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The development of genetically inherited resistance to zinc in laboratory-selected generations of the earthworm *Eisenia fetida*

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"Capsule": *Zinc resistance in populations of the worm* Eisenia fetida *was demonstrated.*

Abstract

The capacity of species to adapt both physiologically and genetically to contaminants may allow populations to persist in polluted environments. Such 'adaptation' can have important implications for risk assessment, since it may mean that prediction based on extrapolation of toxicity studies with naïve populations may prove invalid for long-term contaminated sites. To investigate the evolution of zinc resistance in *Eisenia fetida*, worms from a previously unexposed population (parent) were selected and reared over two generations (F1, F2) while exposed to zinc in the laboratory. Relative sensitivities of unexposed and selected generations were then compared by exposing parent, F1 and F2 individuals to zinc in contact filter papers tests. Calculation of effect concentrations from this work indicated differences in sensitivity to zinc for successive generations, with higher toxicity values (LC_{50}, LC_{90}, LC_{99}) found for the selected worms. The increases in resistance found for F1 and F2 worms were confirmed in a discriminating dose study. In addition to comparing the sensitivities of the parent, F1 and F2 generations for zinc, toxicity tests were also conducted with copper to assess if there was evidence of cross-resistance between the two metals. Results indicated similar increases in resistance to copper to those found for zinc. Mechanisms underlying the increased metal resistance were studied in toxicokinetic experiments. Results indicated no clear trends between the three generations indicating that physiological responses, other than differences in kinetic parameters, are responsible for the increased resistance found in the selected worms. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

For terrestrial invertebrates, over 50 papers concerning the evolution of metal tolerance have been published (Posthuma and Van Straalen, 1993). However, conclusive evidence for the presence of genetically based resistance has only been detected in the fruit fly *Drosophila melanogaster* and four soil invertebrate species. For *D. melanogaster*, Maroni et al. (1987) discovered metal-specific resistance in selected populations resulting from the induction of metal-specific metallothioneins (Bonneton and Wegnez, 1995). Furthermore, Shirley and Sibly (1999) detected increased cadmium resistance that was conferred by a single gene located on

the X chromosome. Among soil invertebrates, Donker and Bogert (1991) found genetic adaptation to cadmium in exposed populations of *Porcellio scaber*. Tranvik et al. (1993) found life-history divergence for clean and exposed populations of the springtails *Onychiurus armatus* and *Isotoma notabilis*, while one of the most detailed studies of metal resistance, Posthuma et al. (1992, 1993a, b), noted evolutionary changes in excretion rate, equilibrium concentration and life-history characteristics of the F1 and F2 progeny of *Orchesella cincta* from contaminated sites.

For earthworms, indirect evidence for metal tolerance is indicated by the fact that worms can be collected from soils containing residue levels that exceed effect concentrations for key life-cycle traits. For example, Spurgeon and Hopkin (1996a, 1999a) and Corp and Morgan (1991) found *Lumbricus rubellus* in soils containing 280 µg Cu g^{-1} and 3490 µg Zn g^{-1}, and 816 µg Cu g^{-1} and

183,000 µg Zn g^{-1}, respectively. This compares with the cocoon production EC$_{50}$s for copper of 80 µg Cu g^{-1} found by Ma (1988) and an EC$_{50}$ for zinc of 348 µg Zn g^{-1} found by Spurgeon and Hopkin (1996a) for this species (EC$_{50}$ = concentration resulting in a 50% reduction of the measured effect in the population). Despite the circumstantial evidence, Morgan and Morgan (1988) give the only data explicitly indicating resistance in earthworms. In this study, a lower proportion of lead was found in the 'rest' fraction (excluding anterior and posterior alimentary canal) of *Lumbricus rubellus* collected from a contaminated site when compared to reference site worms exposed to similar concentrations. The lower lead levels found in sensitive tissues suggest that polluted site worms show greater efficiency for lead storage and detoxification. Further studies designed to indicate metal tolerance in earthworms by Marino and Morgan (1998), Corp and Morgan (1991) and Bengtsson et al. (1992) have failed to find clear inter-population differences.

