Arch. Environ. Contam. Toxicol. 37, 332–337 (1999)

ARCHIVES OF
Environmental
Contamination
and Toxicology

Tolerance to Zinc in Populations of the Earthworm *Lumbricus rubellus* from Uncontaminated and Metal-Contaminated Ecosystems

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Received: 8 September 1998/Accepted: 21 March 1999

Abstract. Zinc tolerance in *Lumbricus rubellus* populations from two metal-polluted (smelter and mine) sites was studied by comparing the effects of zinc with responses in a reference site strain. For the study, adult worms were collected directly from the field. Thus, no attempt was made to differentiate between tolerance resulting from population-level genetic adaptation or phenotypic plasticity in metal physiology. To compare relative sensitivity for zinc, worms from the three populations were exposed in laboratory tests. Effects on survival, weight change, cocoon production, and internal zinc levels were measured. Prior to exposure, it was anticipated that worms from the metal-contaminated sites would show substantially increased tolerance to zinc. This was not the case for all measured parameters. Thus, although differences in the shape of the dose-response relationships for survival and cocoon production were found, substantial variations in measured responses, effect concentrations, or zinc accumulation rates were not apparent. Overall, therefore, zinc tolerance is unlikely to be a major factor influencing the distribution of *L. rubellus* in contaminated regions.

Numerous studies have demonstrated tolerance (better performance than controls in contaminated environments) to metals in soil invertebrate species (Posthuma and Van Straalen 1993). However, conclusive evidence for resistance (inheritable tolerance) remains relatively rare, with only four examples reported in the literature. Donker and Bogert (1991) found genetic adaptation to cadmium in metal-exposed populations of the isopod *Porcellio scaber*, while Donker *et al.* (1996) found differences in zinc assimilation and accumulation in clean and polluted site populations of this species. Tranvik *et al.* (1993) found life-history divergences for clean and exposed populations of *Onychiurus armatus* and *Isotoma notabilis*. In addition, Posthuma (1990) found increased cadmium tolerance in the growth response of F1 progeny of *Orchesella cincta* from metal-contaminated sites. Further studies with this species indicated evolutionary changes in excretion rate, equilibrium concentration, and life history characteristics (Posthuma *et al.* 1992, 1993).

For earthworms, published studies have so far failed to find clear tolerance traits. This is in spite of the fact that earthworm populations can persist in soils containing metal levels that exceed effects concentrations for key life history parameters (Posthuma and Van Straalen 1993). Despite circumstantial evidence for metal tolerance, the only experimental data to explicitly indicate adaptation in earthworms is given by Morgan and Morgan (1988). In this study, a lower proportion of lead was found in sensitive tissues of *Lumbricus rubellus* collected from a metal-contaminated site when compared to reference site worms exposed to similar lead concentrations. The lower lead levels found in the polluted site populations suggest that these worms may possess more efficient lead storage and detoxification systems. Further studies designed to indicate metal tolerance in field populations have failed to find significant interpopulation differences in uptake, protein elution patterns, or sensitivity for effects on mortality (Corp and Morgan 1991; Bengtsson *et al.* 1992; Marino and Morgan 1999).

That investigations with field populations of earthworms have failed to demonstrate adaptation may be due to the priority given to studies with cadmium and lead. A recent study by Spurgeon and Hopkin (1995) concluded that these metals are unlikely to play a major role in the elimination of earthworms from soils around a smelting works. Instead it is the high concentrations of zinc in soils in the area that is primarily responsible for limiting earthworm distribution (Spurgeon and Hopkin 1995; Spurgeon 1997). Thus, at many metal-contaminated sites, it would be expected that greatest selection pressure will be for the development of resistance to zinc. It is therefore responses to this metal that will be the main focus of this study.

