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Dose-Dependent Effects of Dietary Pb and Zn on Feeding and Growth Rates of the Landsnail *Helix engaddensis*

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Dose-dependent effects of dietary lead and zinc on feeding and growth rates of adult *Helix engaddensis* snails were studied over a 1-month period followed by a 2-week recovery period. Snails were fed on an artificial diet containing the following lead or zinc concentrations: 0, 20, 100, 500, 2500, and 12,500 µg/g dry food. At the end of the 6 weeks, mortality rate among snails fed on Pb-contaminated diet was 18.3% (11/60) and the respective value for Zn-contaminated diet was 50% (30/60). Both metals significantly reduced growth and feeding rates. Snails were found to be sensitive to zinc but tolerant to lead. During the recovery period, snails fed on Zn-contaminated diet failed completely to feed or grow normally, whereas snails fed on Pb-contaminated diet showed signs of slow improvement in terms of feeding and growth rates. The NOEC and LOEC for lead were 100 and 500 $\mu g \cdot g^{-1}$, respectively, while the respective concentrations for zinc were 20 and 100 $\mu g \cdot g^{-1}$. © 2001 Academic Press

Key Words: landsnails; *Helix engaddensis*; lead; zinc; feeding; growth; toxicity.

INTRODUCTION

Snails and some other invertebrates concentrate heavy metals in their tissues and are considered as good test animals for studying the kinetics of metal accumulation and detoxification (Berger and Dallinger, 1989; Gomot, 1997). The kinetics of metal accumulation and detoxification is still a subject of discussion and there is a lack of consensus regarding metal toxicity in snails (Laskowski and Hopkin, 1996b). Berger and Dallinger (1989) found that terrestrial snails might regulate some metals assimilated from food. Van Straalen *et al.* (1987) suggested that nutritional metals might be regulated, while xenobiotic metals are accumulated.

Using snails in toxicity bioassays is an attractive method, since snails are easy to culture in the laboratory and can be fed on artificial diets with the desired amounts of metals and they respond quickly to metal contamination in the range of sublethal doses. However, this might be complicated by the fact that snails fed on diets supplemented with metals may decrease food consumption or even estivate and stop feeding and hence, decrease growth rates (Simkiss and Watkins, 1990; Laskowski and Hopkin, 1996b). Beeby (1985) did not observe any aversion of *Helix aspersa* to lead-contaminated diet. In contrast, Simkiss and Watkins (1990) suggested that *H. aspersa* is able to detect high concentrations of Zn in the diet and consequently, reduce feeding rate of highly contaminated food. According to Gomot (1997), the mechanism involved in the inhibition of growth of snails fed on metal-supplemented food is still unknown. In a study on the effect of cadmium on the growth of the snail *H. aspersa*, she suggested that growth inhibition could be due to inhibition of the production of a growth hormone essential for the growth of *Helix*. Szücs *et al.* (1994) suggested that chronic exposure of neurons of the nerve collar of the snail *Lymnaea stagnalis* to Cd can irreversibly modify the structure of the Ca channel.

The purposes of the present study were to evaluate the dose-dependent effects of dietary Pb (a nonessential metal) and Zn (an essential metal) on growth and feeding rates of the snail *Helix engaddensis* and examine the suitability of this snail to be used in laboratory short-term toxicity bioassays; to estimate the concentrations of Pb and Zn (EC) that reduce growth and feeding rates by 50, 75, and 100% compared to controls and to determine the no-observed-effect concentration (NOEC) and the lowest-observed-effect concentration (LOEC) for both metals; to compare the sensitivity of the snail to both metals; to check whether feeding and growth inhibition caused by both metals is reversible or not when the metals are no longer added to the diet of the snail; and to compare results of a previous study (Swaileh and Ezzughayyar, 2001) on the effects of Cd and Cu on the same species under the same conditions.