The absence of conclusive evidence for resistance in terrestrial oligochaetes is in contrast to aquatic species. Klerks and Levinton (1993) found increased cadmium tolerance in exposed populations of *Limnodrilus hoffmeisteri*. This was conserved for animals reared in clean sediment for two generations and could be induced in four or five generations by selection (Klerks and Levinton, 1989). That investigations with terrestrial oligochaetes have so far 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 be responsible for the elimination of earthworms from soils around a smelting works. Instead, reductions in earthworm number are probably due to the effects of zinc (Spurgeon and Hopkin, 1995; Spurgeon, 1997). As a result, in many contaminated areas, greatest selection pressure will be for the development of resistance to zinc, with lower selection pressure for other metals. It is, therefore, resistance to this metal that is the main focus of this study. In particular, emphasis is given to examining the extent (if any) of adaptation in worms selected over two generations in a laboratory selection experiment, determining if any differences in sensitivity could be explained in terms of zinc kinetics and assessing the magnitude of copper cross-tolerance. The work used established toxicological methods.

2. Materials and methods

2.1. Choice of earthworm species and test system

The evolution of zinc resistance in earthworms was examined in laboratory selection experiments using *Eisenia fetida*. This species was selected since it exhibits a higher reproductive rate and shorter generation time than soil-inhabiting species such as *Lumbricus rubellus* and could, therefore, be more readily cultured. The experiment used worms from a previously unexposed population reared between generations in a clean environment. Thus, any resistance that developed could be attributed to population level genetic adaptation.

Previous work examining the toxicity of zinc to *E. fetida* has indicated steep dose–response relationships for effects on survival and cocoon production (Spurgeon and Hopkin, 1996b). As a result, it was necessary to precisely control exposure during the selection phase of the study. To ensure that selections were conducted at the correct zinc concentration, the contact filter paper test was used for all exposures (OECD, 1984). This method was selected, because it is less vulnerable to within-system heterogeneity than soil-based tests and thus allows consistent dosing for all exposed worms. The contact filter paper test offers a relatively easy method of accurately dosing earthworms. However, exposure is less representative of the field situation than soil-based tests. In the field, uptake can be through dermal or intestinal routes (Van Gestel, 1992). However, in the contact filter paper test only dermal exposure is considered. The consideration of effects due only to passive exposure was not felt to be problematic, however, since a number of studies have indicated that the exposure of earthworms to metals occurs either directly from pore water or indirectly through a pore water-related route (Spurgeon and Hopkin, 1996b; Janssen et al., 1997; Peijnenburg et al., 1997).

For the contact filter paper test, single earthworms were exposed to the chemical for 48 h in glass vials lined with strips of standard filter paper (Whatman No. 1) treated with different concentrations of the test chemical. Zinc was added to each vial as a solution of the nitrate salt (Zn(NO$_3$)$_2$.6H$_2$O). The exposure concentrations used for the test were calculated on the basis of the soluble zinc levels present in the initial solution rather than the potentially innacurate unit of µg cm^2 recommended by the OECD (1984). To add the chemical, solutions of zinc nitrate were made to the correct test concentration. Vials lined with filter paper strips were then dosed individually with 1 ml of the relevant zinc solution. This was allowed to dry before 1 ml of deionised water was added. Finally, a single earthworm of the correct life stage and weight range that had been starved of food for 48 h was added to each vial. Each vial was sealed with a perforated lid and kept at 20°C in constant darkness for 48 h.

2.2. Selection, rearing and calculation of dose–mortality relationships

The initial stage of the selection process, a zinc toxicity test, was conducted to determine a baseline zinc dose–response relationship for *E. fetida*. The exposure

concentrations used were 48.3, 72.5, 84.6, 96.7, 109, 121, 133, 145, 169, 193, 216, 242, 266 and 290 µg Zn ml^{-1}, with 30 vials for each zinc level. Prior to the test, juvenile worms with individual weights of between 120 and 260 mg were taken from the previously unexposed 'parent' generation and starved for 72 h. During this period, they voided organic material from their guts that could reduce zinc availability in the test system. Juvenile worms were used for all exposure and selection phases of the study, since it is known that adult worms store sperm in their spermacathecae which could reduce the validity of the selection process (Meyer and Bouwman, 1997). After starvation, worms were transferred to the individual filter paper-lined glass vials treated with the relevant zinc concentration. Worms were maintained for 48 h at 20°C after which the number of dead worms at each concentration was counted.