Materials and Methods

To determine if earthworm population at polluted sites exhibit increased tolerance to zinc, the effects of this metal on the survival, weight change, and cocoon production of three field strains of *L. rubellus* were measured in laboratory tests. The populations selected for the study were collected from a reference (control) and two polluted (smelter and mine) sites. For the study, adult worms were collected

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directly from the field. Thus, no attempt was made to differentiate between tolerance resulting from phenotypic plasticity of individuals or population-level genetic selection that results in an increase in the frequency of tolerant genotypes.

Both the intensity and duration of exposure can influence the development of tolerance. Thus, the strains used in this study were obtained from sites with divergent (and well-documented) histories of metal contamination. The reference strain was collected from the campus of the University of Reading, UK (Ordnance Survey Grid Reference SU 737714). Previous work at this site has found metal levels to be within the range of an uncontaminated soil (Spurgeon and Hopkin 1995, 1996a). The smelter strain was collected approximately 3 km from a smelting works situated at Avonmouth, UK (Hallen Wood) (Ordnance Survey Grid Reference ST 554802). This site has been subject to aerial deposition of cadmium, copper, lead, and zinc since 1929 and is known to contain zinc levels in excess of clean soil values (Spurgeon and Hopkin 1996a). The mine strain was obtained from Shipham, UK (Ordnance Survey Grid Reference ST 451573). This site is contaminated with very high levels of cadmium, lead, and zinc as a result of metal extraction and processing during the sixteenth and seventeenth centuries.

At each of the selected sites, sufficient (200) adult *L. rubellus* were collected by digging and hand-sorting. This method was used because it is less likely to result in damage to worms than chemical or electrical extraction. Worms were returned to the laboratory on own site soil and stored at 3°C until required for testing. Soil samples were also collected from each site by removing the top 2-cm layer of the soil profile (below the litter horizon). These soils were returned to the laboratory, where they were dried and used to measure pH, percent loss on ignition and total cadmium, copper lead and zinc contents (by flame atomic absorption spectophotometry [AAS] of nitric acid digests) (Hopkin 1989).

Edwards (1983) developed a soil-based method for measuring the toxicity of chemicals to earthworms. This technique, based on the use of an artificial soil, was adopted by the OECD (1984) and the EEC (1985) and has been widely used for toxicity tests with earthworms (Reinecke 1992; Van Gestel and Van Straalen 1994). The OECD test allows exposure under standardized conditions. However, previous experiments to measure metal toxicity for *L. rubellus* in OECD soil have indicated some problems, in particular high control mortality, when using the procedure (Spurgeon and Hopkin 1996a). Thus for the current study, artificial soil was not used. Tests were instead conducted with a medium based on commercially available sandy loam topsoil. Analysis of this soil indicated a loss on ignition of 9.9%, a value somewhat lower than the organic matter levels found in the soils at the three selected sites (Table 1). Thus to increase organic content, 20% by weight of finely ground *Sphagnum* peat was added. This soil/peat mixture has been found to be suitable for the long-term maintenance of *L. rubellus.*

Worms used for testing were first preexposed to uncontaminated soil for 7 days at 15°C. After this time they were removed; six worms were weighed and added to each test replicate (plastic boxes, 220 mm $\times$ 160 mm $\times$ 80 mm). Zinc concentrations of 0, 190, 350, 620, 1,200, 2,000, and 3,600 µg Zn g^{-1} were used in all tests, with four replicates for each concentration. For the zinc-treated soils, solutions of the nitrate salt (Zn $NO_3 \cdot 6H_20$) (BDH chemicals, Poole, Dorset, UK) were mixed with 1 kg of the dry medium to give the required water content (65% wet weight) and metal concentrations in test soils. The same volume of distilled water was added to controls.

