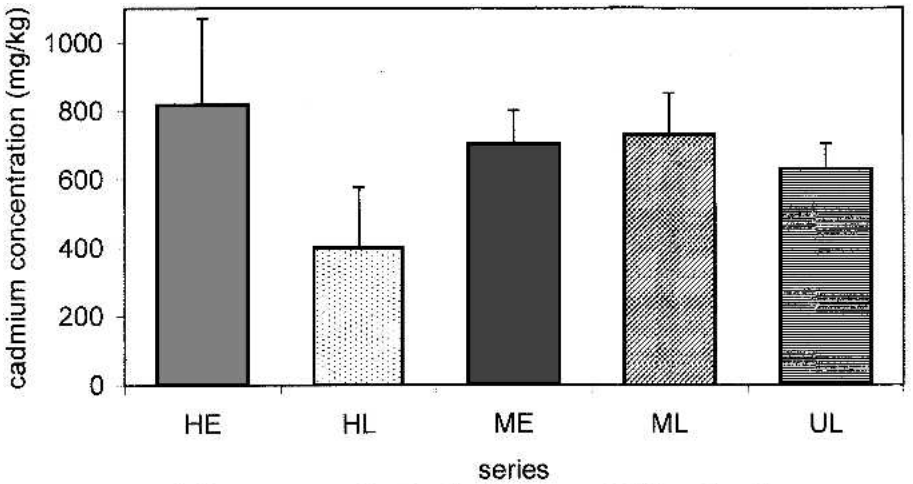

Figure 1. Cadmium concentration (mg/kg dry mass ±SD) in cultured *Dendrobaena octaedra* exposed to cadmium early (E) and late (L). Animals taken for culture from populations: H - inhabiting heavily polluted, M - moderately polluted and U - unpolluted forests . N = 3.

ELMER). Accompanying every run were five blank samples of nitric acid and three samples of standard reference material (bovine liver BCR No. 185, PROMOCHEM GmbH, Germany; certified concentrations: Cd = 0.298 ± 0.035 mg kg^{-1}).The data were subjected to ANOVA with the Tukey HSD range test or T-test. In the case of variance heterogeneity the Kruskall-Wallis test was used (Sokal and Rohlf 1981). The results are expressed as means ± SD.

RESULTS AND DISCUSSION

The cadmium level was measured in adult individuals (Fig. 1). There were no significant differences in cadmium concentration neither between all groups ($F = 3.076$, $p = 0.068$) nor between animals cultured from the cocoon stage in polluted soil (E) and those transferred to cadmium-spiked soil after some months of culture in clean soil (L) ($F = 3.321$, $p = 0.091$).

The observed lack of differences in cadmium concentration between animals cultured from the cocoon stage in cadmium-spiked soil (E) and those transferred to cadmium-spiked soil after one year of culture in clean soil (L) suggests that the cadmium accumulation reached equilibrium. In *Eisenia fetida*, Reinecke and Reinecke (1997) similarly found no difference in cadmium concentration after 90 and 120 weeks of exposure. Spurgeon and Hopkin (1999) reported a lack of equilibrium for nonessential metals, but probably this was due to the shorter duration of exposure to cadmium in that study.

It is known that cadmium negatively influences growth in the earthworm (Van Gestel et al. 1991). In the present study, growth was reduced in response to cadmium application. In the first months of culture (June –July 97, November 97 – February 98), animals cultured in clean soil (L) grew noticeably ($p<0.05$) faster than