After completion of the parent generation toxicity test, LC$_{50}$ (LC$_{50}$ = median lethal concentration), LC$_{90}$ and LC$_{99}$ concentrations and log–probit slope parameters were calculated by probit analysis using the MicroProbit 3.0 statistical software package. Calculation of these effect concentration values preceeded the selection of the parent generation worms. For selection, juvenile parent generation worms of a similar size range as used in the initial dose–response experiments (to avoid any effects due to differences in the surface:volume ratios of small and large worms) were exposed to the LC$_{90}$ concentration in contact filter paper vials. The aim of this exposure was to select the most tolerant 10% of the parent population from which to breed the F1 generation. For the selection, 660 parent worms were exposed to the LC$_{90}$ of 159 µg Zn ml^{-1}. All animals that survived were incubated on clean paper for 48 h, transferred to clean cultures, reared to adulthood and allowed to produce cocoons from which F1 worms were hatched and grown. When sufficient F1 juveniles of the specified weight range were available, a second contact filter paper test was conducted with the F1 generation. Exposure concentrations of 48.3, 72.5, 84.6, 96.7, 109, 121, 133, 145, 169, 193, 216, 242, 266 and 290 µg Zn ml^{-1}, were used, with 30 replicates for each concentration.

Completion of the dose–response study for the F1 generation allowed calculation of LC$_{50}$, LC$_{90}$ and LC$_{99}$ values using the MicroProbit 3.0 package. The calculated LC$_{90}$ value was used to select the most resistant F1 worms from which the F2 generation would be bred. For the selection, 600 juvenile F1 worms of the specified weight range were exposed to a zinc concentration of 185 µg Zn ml^{-1} in contact filter paper vials. All animals that survived were incubated on clean paper for 48 h, reared to adulthood in clean cultures and used to breed the F2 generation. The final stage of the study was to conduct a contact filter paper test for the F2 generation worms. For this test, juvenile F2 worms were exposed to a zinc concentration series of 48.3, 72.5, 84.6, 96.7, 109,

121, 133, 145, 169, 193, 216, 242, 266, 290, 314, 338 and 363 µg Zn ml^{-1}. Thirty vials were used for each concentration. The results of this test were used to calculate LC$_{50}$, LC$_{90}$ and LC$_{99}$ values for the F2 generation.

2.3. Sensitivity comparisons using discrimination dose

In addition to comparing the relative sensitivities of parent and F1 and F2 selected generations to zinc in dose–response studies, resistance development was also studied using the discriminating dose method. This technique, which is a powerful tool for identifying resistance has been widely used for monitoring tolerance to biocides in pest species (Ffrench-Constant and Roush, 1990; Gilbert et al., 1996). For the assay, selected generations are exposed to the LC$_{99}$ calculated for a susceptible population. Thus, in the current study, 100 juvenile worms with individual weights of between 120 and 260 mg from the parent, and the two selected (F1, F2) generations were exposed to the LC$_{99}$ of 198 µg Zn ml^{-1} determined for the parent worms. The percentage of worms surviving after 48-h exposure to this dose was determined and used to compare population sensitivities.

2.4. Estimation of copper cross-tolerance

A number of studies have demonstrated the presence of cross-resistance to chemically related and diverse pesticides in invertebrates (Sauphanor and Bouvier, 1995; Bisset et al., 1997). To examine if such cross-resistance also occurs for species selected using individual metals, a series of copper toxicity tests was conducted with parent, F1 and F2 worms exposed to zinc using the contact filter paper protocol. For the test, 30 replicate worms of the correct life stage and weight from the parent and two selected populations were exposed to copper concentrations of 2.56, 5.13, 10.3, 20.5, 30.8, 41, 51, 62, 82, 103, 123 and 144 µg Cu ml^{-1} added as a solution of the nitrate salt. Results from these tests were compared with the results from the zinc exposures to determine if any increases in zinc resistance in the selected generations were also present for copper. If similar increases in resistance for the two metals were found in the selected generations, this would indicate cross-resistance for the two metals. However, if increases in copper resistance were not found, this would indicate that the mechanisms underlying any increases in zinc resistance are specific for this metal.