MATERIALS AND METHODS

Collection and Culture of Snails

H. engaddensis is one of the common landsnails in Palestine. It is smaller in size than European *Helix* species. Adult

TABLE 1
Nominal Treatments and Actual (Measured) Concentrations of Lead and Zinc (Mean ± SE, $n = 3$) in the Experimental Food and Average Weight (g ± SE) of Snails in Each Group at the Start of the Experiment

| | Metal concentration in the diet ($\mu g \cdot g^{-1}$) | | |
Group and metal	Nominal	Actual	Average weight
G1–Pb	0(control)	1.25 ± 0.250	5.11 ± 0.10
G2–Pb	20	19.90 ± 0.33	5.16 ± 0.15
G3–Pb	100	96.20 ± 2.60	5.20 ± 0.08
G4–Pb	500	454.5 ± 11.1	5.18 ± 0.10
G5–Pb	2500	2274 ± 141.2	5.10 ± 0.18
G6–Pb	12,500	12104 ± 519	5.15 ± 0.17
G1–Zn	0 (control)	14.60 ± 1.50	7.38 ± 0.28
G2–Zn	20	30.90 ± 2.30	7.29 ± 0.32
G3–Zn	100	104.1 ± 6.00	7.37 ± 0.29
G4–Zn	500	521.7 ± 35.0	7.41 ± 0.52
G5–Zn	2500	2517 ± 151.1	7.40 ± 0.34
G6–Zn	12,500	12615 ± 415	7.49 ± 0.35

snails were collected from a house garden in Qalqilia city and cultured in the laboratory in large glass aquaria. Snails were fed on carrots and lettuce and kept at room temperature. Before starting the experiment, snails of similar weight were selected, cleaned, and kept in transparent plastic boxes (size $17 \times 13 \times 7$ cm). Each box contained 10 snails (Table 1). Boxes were perforated on the sides to allow proper aeration. The bottom of each box was covered by a thin sponge soaked with deionized water to keep 100% humidity. The experiments were run in a growth chamber at 15°C and 16/8-h light/dark period. Before starting the experiments, snails were offered an artificial control food for 3 days in order to acclimatize.

Preparation of food

Metal stock solutions (1 g/L) were prepared using lead nitrate ($Pb(NO_3)_2$) and zinc chloride ($ZnCl_2$). An artificial food containing ca. 5% dry mass was prepared by mixing 4 g Cerelac—with vegetables—baby food (Nestlé, Belgium) and 1 g agar (Sigma, St. Louis) with solutions containing the required concentrations of metals (Table 1) to give 100 ml of agar medium. A fungicide (*p*-hydroxy benzoic acid methyl ester = methyl paraben, Sigma) was added to the solutions as 0.3 ml/100 ml food. Each 100 ml of medium was divided equally between four Petri dishes (25 ml/dish). After cooling, Petri dishes were kept in the refrigerator. Control food was prepared the same way except distilled water was used instead of the metal solutions above. From each treatment three Petri dishes and food were taken randomly and dried in an oven at 60°C until constant weights were observed. Thereafter, dried food was ground to powder and sub-

samples of 0.2 g were taken from each treatment and digested using a mixture of 1:1, nitric:perchloric acids (Suprapur, Merck) until the mixture became clear. Samples were diluted with deionized distilled water and volumes were adjusted to 25 ml in volumetric flasks. Finally, concentrations of Pb and Zn were measured (Table 1) using a flame atomic absorption spectrophotometer (Water and Environmental Engineering Department, Birzeit University).

Feeding of Snails

Snails were offered food *ad libitum*. Boxes were examined daily and food was offered as required. Once every 7 days, the snails were weighed and the boxes were cleaned. Unconsumed food that remained in the Petri dishes was freed of feces and dried in an oven at 60°C until a constant weight was obtained. Knowing that the dry mass of food in each dish was about 1.25 g, the dry weight of consumed food was calculated for each group on a weekly basis. During the first 4 weeks of the experiment, snails were fed metal-contaminated food. Thereafter, all groups of snails were fed food with no metals added (control food) for another 2 weeks.