After addition of metal solutions, soils were left to stabilize for 1 week prior to the introduction of earthworms. Containers were covered to prevent water loss and kept for 42 days at 15 $\pm$ 2°C under constant light. In all tests, a suitable food (finely ground fresh horse manure, dried and rewetted to 75% water content) was added to increase cocoon production (Reinecke and Viljoen 1990; Spurgeon *et al.* 1994; Van Gestel and Van Straalen 1994). To increase food palatability, the manure was mixed with a small volume of test soil, since *L. rubellus* incorporate substantial quantities of inorganic matter in their diet (Piearce 1978; Holmstrup *et al.* 1991). Two grams dry weight of manure was added weekly to each replicate.

Parameters measured in the three tests were mortality, weight change relative to initial weight, and cocoon production rate. To measure survival, the number of earthworms alive in each experimental container was counted after 14, 28, and 42 days. Weight change was assessed by comparing mean weight at the end of the experiment to mean initial weight to determine percentage change over the duration of the test. Cocoon production was measured by wet-sieving soils at the end of the test. The total number of cocoons present in each container was counted and could then be compared to survival data to allow a cocoon production rate (cocoons/worm/week) to be calculated. Cocoon production rate was calculated by assuming that any worms that died did so at the midpoint between respective sample intervals.

In addition to measuring effects on life-cycle parameters, earthworm zinc concentrations were also measured for all populations both prior to exposure (collected at the end of the preexposure phase) and at the end of the test. Analysis was conducted using a method adapted from Hopkin (1989). Earthworms were first starved for 72 h on moist filter paper to remove any soil present in their guts. This starvation period was sufficient to remove all soil present in the gut, since Hendriksen (1991) found food retention times of 9 to 15 h and 3 to 6 h for the related species *Lumbricus festivus* and *Lumbricus castaneus,* respectively. Individuals were then digested in nitric acid and analyzed for zinc content by flame AAS.

Significant differences in mortality, growth, and cocoon production rate were calculated using ANOVA. When differences were found, Tukey's multiple comparison test was used to determine differences between specific treatments. The dose-response curves for survival and cocoon production for each strain were compared using the Genstat statistical software system. For survival, separate curves were compared using a Chi-square test of the logistic regression, while for cocoon production an F-test of the logistic curves was applied. In addition to comparing dose response fits, collected lethal and sublethal data was also used to calculate LC_{50} and EC_{50} values. LC_{50}s with 95% confidence intervals (95% CIs) using the log-probit method, while the linear interpolation technique (Norberg-King 1993) was used to determine EC_{50} values. In some cases, 95% CIs could not be calculated as only one data point showed a partial effect. In these circumstances, toxicity values are stated without respective confidence intervals (N/A in text and tables). For calculation of weight change EC_{50}s, weight loss values would be required for all concentrations. However for the containers in which no worms survived after 42 days, such data was not available. Thus to allow weight change EC_{50}s to be calculated, a weight loss of 50% was assumed in the containers with no worms alive at 42 days. This value was selected since it estimates the maximum weight loss that can occur before mortality is guaranteed. Weight changes at all other concentrations were calculated relative to this value. Mean earthworm zinc concentrations were compared using a two-way ANOVA with zinc concentration and strain as the variable factors.

Results

Analysis of soils from the locations at which *L. rubellus* were collected indicated elevated metals levels at the smelter and mine sites when compared to reference values. Highest levels of all metals were found in mine soils, with the cadmium, lead, and zinc concentrations at this site higher than those in the other soils by at least an order of magnitude (Table 1). For the smelter soil, cadmium, lead, and zinc concentrations exceeded reference values by at least a factor of 10, whereas copper levels were higher by a factor of 4. Metal levels in the reference site were within the range found in uncontaminated soils (Merian 1991). Comparisons of soil abiotic characteristics indicated higher soil pH at the two polluted sites (Table 1). High soil pH