2.5. Zinc kinetics in parent and selected generation worms

In addition to the zinc and copper dose–response and zinc-discriminating dose studies, toxicokinetic experiments were conducted to determine if differences in

sensitivity between the parent and selected generations could be explained by variations in zinc accumulation and elimination rates. To assess zinc uptake and excretion, time series exposures were undertaken for the three generations. To measure uptake, 280 juvenile *E. fetida* weighing between 120 and 200 mg (fresh weight), that had been starved for 72 h, were individually exposed to zinc in contact filter paper vials. The zinc concentration used in the uptake phase was 48.4 µg Zn ml⁻¹. This concentration was selected, since it was considered to be the highest concentration at which no mortality of worms would be recorded over the duration of the study. It was considered to be important to avoid mortality during the exposure phase, because any worms that died would be likely to have the highest zinc accumulation rate, since a link between body burden and toxic effect has been found in the experimental studies for soil invertebrates described by Van Straalen (1996) and Posthuma et al. (1998). Thus, if a concentration resulting in partial mortality had been used, these rapidly accumulating worms would have been missed from the sample. At selected time intervals (1, 2, 3, 4, 6, 9, 12, 15, 18, 24, 30, 36 and 48 h from the start of the experiment), eight worms were removed from the test containers, washed in distilled water, killed by immersion in liquid nitrogen and stored at −20°C for subsequent measurement of zinc concentrations.

After 48 h exposure, all remaining worms were transferred to clean containers to allow zinc excretion. At selected time intervals (1, 2, 3, 4, 6, 9, 12, 15, 18, 24, 30, 36, 48, 60, 72, 96 and 168 h), eight worms were removed, washed in distilled water, killed by immersion in liquid nitrogen and stored at −20°C. Analysis of zinc concentrations was conducted using a method adapted from Hopkin (1989). Whole worms were placed into acid-washed test tubes and dried to constant weight. Two millilitres of Analar-grade nitric acid was added to each tube and the solution heated until all tissue had been digested. Once cool, the digest was diluted to 10 ml with double-distilled water and analysed for zinc by flame atomic absorption spectrophotometry (AAS).

To model metal kinetics and allow accumulation and excretion rates to be calculated, separate models were fitted to the data from the accumulation and elimination phases. Previous studies of the uptake of metals by soil arthropods (Janssen et al., 1991) and earthworms (Posthuma and Notenboom, 1996; Marinussen et al., 1997; Spurgeon and Weeks, 1998) have indicated that uptake can be described by a one-compartment model. Thus, to model changes in earthworm zinc concentrations over the uptake phase, least-squared fitting of the data was conducted in the SAS statistical software system according to Eq. (1):

$$\text{Accumulation phase}: Q_t = C_r + \frac{a}{k}[1 - e^{-kt}], \qquad (1)$$

where: Q_t = concentration of zinc in the animal (µg Zn g⁻¹); t = time (h); C_r = concentration of residual zinc (µg Zn g⁻¹); a = accumulation rate (µg Zn g⁻¹ h⁻¹); and k = excretion rate (h⁻¹).

For the calculation, C_r was not estimated iteratively as zinc is an essential metal and is thus present in worms even in uncontaminated systems. Instead, values of C_r measured prior to the start of the exposure ($t = 0$) were used in all calculations.

Metal kinetics for the excretion phase of the study were modelled separately using a first-order decay with residual model according to Eq. (2):

$$\text{Elimination phase}: Q_t = a(e^{-kt}) + C_r, \qquad (2)$$

where: Q_t = concentration of zinc in the animal (µg Zn g⁻¹); t = time (h); C_r = concentration of residual zinc (µg Zn g⁻¹); a = accumulation rate (µg Zn g⁻¹ h⁻¹) and k = excretion rate (h⁻¹).

For the statistical analysis of pollutant kinetics, it is usual to use body burdens rather than tissue concentrations, since the latter can be affected by rapid changes in the weight of the organism. However, in the present study, the short accumulation period meant that substantial weight loss was unlikely. Thus, kinetic trends and parameters have been determined on a concentration basis to account for any variations resulting from the different body weights of the sampled worms.

3. Results

The contact filter paper test used to determine the toxicity of zinc for the parent generation gave an LC₅₀ value [with 95% confidence intervals (CIs)] of 122 (112–131) µg Zn ml⁻¹ and LC₉₀ and LC₉₉ values of 159 (148–176) and 198 (179–231) µg Zn ml⁻¹, respectively (Table 1). A slope parameter of 11.1 ± 0.9 was calculated for the log–probit regression in this generation. To select worms that would be used for breeding of the F1 generation, 660 juvenile worms were exposed to the LC₉₀ concentration of 159 µg Zn ml⁻¹ in contact filter paper tests. In total, 49 worms survived selection. This indicates that the most tolerant 7.4% of the population were used for production of the F1 generation.