Statistical Analysis

All statistical tests were performed using SYSTAT for Windows 5.02 (SYSTAT, Inc., Evanston, IL, 1993). $P \leq 0.05$ was used in all tests to determine statistical significance. Each week, average weights of groups were tested for differences using ANOVA test. Thereafter, Tukey test was performed for pairwise comparisons between groups. The growth coefficient for each group was calculated weekly as mean weight of that group $\times 100$/mean weight at the start of the experiment (Gomot, 1997). The values of the growth coefficient obtained each week were plotted against log values of the concentrations of metals used in the experiment. The straight-line equation obtained enabled the calculations of the estimated concentrations (EC_G) that reduce growth rate by 50, 75, and 100% compared to controls. Feeding rates were calculated as the average dry food (mg) consumed by every snail each week. Food consumption values were transformed into percentages of food consumption compared to that of the control group, which was considered 100%. Percentages were plotted against the log of the concentrations of each metal studied. This semi-log regression enabled the calculations of the estimated concentrations (EC_F) that reduce food consumption by 50, 75, and 100%.

RESULTS

Mortality

During the 6 weeks of the experiment, 41 snails of 120 died. From the snails fed on Pb-contaminated diet, 11 death

cases were reported. These were distributed over all groups except the control. In groups 2 and 3, one death case was observed per group, while death rate in groups 4, 5, and 6 was 3 snails per group. The death cases observed among snails fed on Zn-contaminated food were obviously higher (30 snails) and were from all groups. The cases were as follows G1 = 1, G2 and G3 = 4 each, G4 = 5, and G5 = 6. Moreover, all snails in group 6 died during the sixth week of the experiment.

Growth

Effect of dietary Pb on the growth of *H. engaddensis* was obviously dose-dependent (Fig. 1). The inhibitory effect of Pb on growth of snails became distinct after 2 weeks of exposure, as the average weight of group 6 (12,500 μg Pb/g food) became statistically less than that of controls, whereas inhibitory effect on other groups was not significant during the first 2 weeks. During the third and fourth weeks of exposure, growth inhibition started to be obvious. Snails in groups 4, 5, and 6 estivated most of the time during the exposure period of the experiment and reduced or refused feeding. Snails in other groups, exposed to Pb concentra-
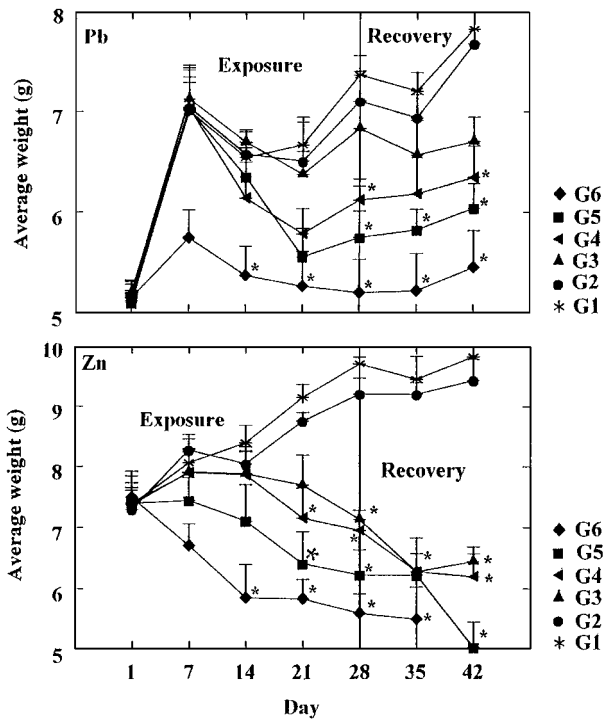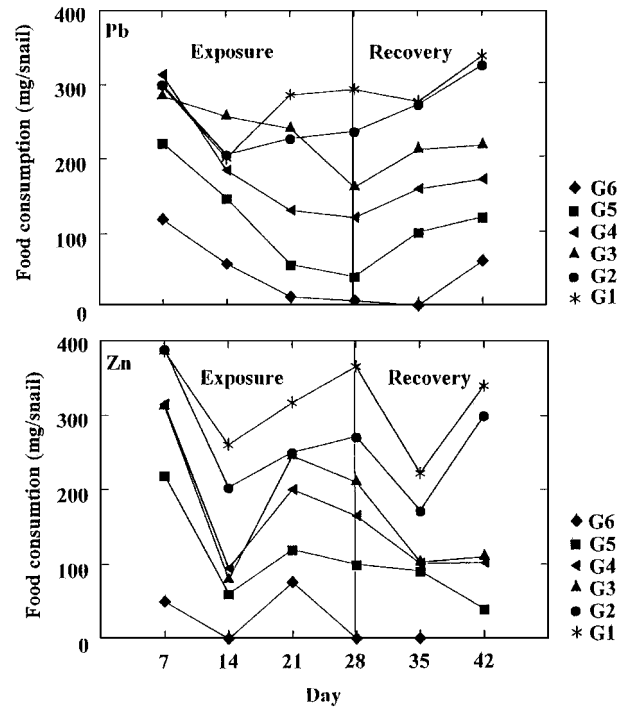