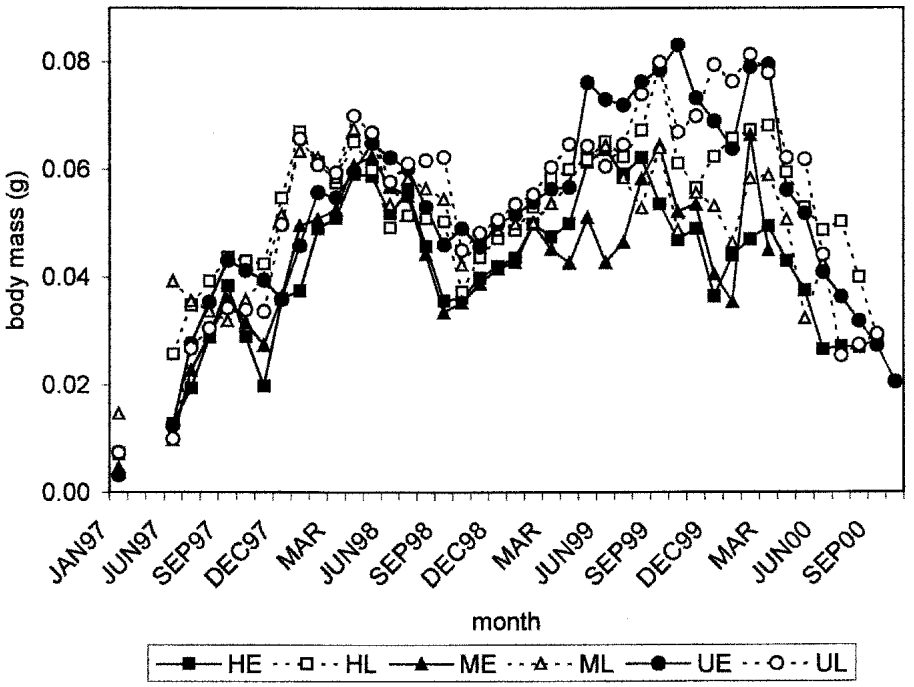

Figure 2. Growth of *Dendrobaena octaedra* during culture. Animals taken for culture from populations: H - inhabiting heavily polluted, M – moderately polluted and U - unpolluted forests, exposed to cadmium early (E) and late (L).

animals cultured in cadmium-spiked soil (E). In January 1998, immature animals of the L series were transferred from clean soil to cadmium-spiked soil; slower growth in the following months resulted (from March 98, $p>0.05$). In that treatment the body mass of L animals approached that of the E series (Fig. 2). This observation, along with findings on the parent generation (Rożen in press), show that growth is a cadmium-sensitive parameter and that the negative effect of cadmium on growth is independent of the age of the exposed animals.

Age-dependent effects of cadmium were observed in reproduction. Cadmium applied early in the earthworms' life delayed maturation (appearance of sexual characters: clitellum and tubercula pubertatis). In each population, animals cultured in cadmium-spiked soil (E series) matured later than those transferred to cadmium-spiked soil after one year of culture in clean soil (L series). Individuals of HE matured 4.7 months later than HL ($t = 2.69$, $p = 0.03$), ME 2.9 months later than ML (nonsignificant difference) and UE individuals 8 months later than UL ($t = 3.4$, $p = 0.003$), reaching similar weights. Mature individuals started to reproduce at the same age ($F = 2.2$, $p = 0.07$) (Tab. 2). Body mass at first reproduction did not differ between series. An experiment on *Lumbricus rubellus* (Ma 1982, cit. after Klok and De Roos 1996) yielded a similar finding - animals differently dosed with copper reproduced at the same body mass.

Not all earthworms laid cocoons. In the treatments with later exposure to cadmium, more of the animals initiated reproduction. Only 20% HE but 51.6% HL, 19.2% ME but 48.8% ML, and 9.1% UE but 45% UL began to reproduce. This means that the negative influence of cadmium on reproduction was stronger under early (E series) than under late (L series) application.
There was no clear influence of cadmium exposure time on cocoon production. Mean total cocoon production per reproducing individual during the whole life was similar ($F=1.29$, $p=0.28$) for the L and E series, and lower only for ME

Table 2. Mean body mass and age at maturity (appearance of clitellum) and at reproduction, cocoon production and mean survival time of *Dendrobaena octaedra* in laboratory culture.

Series	Maturity			First reproduction			Cocoon production	Survival	
	n	Body mass (g)	Mean age (months)	N	Body mass (g)	Mean age (months)	Cocoons/ individual	n	Months
HE	17	0.0768 ±0.0105	29.2 ±6.9	8	0.0768 ±0.0106	33.5 ±1.4	7.6 ±4.5	40	26.6 ±11.2
HL	22	0.0785 ±0.0100	24.4 ±6.2	16	0.0757 ±0.0134	32.7 ±5.3	6.7 ±4.6	31	33.7 ±8.5
ME	9	0.0591 ±0.0210	23.2 ±3.3	5	0.0618 ±0.0205	28.6 ±5.6	3.8 ±1.7	20	23.3 ±12.8
ML	34	0.0769 ±0.0171	20.3 ±5.1	21	0.0769 ±0.0171	30.1 ±6.8	8.9 ±3.8	44	30.3 8.5
UE	4	0.0826 ±0.0225	34.2 ±5.0	2	0.0826 ±0.0225	35.0 ±1.4	9.5 ±4.9	22	16.5 ±11.5
UL	19	0.0808 ±0.0119	26.2 ±4.2	12	0.0750 ±0.0174	35.1 ±3.8	6.6 ±5.4	25	33.5 ±8.7