The contact filter paper test using F1 juvenile worms gave an LC₅₀ of 135 (124–145) µg Zn ml⁻¹. Thus, the resistance ratio of LC₅₀ values for the F1 and parent generations worms was 1.11:1. Selection also resulted in higher LC₉₀ and LC₉₉ values of 185 (172–203) and 240 (217–278) µg Zn ml⁻¹. In addition to the effects of selection on calculated effect concentrations, a difference in the slope of the log–probit regression was also found. The calculated slope parameter in the F1 test was 9.3 ± 0.6, indicating a shallower slope than for the parent generation test. To select for worms that would

Table 1
LC_{50}, LC_{90}, LC_{99} [with 95% confidence intervals (CIs)] and the log–probit slope values calculated for three laboratory strains of *Eisenia fetida* in toxicity tests with (a) zinc and (b) copper[a]

Strain	LC_{50} (μg ml^{-1})	LC_{90} (μg ml^{-1})	LC_{99} (μg ml^{-1})	Slope $\pm$ SE
(a) Zinc				
Parent	122 (112–131)	159 (148–176)	198 (179–231)	11.1 ± 0.9
F1	135 (124–145)	185 (172–203)	240 (217–278)	9.3 ± 0.6
F2	144 (129–158)	225 (205–251)	324 (286–381)	6.6 ± 0.4
(b) Copper				
Parent	23.7 (14.5–31.6)	47.1 (36–62.6)	82.2 (61.9–133)	4.31 ± 0.39
F1	26 (19–32.2)	49.2 (40.6–60.7)	82.8 (66.3–116)	4.63 ± 0.41
F2	28.9 (21.4–35.9)	61.1 (50–78)	112.7 (87.8–163.2)	3.93 ± 0.39

[a] Parent worms are a susceptible strain, while F1 and F2 are bred from individuals selected on the basis of zinc tolerance (see text).

be used for the production of the F2 generation, 600 F1 juveniles were exposed to a zinc solution of 185 μg Zn ml^{-1}. In total, 12% of the exposed worms survived selection.

Exposure of the F2 generation gave an LC_{50} of 144 (129–158) μg Zn ml^{-1}. Thus, the ratios of LC_{50}s for this generation compared to parent and F1 generation worms were 1.18:1 and 1.07:1, respectively. The LC_{90} and LC_{99} values of 225 (205–251) and 324 (286–381) μg Zn ml^{-1} were also higher than the values found for the parent and F1 generations. Slope parameters for the log–probit regression indicated a shallower slope (6.6 $\pm$ 0.4) for the F2 worms when compared to both the parent and F1 values. To assess if there was an effect of selection on survival responses of the control, F1 and F2 generations, dose–response fits for survival in each of the three generations were compared using a chi-square test of the logistic regressions. Analysis indicated a significant difference in measured relationships ($\chi^2 =$ 100.87, $p < 0.001$). Thus, a clear effect of selection on the shape of dose–response fits was found for the three generations.

The increased resistance to zinc found in the F1 and F2 generations as indicated by differences in the shapes of the logistic regressions and by higher effect concentrations was supported by the results of the discriminating dose tests. Exposure of 100 worms from the parent, F1 and F2 generations to the LC_{99} determined in the parent generation test resulted in survival of

3% of worms for the parent generation, 8% in the F1 selected worms, and 19% of the F2s.

Results of the toxicity tests to determine the effects of copper on the survival of parent, F1 and F2 worms indicated increases in respective LC_{50}, LC_{90} and LC_{99} values and lower slope parameter values in successive generations (Table 1b). Resistance ratios for copper LC_{50}s were 1.09:1 (F1:parent), 1.21:1 (F2:parent) and 1.11:1 (F2:F1), respectively. Thus, the resistance ratios found for copper were comparable with those found for zinc. Calculated slope parameters in the copper test did not show the same consistent trend for decrease in the selected generations, although the lowest slope value was found for the F2 generation (Table 1).

