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Bradybaena similaris (Férrusac) Shell as a Biomonitor of Copper, Cadmium, and Zinc

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Many studies have used mollusks as indicators of metal pollution in aquatic and terrestrial environments (Coughtrey and Martin 1977; Russell et al. 1981; Beeby and Eaves 1983; Berger and Dallinger 1993; Nelson et al. 1995; Rainbow 1995). For example, monitoring programs such as the U.S. Environmental Protection Agency's (EPA) Mussel Watch utilize blue mussels, *Mytilus edulis*, as an indicator to examine marine pollution (Farrington et al. 1983; Nelson et al. 1995). Mollusks have been favored in these studies because mussels of aquatic environments have been shown to bioconcentrate many inorganic contaminants, mainly heavy metals. Mollusks are said to store these metals by factors of 10^2 to 10^5 above seawater concentrations (Williamson and Evans 1972; Beeby and Eaves 1983; Farrington et al. 1983; Nelson et al. 1995). Several authors claim that snails could accumulate substantial amounts of heavy metals in a polluted environment. They have also suggested that the snails may represent a critical pathway for the food-chain transport of heavy metals (Popham and D'Auria 1980; Beeby and Eaves 1983; Berger and Dallinger 1993; Laskowski and Hopkin 1996; Gomot 1997). Coughtrey and Martin (1977), using the terrestrial snail *Helix aspersa*, argued that terrestrial snails of a similar size are suitable for use as a biological indicator of metal pollution.

Studies on terrestrial mollusks, particularly snails, suggest that variation in metal accumulation in their soft tissues is due to the body size, age, and season (Beeby and Eaves 1983). Due to their complex metabolism, the details of complete biochemical relationships between the metal concentration in their environment and in their tissues are not well known. Previous studies have demonstrated that heavy metal elements once accumulated into mollusk soft tissues may be decreased when the mollusk is moved to a noncontaminated environment (Williamson 1980; Beeby and Eaves 1983). However, the advantage of the use of mollusks is that the metabolic elimination of metal accumulated into soft tissues is relatively slow compared with other organisms (Cossa 1989). On the other hand, the shells are known to preserve the accumulated metals within their crystalline calcitic or aragonitic structure, and do not excrete metals even after the death of the organism (Watson et al. 1995). If the metals are accumulated into the shell in proportion to the metals in the environment, the shells of the snails must be fit to be used as a convenient environmental bioindicator rather than their soft tissues. However, the studies on the terrestrial snail shells from this point of view have been very scanty. Additionally, no standardized monitoring methods are yet available for terrestrial snails, for most studies used snails of unknown age and unknown physiological background (Gomot and Pihan 1997). For such reasons, basic information on snails, specifically in regards to the biochemical pathways for uptake of heavy metals in relation to their growth, are necessary for the development of an effective monitoring method.

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The aim of this study was to determine the extent of Cu, Cd, and Zn accumulation in *Bradybaena similaris*, a terrestrial snail. Another goal was to assess whether the snail shell could be used as a monitor of heavy metal pollution in terrestrial environments through comparative studies on bioaccumulation of these metals in both the shells and the soft tissues. In addition, effects of their living environment and heavy metals on their growth were investigated.

MATERIALS AND METHODS

B. similaris was chosen for the reasons that they are easily found and are wide spread throughout the natural environment in Japan, and also because they are relatively easy to cultivate in laboratories. The parent snails were collected from Meguro-ku, Tokyo, Japan. In order to reflect the randomness of snail size in a natural habitat, 20 adult snails were paired to make a mass of 1000 juvenile snails. Then the snails were randomly selected into ten groups, 40 snails in each. The snails were cultivated separately in small acrylic boxes, 100×60×20mm, until the fourth week, followed by cultivation in boxes twice the size of the first. The cultivation was run for a total of 12 weeks at 25℃ under light control of 18L6D. They were given an artificial diet containing Cu, Zn, and Cd. The metal concentrations added to the artificial diets were: control (0 ppm), Cu (2ppm, 4ppm, 8ppm), Cd (0.4ppm, 2ppm, 4ppm), and Zn (2ppm, 4ppm, 8ppm). These concentrations were chosen according to the preliminary studies, which showed that majority of snails given the diets containing metals over 10 to 20ppm did not survive through the cultivation experiments. The snails were weighed once every week to observe the influences of the cultivating environment on snail growth.

Based upon previous artificial diet recipes (Kohno 1976; Laskowski and Hopkin 1996), an artificial diet was synthesized to control the metal concentration. Our method was similar to that of Laskowski and Hopkin (1996), which we deemed appropriate in order to maintain the homogeneity of the metals in each diet as much as possible. First, 1g agar (Wako Pure Chemicals) and 4g powdered base (cat food: $CaCO_3$=3:1) were soaked in 55ml deionized water, and 40ml of each metal solution of various concentrations was added to make the total volume of the diet at 100ml. Cat food was from Natural Recipe®, purchased from a local supermarket. Metals were supplied from Wako Pure Chemicals in the following chemical forms: $Cu(NO_3)_2$, $Cd(NO_3)_2$, and $Zn(NO_3)_2$. Deionized water used here was made by Millipore Elix-3 at 15MΩ/cm. After being boiled and stirred well with a magnetic stirrer, the diet was formed into thin layers in petri dishes. The diet was kept in a refrigerator (4℃) until use.