Table 1. Soil pH, percentage loss on ignition (% loi), and metal concentrations (μg g^{-1}) in soils sampled from sites from which the three *Lumbricus rubellus* field strains were collected

	pH (median)	% Loi	Cadmium	Copper	Lead	Zinc
Clean (Campus)	5.82	20.1 ± 3.4	0.05 ± 0.02	9.92 ± 1.02	21.83 ± 1.82	31.48 ± 2.28
Smelter (Hallen Hill)	7.36	20.7 ± 1.2	5.93 ± 0.24	40.3 ± 2.22	291 ± 54	406 ± 22
Mine (Shipham)	6.19	23.3 ± 1.2	60.8 ± 3.1	51.8 ± 4	10,200 ± 690	6,050 ± 416

All values are means ± SE of six replicates

would be expected to reduce metal availability, particularly in the smelter soil, which had highest pH (Spurgeon and Hopkin 1996b). However despite this reduced availability, metal levels in both polluted soils are high enough to ensure a selection pressure at these sites. Soil %loi was similar at all three sites (Table 1).

Reproductive rates for the reference and smelter worms maintained in the control soil compare favorably with those found in previous studies (Elvira *et al.* 1997), while rates for the mine sites worms were less than half of these values. Comparisons of cocoon production rates for the three strains when maintained in control soil also indicated significant differences between strains. Rates were significantly (t test, $p < 0.05$) lower in mine site worms compared to the smelter and reference populations. The lower cocoon production rates found for the mine worms could be explained by a number of factors relating to previous metal exposure, including past food supply, abiotic conditions, or random genetic variation. A further explanation is the presence of a ''cost of tolerance'' for the mine site worms.

Comparison of dose-response fits for survival of the three tested strains using a Chi-square test of the logistic regressions indicated that the relationships differed significantly ($\chi^2 = 24.82$, $p < 0.001$). Similarly, for cocoon production rate, a comparison of the dose response relationship using an F-test of the logistic curves also indicated significant difference in the shape of the dose-response fits ($F = 13.27$, $p < 0.01$). In addition to comparing dose-response fits for the three strains, the collected lethal and sublethal toxicity data was also used to compare responses and calculate effect concentrations for the three strains.

For worms collected from the reference site, mortality was significantly (Tukey, $p < 0.001$) higher than controls at 2,000 μg Zn g^{-1} (Table 2). Indeed only one worm survived for 42 days in this soil. All worms exposed to 3,600 μg Zn g^{-1} died. A 42-day LC_{50} of 1,424 μg Zn g^{-1} (N/A) was calculated for this strain. Mean weight relative to initial weight increased in worms exposed to 0, 350, and 620 μg Zn g^{-1}, with the greatest increase (11.7%) for worms exposed to 620 μg Zn g^{-1}. Worms exposed to all other zinc concentration lost weight. Calculation of an EC_{50} for the effects of zinc on weight change gave a value of 1,520 μg Zn g^{-1} (N/A). Cocoon production was the most sensitive parameter tested. This agrees with results of previous toxicity tests conducted with *L. rubellus* (Spurgeon and Hopkin 1996a). For the campus worms, the highest rate of cocoon production (2.33 cocoons/worm/week) was found in the control soil, while rates were significantly (Tukey, $p < 0.05$) reduced at 620, 1,200 and 2,000 μg Zn g^{-1}. The 42-day EC_{50} for the effects of zinc on cocoon production on this strain was 600 (515–734) μg Zn g^{-1}.

Mortality of smelter *L. rubellus* was significantly (Tukey, $p < 0.05$) increased at 1,200 and 2,000 μg Zn g^{-1}, and all worms exposed to 3,600 μg Zn g^{-1} died (Table 2). The 42-day LC_{50} for this strain was 1,264 (534–4247) μg Zn g^{-1}. Mean weight change relative to initial weight indicated that worms in all soils lost weight. Lowest weight loss (−14.7%) was for worms exposed to 350 μg Zn g^{-1}, while highest weight loss (−43.8%) was for worms exposed to 2,000 μg Zn g^{-1}, although the high mortality found in this soil may partly account for this reduction. Calculation of an EC_{50} for the effects of zinc on mean weight change gave a value of 1,308 (513–2,120) μg Zn g^{-1}. Highest cocoon production rate of 2.18 cocoons/worm/week was for worms incubated in the control soil. Unlike for the reference worms, cocoon production was not significantly reduced at 620 μg Zn g^{-1}. However, rates were significantly (Tukey, $p < 0.05$) lower at 1,200 and 2,000 μg Zn g^{-1}. The 42-day EC_{50} for the effects of zinc on cocoon production in the smelter strain was 874 (665–1,002) μg Zn g^{-1}.