Toxicokinetic measurements of zinc uptake and elimination were conducted to determine if the increased resistance of the worms found in the selection experiment could be explained from changes in kinetic parameters. For example, if reduced uptake or increased excretion was found in the selected worms, it would be anticipated that this would result in lower body burdens and thus lower toxic effects. Comparisons of time-dependent uptake indicated similar patterns in the three laboratory generations. Body burdens increased rapidly over the initial phases of the exposure and reached an equilibrium concentration after approximately 40 h (Fig. 1a–c).

From the body zinc concentration data it was possible to calculate the kinetic parameters of initial concentration, accumulation and excretion rates, half-lives and theoretical equilibrium concentrations. Value estimates for the three generations are summarised in Table 2. For the uptake phase, calculation of accumulation rates indicated the highest value of 11.7 μg g^{-1} h^{-1} was for the parent worms, an intermediate value of 11.1 μg g^{-1} h^{-1} was estimated for the F2, while the lowest value of 6.2 μg g^{-1} h^{-1} was for the F1 generation. Determination of excretion rates indicated that the highest value was for the parent worms, while values for the F1 and F2 generations were comparable. The excretion rates for the three generations could be used to estimate half-lives for zinc ranging from 9.7 h for the parent generation to 17.9 h for the F1 worms (Table 2). Short half-lives for zinc in *E. fetida* have been confirmed in toxicokinetic studies conducted in a soil test system (Spurgeon and Weeks, 1998), indicating that zinc excretion for this species is faster than for soil-inhabiting worms (Marino and Morgan, 1998). For example, Helmke et al. (1979) used radioisotopes to estimate a half-life of 245 days for zinc in soil-dwelling earthworm species. Estimated theoretical equilibrium concentrations were calculated using the equation $Co + (a/k)$. Estimations for the three generations gave values of 300, 286 and 402 μg Zn g^{-1} for the parent, F1 and F2 worms, respectively.

Comparison of the elimination rates for the excretion phase with those calculated during uptake indicated lower values in all cases (Table 2). Of the three genera-
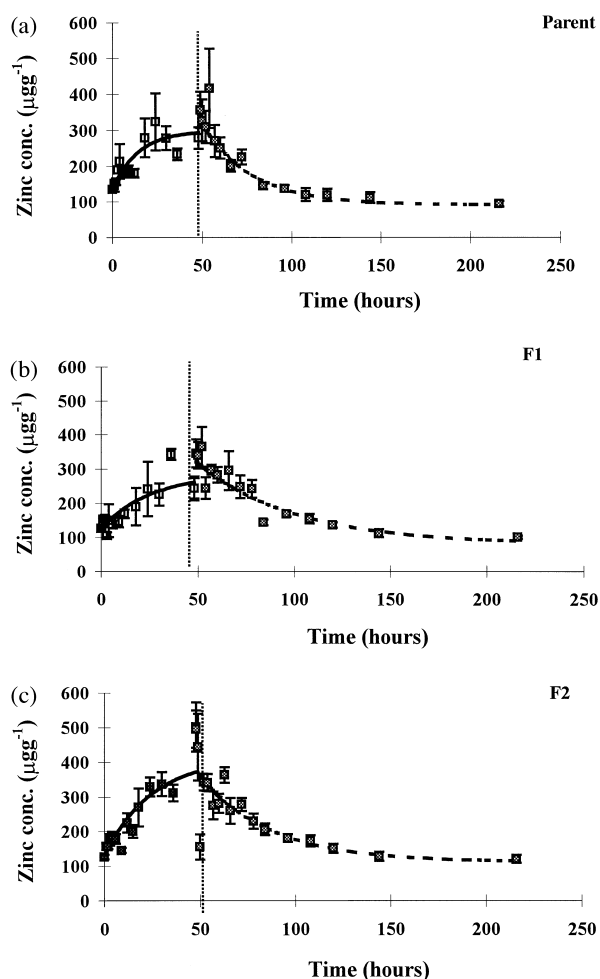
Fig. 1. (a–c) Accumulation and excretion of zinc in parent and two selected generations of the earthworm *Eisenia fetida* exposed to a solution of 48.3 µg Zn l^{-1} in contact filter paper vials. Original data are given as means (of eight replicates) with their standard errors. All values are expressed as µg g (dry wt.)$^{-1}$. The solid line indicates the fitted one-compartment model for the accumulation study; the dashed line indicates the fitted line for the elimination study.

tions, values were highest for the parent worms, intermediate for the F2 worms and lowest in F1s (Table 2). In order to determine the significance of any differences in kinetics between generations in both the uptake and elimination phases, comparisons of curves for the three generations were undertaken using an *F*-test. Results

indicated no significant differences in the shape of the curves in either the uptake or the elimination phase ($p > 0.05$).