FIG. 2. Food consumption of *Helix engaddensis* snails exposed to Pb- and Zn-contaminated food for 28 days (Exposure) and offered control food for another 14 days (Recovery). Metal concentrations were G1 = 0, G2 = 20, G3 = 100, G4 = 500, G5 = 2500, and G6 = 12,500 μg/g dry food.

tions $\leq 100 \, \mu g \cdot g^{-1}$, did not show significant growth inhibition and continued to grow. At the end of the 4 weeks of exposure, average weights of groups 4, 5, and 6 were significantly less than the average weight of the controls. Growth coefficients (Fig. 2) of groups 4, 5, and 6 decreased with time of exposure. However, values did not decrease below 100. The highest dietary concentration of Pb that did not show any inhibitory effect on growth (NOEC) after 4 weeks of exposure was $100 \, \mu g \cdot g^{-1}$ and the lowest concentration that significantly slowed growth rate (LOEC) was $500 \, \mu g \cdot g^{-1}$. At the end of the 4 weeks of exposure, all snail groups were offered control food (no Pb was added) for 2 weeks. Snails did not show significant signs of recovery, although they started to feed and grow slowly (Figs. 1 and 3). Significant differences in average weight between the control group and groups 4 ($500 \, \mu g \cdot g^{-1}$), 5 ($2500 \, \mu g \cdot g^{-1}$), and 6 ($12,500 \, \mu g \cdot g^{-1}$) continued to exist until the end of the experiment.

During the first 2 weeks of the experiment, no significant relationship was observed between growth coefficients and dietary Pb concentrations. Such a relationship became statistically significant only after the third and fourth weeks of exposure (Table 2). Therefore, it was possible to calculate the $EC_{G\,50,\,75,\,100}$ for only the third and fourth weeks of exposure (Table 2). All EC_G values decreased from the third

FIG. 1. Growth curves of *Helix engaddensis* snails exposed to Pb- and Zn-contaminated food for 28 days (Exposure) and offered control food for another 14 days (Recovery). Metal concentrations were G1 = 0, G2 = 20, G3 = 100, G4 = 500, G5 = 2500, and G6 = 12,500 μg/g dry food. Values represent means ± standard errors. *Values significantly different (*P* < 0.05) from the control.
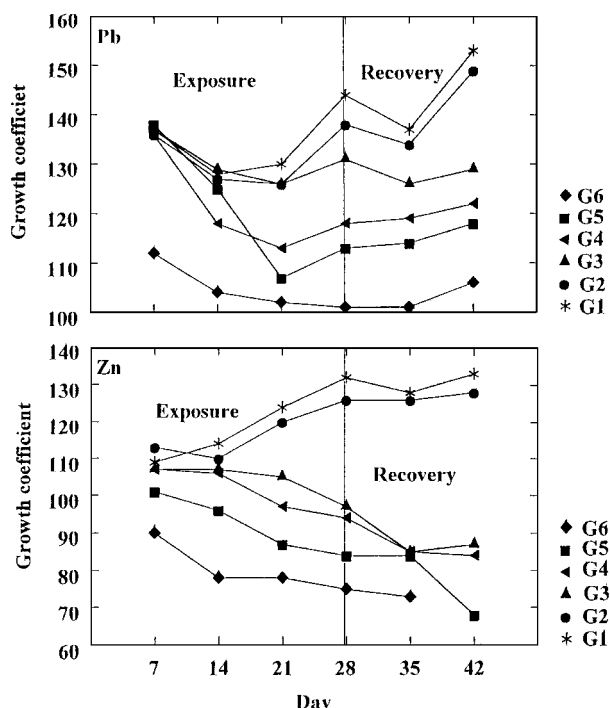
FIG. 3. Growth coefficients of *Helix engaddensis* snails exposed to Pb- and Zn-contaminated food for 28 days (Exposure) and offered control food for another 14 days (Recovery). Metal concentrations were G1 = 0, G2 = 20, G3 = 100, G4 = 500, G5 = 2500, and G6 = 12,500 μg/g dry food.