Table 2. Effects of zinc on the survival, growth, and reproduction of *Lumbricus rubellus* collected from the reference, smelter, and mine sites (mean of four replicates)

Nominal Zinc Concentration	Percent Survival 14 Days	Percent Survival 42 Days	Cocoon Production (cocoons/ worm/week)	% Weight Change Relative to Initial
Reference				
0	100 ± 0	100 ± 0	2.33 ± 0.17	3.4 ± 5.6
190	100 ± 0	100 ± 0	1.82 ± 0.2	−0.2 ± 7.2
350	100 ± 0	96 ± 3	1.89 ± 0.16	5.3 ± 9.8
620	100 ± 0	100 ± 0	1.11 ± 0.1*	11.7 ± 6.9
1,200	92 ± 3	92 ± 3	0.22 ± 0.05*	−9.2 ± 5.2
2,000	8 ± 3*	4 ± 3*	0.02 ± 0.02*	−6.4
3,600	0*	0*	0*	—
Smelter				
0	100	88 ± 6	2.18 ± 0.24	−19.4 ± 4.7
190	96 ± 3	92 ± 3	1.76 ± 0.08	−16 ± 1.6
350	96 ± 3	92 ± 3	1.42 ± 0.22	−14.7 ± 7.3
620	96 ± 3	92 ± 3	1.69 ± 0.14	−28.3 ± 2.8
1,200	79 ± 4	67 ± 3*	0.48 ± 0.07*	−32.2 ± 3.3
2,000	42 ± 9*	29 ± 6*	0.02 ± 0.02*	−43.8 ± 4.4
3,600	0*	0*	0*	—
Mine				
0	100	88 ± 3	0.94 ± 0.14	−12.9 ± 4.6
190	100	92 ± 6	0.94 ± 0.07	−18.3 ± 1.6
350	100	92 ± 3	1.13 ± 0.08	−18 ± 3.2
620	96 ± 3	83 ± 10	0.7 ± 0.08	−19.9 ± 4.6
1,200	100	92 ± 6	0.25 ± 0.02*	−29.8 ± 10.3
2,000	83 ± 3*	33 ± 7*	0*	−42 ± 4.5
3,600	0*	0*	0*	—

Values marked with asterisks are significantly different from controls at $p < 0.05$

The survival of *L. rubellus* from the mine site after 42 days' exposure was significantly reduced at 2,000 μg Zn g^{-1} (Tukey,

$p < 0.001$), and all worms exposed to 3,600 μg Zn g^{-1} died (Table 2). A 42-day LC_{50} of 1,450 μg Zn g^{-1} (N/A) could be calculated for this strain. As for the smelter worms, weight change relative to initial weight indicated a reduction in mean weight at all zinc concentrations. Lowest weight loss was for worms in the control soil (−12.9%), while highest loss (−42%) was for the worms exposed to 2,000 μg Zn g^{-1}, although as for the smelter strain, the high mortality found in this soil may partly account for this reduction. Calculation of an EC_{50} value for the effects of zinc on weight change gave a value of 1,301 μg Zn g^{-1} (N/A). Highest cocoon production rates of 1.13 cocoons/worm/week were for worms exposed to 350 μg Zn g^{-1} and cocoon production was significantly (Tukey, $p < 0.01$), reduced only at 1,200 and 2,000 μg Zn g^{-1} (Table 2). A 42-day EC_{50} of 876 (589–975) μg Zn g^{-1} could be calculated for the effects of zinc on cocoon production in mine site worms.