4. Discussion

Selection of *E. fetida* with zinc over two generations resulted in significant differences in the shape of dose–response curves for survival of worms exposed to zinc and these differences were accompanied by changes in effects concentrations (Table 1). Resistance ratios for LC$_{50}$s between generations were 1.11:1 for the F1 and parent generations and 1.18:1 and 1.07:1 for the F2 and parent and F2 and F1 worms, respectively. Comparisons of LC$_{90}$ and LC$_{99}$ values also indicated lower sensitivity in selected generations. The increased zinc resistance found for selected generations in the dose–response tests was confirmed in a discriminating dose study. Exposure of the three generations to the zinc LC$_{99}$ for parent worms resulted in highest survival for F2 worms with lowest survival in the parent generation.

The significant differences found between regression fits for the selected generations in the dose–response tests reflected changes in calculated slope parameters for log–probit regressions (Table 1). The steepest slope (11.1 ± 0.9) was found in the parent generation, while the lowest slope (6.6 ± 0.4) was for the F2 worms. Changes in log–probit regression slopes are frequently found in populations that have undergone adaptation as a result of the presence of toxic substances. This applies particularly when resistance does not conform to a monogenic model, but is conferred by selection at a number of loci (Chilcutt and Tabashnik, 1995).

The changes in dose–response relationships and the reduced toxicity found for the F1 and particularly F2 worms in the contact filter paper tests clearly suggests that selection has occurred. However, two other factors in addition to zinc concentration, namely pH and nitrate concentration could underlie the selection effect. Analysis of pH in the zinc solutions prepared for dosing the filter paper vials indicated that pH increased from 3.73 in the uncontaminated distilled water to 5.22 at 290 µg Zn ml^{-1}. Earthworms are known to be sensitive to

Table 2
Kinetic parameter estimates for zinc uptake and excretion by the earthworm *Eisenia fetida*[a]

Strain	Accumulation phase				Elimination phase	
	a (µg g^{-1} h^{-1})	k (h^{-1})	Half-life (h)	Equilibrium content (µg g^{-1})	k (h^{-1})	Half-life (h)
Parent	11.7 ± 3.62	0.072 ± 0.027	9.7	298	0.034 ± 0.012	20.4
F1 selected	6.2 ± 1.72	0.039 ± 0.016	17.9	286	0.02 ± 0.008	34.7
F2 selected	11.1 ± 2.18	0.041 ± 0.012	17	398	0.027 ± 0.027	25.7

[a] 'Accumulation phase' values are for worms exposed to 48.4 µg Zn ml^{-1} in contact filter paper tests. 'Elimination phase' values are for worms exposed to 48.4 µg Zn ml^{-1} for 48 h and then moved to a clean environment. For definitions of the parameter, their calculation and details of procedure for their calculation, see Eqs. (1) and (2). Half-life is calculated by ln2/k and equilibrium content by $Co + (a/k)$.

pH. However, survival is improved at neutral pH in comparison to acid conditions (Bengtsson et al., 1986). Thus, it is unlikely that pH is responsible for the dose–response and selection effects observed in this study. Nitrates are known to have direct toxic effects on earthworms. However, comparative studies have indicated that effect concentration exceeded those for zinc by a factor of at least two (Posthuma and Notenboom, 1996). Thus, it appears unlikely that nitrate is primarily responsible for the dose effect and selection responses seen in this study. Instead it is likely that it is a combined effect of zinc and nitrate, such as that demonstrated for copper by Diaz Lopez and Mancha (1994), with the major portion of the effect due to zinc, that underlie the dose–response adaptations found in this study.

In an attempt to determine the physiological nature of the resistance found in selected generation worms, a toxicokinetic study was undertaken for the three generations to determine if the differences in sensitivity between parent and selected generation worms could be explained by changes in zinc kinetics. Results from this study indicated no differences in the shapes of kinetic curves and no clear trends in calculated accumulation or elimination parameters, zinc half-lives or theoretical equilibrium concentrations (Table 2). That the increases in zinc resistance found in the selected generations could not be related to changes in metal kinetic indicates that the adaptation present is conferred by changes in other physiological parameters in the selected earthworm generation. However, at present, it is not possible to be certain as to which mechanisms underlie the increased zinc resistance found.