TABLE 2

Weekly Estimated Effect of Dietary Lead and Zinc (20–12,500 μg · g^{-1}) on Growth of Snail *Helix engaddensis* Fed on Pb- and Zn-Contaminated Food for 4 Weeks

Week	Metal	Inhibition equation	EC_G (μg·g^{-1}) 50	75	100	NOEC (μg·g^{-1})	LOEC (μg·g^{-1})
2	Zn	$y = 28.35 - 10.73\log(x)$ $r = 0.91, P = 0.016$	100	200	440	2500	12,500
3	Zn	$y = 36.77 - 14.59\log(x)$ $r = 0.99, P = 0.001$	50	130	330	100	500
4	Zn	$y = 44.28 - 17.59\log(x)$ $r = 0.98, P = 0.003$	40	115	330	20	100
3	Pb	$y = 40.66 - 9.58\log(x)$ $r = 0.97, P = 0.007$	420	2720	17,550	2500	12,500
4	Pb	$y = 55.71 - 13.16\log(x)$ $r = 0.98, P = 0.001$	360	2500	17,100	100	500

Note. EC_{50G}, estimated concentration that reduces growth rate to 50% compared to the control; NOEC, no-observed-effect concentration; LOEC, lowest-observed-effect concentration.

week to the fourth. For example, the EC_{50G} decreased from 420 μg · g^{-1} after the third week to 360 μg · g^{-1} after the fourth week.

Effect of dietary Zn on growth of snails was also found to be dose-dependent. Growth inhibitory effect of Zn first became significant after the second week of exposure, as the average weight of snails in group 6 (12,500 μg · g^{-1}) became significantly less than that of the control group (Fig. 1). After the third week of exposure to Zn-polluted diets, average weights of snails in groups 4, 5, and 6 (Zn concentration > 500 μg · g^{-1}) became significantly less than that of controls. After the fourth week, all groups, except 2, (Zn concentration = 20 μg · g^{-1}) had average weights that were significantly less than those of controls. Snails were estivating most of the time during the exposure period and feeding activity was reduced or stopped. Growth coefficients decreased regularly with exposure time (Fig. 2). Growth coefficients below 100 were observed for all groups except 1 and 2. The highest concentration of dietary Zn that did not show any growth inhibitory effect (NOEC) after 4 weeks of exposure was 20 μg · g^{-1}, while the lowest concentration that slowed growth rate (LOEC) was 100 μg · g^{-1}. When all snail groups were offered control food by the end of the fourth week, snails did not show signs of recovery and continued to estivate and refuse feeding (Figs. 1 and 3). At the end of the

sixth week, average weights of snails in all groups (except G2) remained significantly less than that of the control.

A significant relationship between Zn concentration in the diet and growth coefficients of groups was first established after the second week of exposure. $EC_{G50, 75, 100}$ were calculated for the second, third, and fourth weeks of exposure. Values decreased strongly from the second to the third week, but slightly from the third to the fourth one (Table 2). For example, the EC_{G50} decreased from 100 to 50 to 40 μg · g^{-1} at the end of the 2, 3, and 4 weeks of exposure, respectively.

Feeding

Feeding rates of snails expressed as milligram dry food/snail/week are shown in Fig. 3. Feeding rate of snails offered metal-contaminated diet was clearly dose-dependent. Effect of dietary Pb on food consumption was noticeable after the first week of exposure (Fig. 3). This was clear by the decrease in feeding rate from 300 mg/snail (control group) to about 100 mg/snail (G6 = 12,500 μg Pb/g food). However, a significant relationship between food consumption and dietary Pb concentration was first established after the third week of exposure (Table 3). During the third and fourth weeks of exposure, food consumption of snails in group 6 decreased to about zero and snails were estivating most of the time. Even after control food was offered, snails in group 6 did not respond directly like other groups, which exhibited a slight improvement in food consumption (Fig. 3). Values of $EC_{F50, 75, 100}$ were estimated for the third and fourth weeks of exposure (Table 3).