Analysis of earthworm zinc levels after preexposure but prior to the start of the toxicity tests indicated higher concentration for both smelter and mine site populations in comparison with reference values (Figure 1A–C). These differences were significant when compared by paired *t* tests, although it should be noted that zinc levels in polluted-site worms showed higher variance than that for control site animals. Comparisons of initial zinc levels in worms collected after preexposure to those in worms collected from the control soil at the end of the experiment indicated no consistent trends. Levels were lower in mine worms, somewhat higher in the smelter tests, and comparable for reference animals. Paired *t* tests indicated no significant ($p < 0.05$) differences between initial and control worm zinc concentrations in any of the tested strains (Figure 1A–C).

Two-way ANOVA indicated a significant influence of both exposure concentration ($p < 0.01$) and strain ($p < 0.001$) on earthworm zinc concentrations. The interaction between the two factors were not significant ($p < 0.05$), suggesting that there are no differences in zinc accumulation patterns between strains except for differences in metal levels per se. Comparisons of the zinc levels for the three strains indicated that at any given soil concentration, internal levels were lower in reference worms than for smelter or mine site animals. These differences were particularly pronounced at the higher zinc concentrations (Figure 1A–C). For control site worms, zinc exposure significantly ($p < 0.05$) increased internal zinc concentrations at 350, 620, and 1,200 μg Zn g^{-1}. However, for smelter and mine site worms, no significant differences ($p > 0.05$) were found in tissue zinc levels at any of the tested concentrations (Figure 1A–C). This was due primarily to the higher variability in internal zinc concentration found in exposed worms from these strains.

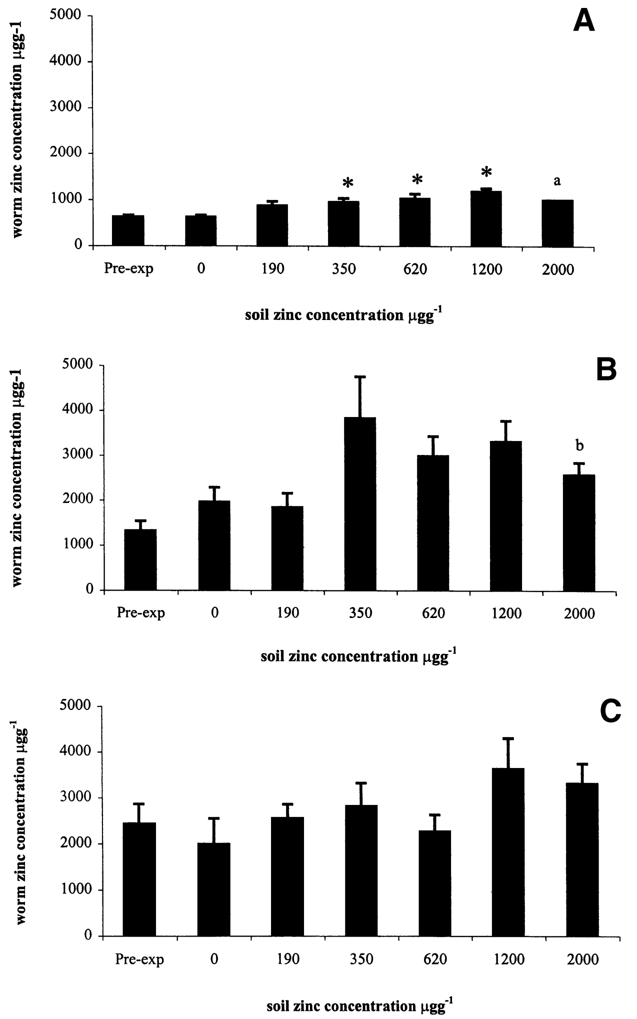

Fig. 1. Mean tissue zinc concentrations ± SE of (A) reference, (B) smelter, and (C) mine strain *Lumbricus rubellus* preexposure (pre-exp) and after exposure to soil with the stated zinc concentration for 42 days. * = significantly different from control soil (Tukey's test, $p < 0.05$), A = only one worm available, B = only seven replicate worms available