In addition to comparing the zinc sensitivity of parent and selected generation worms, copper toxicity tests were also conducted to determine the extent of any cross-resistance between metals. The resistance ratios found for copper were 1.09:1 (F1:parent), 1.21:1 (F2:parent) and 1.11:1 (F2:F1), respectively. Thus, increases in copper resistance were comparable to those found for zinc. That increases in copper resistance in selected generation worms mirror those for zinc suggests that the increased resistance in selected generations are determined by changes in non-metal-specific factors, rather than the presence of increased levels of metal-specific proteins. The presence of metal-specific metallothioneins in worms at contaminated sites has been reported in a number of studies and it has been suggested that the presence of higher levels of these proteins, as a result of the up-regulation of gene expression, may explain population persistence (Morgan et al., 1989; Stürzenbaum, 1997; Stürzenbaum et al., 1998). However, to date, no evidence for zinc storage metallothionein in earthworms has been found. Stürzenbaum (1997) has isolated a non-metallothionein like zinc-binding protein from a polluted site population of *Lumbricus rubellus*.

However, since the structure and function of this protein are currently not known, it is difficult to predict if it plays a role in zinc (and copper) resistance.

The increases in zinc resistance found in this study are relatively small compared to those for cadmium resistance in laboratory-selected generations of the aquatic oligochaete *Limnodrilus hoffmeisteri* (Klerks and Levinton, 1989). Resistance in this species was found to have a high heritability, indicating a rapid development of resistance when this species is exposed in the field. Studies of resistance to pesticides have also indicated that resistance can be induced within a few generations in laboratory-selected animals. For example, Bloch and Wool (1994) found a seven-fold increase in LC_{50} for potato whitefly *Bemisia tabaci* selected by exposure to methidathion over eight generations, while Thomas and Boethel (1995) found a 93-fold increase in the soybean looper, *Pseudoplusia includens* exposed to permethrin over nine generations. Differences in the rate of development of resistance for species exposed to pesticides and metals can be explained by the fact that pesticides have specific modes of action, while metals have multiple-target effects. Thus, pesticide resistance can develop rapidly by selection of specific enzyme systems, such as esterases, while for many metals, with the exception of those such as cadmium that have clearly established metallothionein-based detoxification systems (Stürzenbaum et al., 1998), multi-system adaptation may be required.

Limitations on the extent of zinc resistance in *E. fetida* may be due to the fact that this metal is essential for earthworms. Zinc has a number of important acid catalyst, control ion and structural ion functions (Fraústo da Silva and Williams, 1991). Thus, the metabolism of this metal may be conserved in evolution, resulting in the absence of the phenotypic variability required for selection. Low genetic variability for zinc metabolism and hence susceptibility is suggested by the fact that steep dose–response relationships are frequently found for the effects of this metal on worms (Spurgeon and Hopkin, 1996a, b; Reinecke and Reinecke, 1997). Such steep slopes are considered characteristic for populations exhibiting high homogeneity at loci which determine sensitivity (Roush and Tabashnik, 1990). Unlike zinc, cadmium is not known to have an essential function in earthworms. As a result, it may be possible for populations to support greater phenotypic (and genetic) variability allowing greater scope for selection in the laboratory or field.

Despite the relatively slow rate of development, it would still be anticipated that earthworms at many metal-contaminated sites may exhibit resistance, since the historic nature of many industrial and mine sites allows selection over numerous generations. That earthworm populations persist in many metal-polluted soils is circumstantial evidence for the presence of tolerance in

these populations (Spurgeon and Hopkin, 1996a, b). However, to date, no clear evidence that confirms the presence of tolerance to zinc or indeed any other metal in natural populations of terrestrial oligochaetes has been found. Confirmation of the presence of tolerance in polluted site populations represents the next step for determining the role of resistance in modulating earthworm distribution at contaminated sites. Such work should focus on the status of earthworm at long-term contaminated sites to determine if they differ in sensitivity from naïve populations (Spurgeon and Hopkin, 1996b).

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