Effect of dietary zinc on feeding rates of snails was obvious after the first week of exposure (Fig. 3). Each snail in the

TABLE 3
Weekly Estimated Effect of Dietary Lead and Zinc ($20–12{,}500\,\mu g \cdot g^{-1}$) on Feeding of Snail *Helix engaddensis* Fed on Pb- and Zn-Contaminated Food for 4 Weeks

			EC_F ($\mu g \cdot g^{-1}$)		
Week	Metal	Inhibition equation	50	75	100
1	Zn	$y = 150.8 - 30.61\log(x)$ $r = 0.95, P = 0.015$	1970	12,800	84.4[a]
2	Zn	$y = 96.91 - 23.46\log(x)$ $r = 0.91, P = 0.03$	100	1160	13,500
3	Zn	$y = 114.45 - 21.88\log(x)$ $r = 0.96, P = 0.009$	880	12,250	170[a]
4	Zn	$y = 109.30 - 25.46\log(x)$ $r = 0.99, P = 0.001$	210	2046	19,600
3	Pb	$y = 129.06 - 30.55\log(x)$ $r = 0.96, P = 0.009$	390	2550	16,700
4	Pb	$y = 114.58 - 28.18\log(x)$ $r = 0.99, P = 0.001$	190	1500	11,600

Note. EC_{50F}, estimated concentration that reduces feeding rate to 50% compared to the control.

[a] Concentrations in $mg \cdot g^{-1}$.

control group consumed about 400 mg dry food, while this figure decreased to about 200 mg in group 5 and to about 50 mg in group 6. Even when snails were offered control food, no signs of feeding improvement were noted. Snails from all groups continued to die and living snails were either estivating or consuming very little food. Exposure of snails to 12,500 μg Zn/g food for 4 weeks was enough to kill 100% of the snails in group 6. A statistically significant relationship between feeding rate and dietary metal Zn concentration was significant from the first week on. Values of $EC_{F50,\,75,\,100}$ are shown in Table 3.

DISCUSSION

During the 6 weeks of the experiment, death rates of *H. engaddensis* snails fed on Pb- and Zn-contaminated diet ($20–12{,}500\,\mu g \cdot g^{-1}$) were 18.3 and 50%, respectively. Compared to results of a previous study on the same species (Swaileh and Ezzughayyar, 2000), death rate among snails fed on Pb-contaminated diet (18.3%) is close to that among snails fed on Cd-contaminated diet (16.7%), whereas mortality rates among snails fed on Zn-contaminated diet (50%) was greatly higher than that among snails fed on Cu-contaminated diet (1.7%). These figures look higher than figures reported by some authors for other species. For example, Laskowski and Hopkin (1996b) studied the effect of Zn, Cu, Pb, and Cd on fitness of *H. aspersa*. Mortality rate was 6.7% among juveniles and 1.9% among adults during the 4 months of exposure to diet contaminated with Pb and Zn at concentration ranges similar to those used in the current study.