Discussion

The sites from which *L. rubellus* field strains were collected differed substantially in both the duration and intensity of zinc selection pressure. At the reference site, zinc levels were within the range found in uncontaminated soils. Thus no selection pressure for zinc tolerance exists at this site. For the smelter site, zinc (and cadmium, copper, and lead) concentrations are above levels found in uncontaminated soils. A zinc selection pressure may therefore be present at this site. The metal contamination present around the smelter results from the aerial deposition from a factory that has been in operation for 70 years, suggesting that selection of worms has occurred over many tens of generations. Highest zinc levels were present in the mine soil. Thus, it would be anticipated that highest selection pressure for zinc tolerance would occur at this site. High metal levels at the mine site result from ore extractions that took place approximately 300 years ago. Thus earthworm populations have been exposed to high zinc levels for hundreds of generations.

Exposure of *L. rubellus* at the smelter and mine sites to elevated zinc concentrations over tens and hundreds of generations respectively, resulted in significant differences in the shape of survival and cocoon production dose-response fits. Despite the differences found for the dose-response relationships, comparisons of the effects of zinc on survival indicated no obvious differences between the three strains (Table 2). For cocoon production rate, smelter and mine site worms did appear marginally more tolerant than the reference population. For example, at 620 μg Zn g^{-1}, no significant effects were found in the two polluted site populations, while cocoon production was significantly reduced in campus worms (Table 2). However, comparisons of cocoon production rate EC_{50}s indicated that, although values for the smelter and mine site strains of 874 (665–1,002) μg Zn g^{-1} and 876 (589–975) μg Zn g^{-1} were slightly higher than the value of 600 (515–734) μg Zn g^{-1} for the reference population, there was substantial overlap of 95% CIs for the three values. This suggests that there are no clear differences in sensitivity of the three strains for the effects of zinc on cocoon production, as well as for survival.

Prior to exposure, it was anticipated that worms from the metal-contaminated sites would show substantially increased tolerance to zinc. Expectations of tolerance were based on the fact that sufficient earthworms could be collected from the mine and smelter sites despite the high concentrations of metals present. Additionally, the results from a laboratory selection experiment conducted by Spurgeon and Hopkin (submitted) indicated significant differences in the survival of successive generations of *Eisenia fetida* in contact filter paper tests. Although the rate of development of zinc resistance found in the selection study was relatively slow, Spurgeon and Hopkin (submitted) concluded that genetic-based tolerance would be expected in earthworms at metal-polluted sites, since the historic nature of the contamination present would allow selection over numerous generations. Despite this evidence suggesting the presence of zinc resistance in long-term exposed earthworm strains, the only indication of increased tolerance found was in the shape of the dose-response curves and in the cocoon production responses of the three strains at 620 μg Zn g^{-1}. Clear differences in survival responses, effect concentrations, and zinc accumulation rates were not found (Table 2). How is this the case? A number of factors can be identified that may limit the development of tolerance in *L. rubellus.*

Firstly, work comparing zinc toxicity in contaminated field and laboratory soils has indicated that effects occur at higher concentrations in field tests (Spurgeon and Hopkin 1995; Smit and Van Gestel 1996). Thus, selection pressure at metal-contaminated sites may not be as great as anticipated from comparison with laboratory tests. Differences in zinc toxicity in field and laboratory soils can be attributed to the effects of a number of factors on bioavailability. These include soil properties, aging time, and the form of the contaminant (Spurgeon and Hopkin 1995, 1996b; Smit and Van Gestel 1996; Crommentuijn *et al.* 1997). In particular, the fact that zinc is present at polluted sites over tens or hundreds of year, rather than added as a solution of a soluble salt, with only a short time allowed for sorption, is important in reducing zinc toxicity in the field (Smit *et al.* 1997).