Mean ± SD, n = number of animals,
Animals taken for culture from populations: H - inhabiting heavily polluted, M - moderately polluted and U - unpolluted forests, exposed to cadmium early (E) and late (L).

individuals (Tab. 2). Maximum cocoon production of animals from cadmium-spiked soil was similar between series and populations: 14 for HE, 15 for HL, 6 for ME, 13 for ML, 13 for UE and 18 for UL. Cocoon production of animals cultured in cadmium-spiked soil has been found to be approximately half of their production in clean soil (Rożen 2006). Therefore the negative impact of cadmium on individual's reproduction is independent of the age of immature specimens at

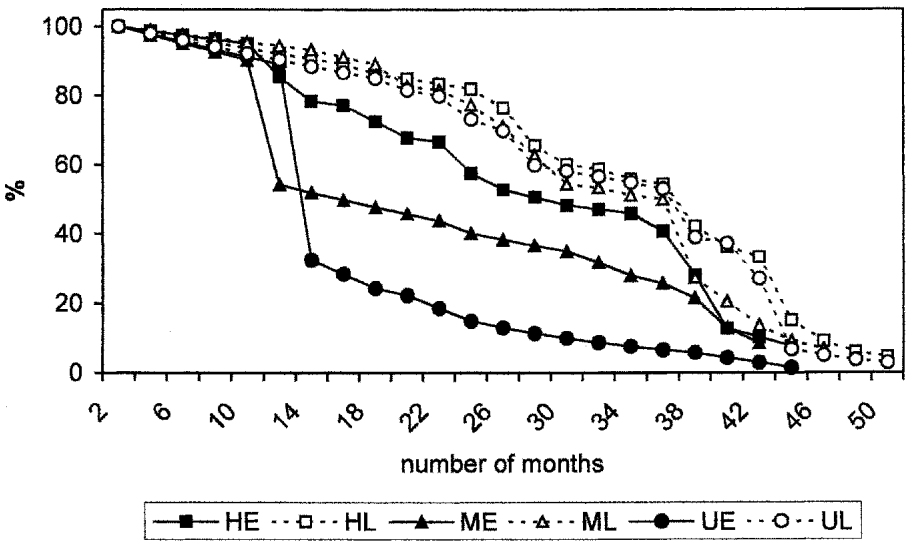

Figure 3. Survival (%) of *Dendrobaena octaedra* during culture. Animals taken for culture from populations: H - inhabiting heavily polluted, M – moderately polluted and U - unpolluted forests, exposed to cadmium early (E) and late (L).

the time of dosing. On the population level, early cadmium intoxication has a stronger detrimental effect, increasing the proportion of nonreproducing individuals. Reproductive allocation (weight of young divided by weight of females) describes the reproductive effort of animals. Reproductive allocation was 19.8% for HE, 19.8% for HL, 7.9% for ME, 23.8% for ML, 10.9% for UE and 20.1% for UL. The only statistically significant difference was between ME and ML ($t = 2.89$, $p < 0.01$). Age at cadmium dosing affected survival (Fig. 3). The differences were caused by high mortality of the youngest individuals exposed to cadmium. Older specimens transferred to cadmium-spiked soil (L) responded to pollution with slower growth but not with increased mortality.

The mean survival of E individuals was significantly shorter than those of the L series ($F = 9.4$, $p < 0.0001$) (Tab. 2). This accords with earlier suggestions that the critical period in earthworms' life are the first months after hatching (Klok and De Roos 1996; Klok et al. 1997).

The forest of origin affected the survival of individuals dosed with cadmium early in life. Animals cultured from individuals taken from the heavily polluted forest survived significantly longer than those cultured from individuals taken from the unpolluted forest (Gehan-Wilcox test 2.83, $p < 0.05$) (Rożen 2006). No such effect was found in earthworms dosed later.

From the present study it is possible to conclude that: i. heavy-metal sensitivity within the juvenile life stage is age-dependent; ii. maturity time, the proportion of reproducing individuals, and survival are the life history parameters whose sensitivity is related to age at exposure to cadmium; iii. growth and cocoon production are life history parameters sensitive to cadmium pollution but apparently independent of the animals age at exposure.

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