Both Zn and Pb in the diet were found to inhibit growth of *H. engaddensis* in a dose-dependent manner. The inhibitory effect of the two metals became statistically significant during the second week of exposure, especially when highest concentrations are considered. With increasing exposure time, the growth inhibitory effect of the metals became more obvious and at least 28 days of exposure were necessary to assess the effect of different dietary metal concentrations on growth of *H. engaddensis*. No effect of dietary Pb on growth of snails at concentrations below 100 $\mu g \cdot g^{-1}$ was observed during the 28 days of exposure. While 100 μg Zn/g food was enough to negatively affect growth. The EC_{G50} after 4 weeks of exposure were 40 and 360 for Zn and Pb, respectively. Moreover, the growth coefficients of snails fed on Pb-contaminated diet remained above 100 throughout the period of exposure, while some growth coefficients for those fed on Zn-contaminated diets (concentration $\geq 2500\,\mu g \cdot g^{-1}$) decreased below 100, indicating negative growth that might be due to toxicity. Therefore, *H. engaddensis* snails seem to be more sensitive to Zn than Pb. Laskowski and Hopkin (1996b) studied the effect of Cu, Cd, Zn, and Pb on fitness in snails *H. aspersa*. They observed that Zn was the only one among the four metals that affected growth rate. Moreover, when they mixed the four metals together in one treatment, they found that toxicity was clearly dominated by the effect of Zn. Their study revealed that *H. aspersa* was very insensitive to lead. In a previous work, Swaileh and Ezzughayyar (2000) studied the effects of dietary Cu and Cd on *H. engaddensis*. The EC_{G50} after 4 weeks of exposure was calculated as 100 $\mu g \cdot g^{-1}$ for both metals. Comparing this concentration with that of Zn ($40\,\mu g \cdot g^{-1}$) and Pb ($360\,\mu g \cdot g^{-1}$) in the present work emphasizes the high toxic effect of Zn compared to other metals. The high value of EC_{G50} for lead is in agreement with the results of Laskowski and Hopkin (1996b) that *Helix* is very insensitive to lead.

The frequent estivation of snails fed on metal contaminated food is a well-known phenomenon. It is not clear, however, whether estivation and food rejection are due to toxicity or simply because snails have the ability to detect polluted food and thus, take action. According to Simkiss and Watkins (1990), *H. aspersa* is able to detect high concentrations of Zn in the diet and reduce its feeding rate if contamination is too high. Gomot et al. (1992) and Gomot (1997) suggested that cadmium may disturb the function of the neurosecretory cells that secrete a growth hormone in the genus *Helix*, causing "growth stoppage." Szücs et al. (1994) found that acute exposure to Cd of neurons of the nerve collars of *L. stagnalis* can reversibly block the Ca channels, whereas, chronic exposure can irreversibly modify the structure of the channel. In the previous work on *H. engaddensis* (Swaileh and Ezzughayyar, 2000), snails reduced or completely refused to consume Cu- and

Cd-contaminated food during the 4 weeks of metal exposure. When offered control food during the fifth and sixth weeks, snails fed on Cd-contaminated diet failed to consume food and growth inhibition continued. On the other hand, snails fed on Cu-contaminated diets started feeding immediately when control food was offered and within 2 weeks, all groups had average weight similar to those of controls. Therefore, It was assumed that snails were irreversibly toxified by Cd-contaminated diets, while snails fed on Cu-contaminated diets were actually starving, as they were refusing polluted food. In the present work, snails fed on Zn-contaminated diet behaved in a manner similar to those fed on Cd-contaminated diet. Snails fed on Pb-contaminated diet responded to control food slowly, and it is possible that 2 weeks of recovery did not allow enough time for the snails to gain weight and equivalent to those of controls. This might be because elimination of accumulated metals (i.e., Pb) proceeds slowly. Cu could have a special efficient metabolic removal (or accumulating) process, as it is required for the synthesis of hemocyanin. Depending on the metal encountered, it can be assumed that growth inhibition estivation in *Helix* fed metal-contaminated diets could be due either to their ability to recognize and reject polluted food (starvation) or to irreversible toxicity.

CONCLUSIONS

At sublethal doses of Pb and Zn, the landsnail *H. engaddensis* was found to respond quickly to metal-polluted diets. Therefore, these snails might be suitable for short-term laboratory toxicity testing of heavy metals.

Zinc was found to be of higher toxicity to *H. engaddensis* than Pb, although both metals inhibited feeding and growth in a dose-dependent manner. However, when offered control food, snails fed on Zn-contaminated diet failed completely to respond and continued negative growth. This is assumed to be due to an irreversible toxicity. Snails fed on Pb-contaminated diet responded slowly to control food but the 2 weeks of recovery were not enough for snails to grow and reach weights to match those of controls. Therefore,

growth inhibition caused by Pb-polluted diet might be reversible but in a slow rate, which could be linked to elimination of the metal. The NOEC and LOEC for dietary Pb were 100 and 500 $\mu g \cdot g^{-1}$ while the respective values for Zn were 20 and 100 $\mu g \cdot g^{-1}$.

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