In addition to the direct effects of soil properties and aging time on metal bioavailability, indirect effects such as earthworm behavior can also reduce exposure. *L. rubellus* live in permanent horizontal burrows, the walls of which have been found to contain high levels of organic matter, mucus, and bacteria (Schrader 1994; Stehouwer *et al.* 1994; Springett 1997; Tiunov *et al.* 1997). That earthworms spend much of their lives in contact with the organically enriched burrow walls rather that the less well-buffered surrounding soil may mean that they are exposed to lower metal levels than would be predicted, even from analysis of bioavailable concentrations in homogenated soil samples.

Differences between species could also explain why *L. rubellus* at polluted sites fail to exhibit the increases in resistance predicted from a laboratory selection experiment with *E. fetida* (Spurgeon and Hopkin submitted). Comparisons of zinc levels in these two species indicated marked differences. For example, Spurgeon and Hopkin (1996b) found zinc concentration of 87.4–117.4 μg Zn g^{-1} in *E. fetida* maintained for 3 weeks in uncontaminated artificial soil. This compared with the mean value of 621 μg Zn g^{-1} and 614 μg Zn g^{-1} found in the field-collected and preexposed reference site worms. Differences in zinc accumulation patterns have also been found for the two species. For *L. rubellus,* Marino and Morgan (1999) found that tissue zinc concentrations increased gradually for worms incubated for 90 days in mine site soils containing approximately 20,000 μg Zn g^{-1}. For *E. fetida,* Spurgeon and Hopkin (1999) found that earthworm zinc levels reached equilibrium after only a few days in smelter-contaminated soils containing 7,310 μg Zn g^{-1} and 43,300 μg Zn g^{-1}. Although these studies were conducted in different soil and comparisons must be treated with caution, they do suggest the presence of metabolic and toxicokinetic differences between species. Such differences could mean that adaptation is precluded in *L. rubellus,* whereas it can develop slowly in *E. fetida.*

A further factor that may reduce selection pressure at heavily contaminated sites is that soil metal levels frequently exhibit spatial variability. This heterogeneity results in variability in the exposure of worms and can be an important for determining survival probability in exposed populations (Marinussen and Van Der Zee 1996). At the smelter site, a high level of heterogeneity would not be expected because contamination results from aerial deposition. However at the mine site, it would be anticipated that zinc levels might show highly patchy distribution dependent on historic use. The high variability found in initial zinc concentrations in smelter and mine site worms, suggest that heterogeneity in metal distribution may be an important factor influencing exposure and hence selection pressure at these sites.

Previous studies designed to indicate metal tolerance in field populations of earthworms have also failed to find pronounced interpopulation differences in sensitivity for metals including cadmium, copper, and lead (Corp and Morgan 1991; Bengtsson *et al.* 1992; Marino and Morgan 1999). Thus, it appears that the marginal increases in tolerance found in this study might be representative for earthworms exposed to a range of metals at a variety of metal-contaminated sites. Clearly from the discussion above, a number of factors can explain the absence of strong tolerance traits in earthworm populations at metal-contaminated sites. Whatever the underlying reasons, the results of this (and previous) studies suggest that tolerance, both as a result of population level genetic adaptation and phenotypic plasticity of individuals, is unlikely to be a major factor influencing the distribution of *L. rubellus* in metal-contaminated regions.

Acknowledgments. Thanks go to Dr. Tim Sparks for help with the statistics. The work was conducted at Reading University while DJS was holding research grant from the Leverhulme Trust. DJS is currently supported by a NERC Advanced Fellowship.

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