The metal concentration of each diet was monitored by ICP-AES, Seiko Instruments SPS7700, occasionally prior to use. Cu and Cd were not detected in the control diet. However, Zn concentrations were 0.5ppm. Also, measured metal concentrations in each diet were within the expected concentrations±0.1ppm. Thus, for convenience, the expected values were applied to discuss the results. After 12 weeks of cultivation, the shells of the snail were separated from their bodies. Then, the bodies were dissected into three parts: mantle, liver, and the rest of the body (which is abbreviated as RB hereafter). The shell was carefully washed by an ultrasonicator in ultra-clean deionized water prepared with a Millipore Elix-3, vacuum-dried, and powdered in an agate mortar with an agate pestle. Since some body parts were <5mg, too scarce to be analyzed alone, the parts were vacuum-dried, weighed one by one, mixed, and then

homogeneously powdered to obtain the mean metal concentration of each body part.

A portion of the powdered parts was weighed and digested by acids in a polytetrafluoroethylene (PTFE) beaker with a PTFE watch glass overnight: the shells by nitric acid, body parts by nitric acid with perchloric acid (HNO_3: $HClO_4$=10:1v/v). The purity grade HNO_3 and $HClO_4$ (toxic metal analysis grade, Wako Pure Chemicals) were chosen to avoid high background metals. Deionized water used was made by a Millipore Elix 3 at 15MΩ/cm for washing and a Millipore ICP-MS 18MΩ/cm for analysis. All glassware and PTFE-ware were first boiled in aqua regia followed by boiling in ultra-clean water (Elix-3), and then dried for use. The plastic containers were ultrasonicated in diluted HNO_3 followed by ultrasonication in ultra-clean water, and then dried. After evaporation of acids, the samples were diluted by super-clean water and analyzed by a Hewlett-Packard ICP-MS, HP4500.

RESULTS AND DISCUSSION

The order of metals accumulated in the soft tissues were Zn>Cu>Cd for the liver, Cu>Zn>Cd for RB, and Cu>Zn≧Cd for the mantle. The concentrations of Cu in each part of the snail (shell, mantle, liver, RB) are presented in Fig. 1. As illustrated in Fig. 1c, the highest concentration of Cu was in the liver and RB. In these two parts, the Cu behavior was similar, with an exception that the level of Cu in RB is approximately 1.5 times higher than that of the liver. The next highest was in the mantle, and the least, in the shell. The highest Cd concentration was found in the liver (Fig. 2). Unlike the results obtained for Cu, the Cd concentration in the mantle showed an almost proportional increase with increasing Cd concentration in the diet in the range from zero to 4ppm Cd diet. Overall, the concentration of Cd in each snail part was half that of Cu. Zn accumulation in the liver was the highest among the metals investigated in this study (Fig. 3).

A finding of the present study is the apparent correlation that has been observed between Cu and Cd in snails' shells and other parts of the body (Figs. 1-2). Among the data presented in the results, metal contents of the body parts of snails fed with Cu and Cd were nearly proportional to the concentration of each metal in their diet, except for Cu in the mantle, as mentioned by Odzak et al. (1994) and Puente et al. (1996) for *Mytilus provincialis*. In our study, *B. similaris* accumulated more of essential elements than the nonessential element, Cd (Fig. 1-3). These results were in accordance with the results obtained by Williamson (1980) with *Cepaea hortenosis,* a terrestrial snail, in which a high level of zinc was observed in its soft tissues, particularly in the digestive gland. Laskowski and Hopkin (1996) observed similar uptake of both elements in the soft tissues of adult *H. aspersa.* Cu and Zn are among the essential elements, and are known to be immobilized in membrane-limited vesicles through a detoxification mechanism in bivalves (Puente et al. 1996). In addition, certain proteins are known to detoxify metals, and Cd is principally eliminated via the kidneys and detoxified by proteins such as metallothioneins (Cossa 1989). Dallinger et al. (1989) stated that their high capacity for metal accumulation and storage is attributed to the induction of metal-binding proteins belonging to the metallothioneins. Moreover, these are probably responsible for the long half-life of Cd in the snails (Williamson 1980; Berger and Dallinger 1993). Evidence with other mollusks indicates a possibility for the metals (Cu, Cd, and Zn) in *B. similaris* to be detoxified in the same or similar manner and explains why metals are accumulated in the liver rather than the mantle. However, the biological function of the metallothioneins is yet a subject of discussion.

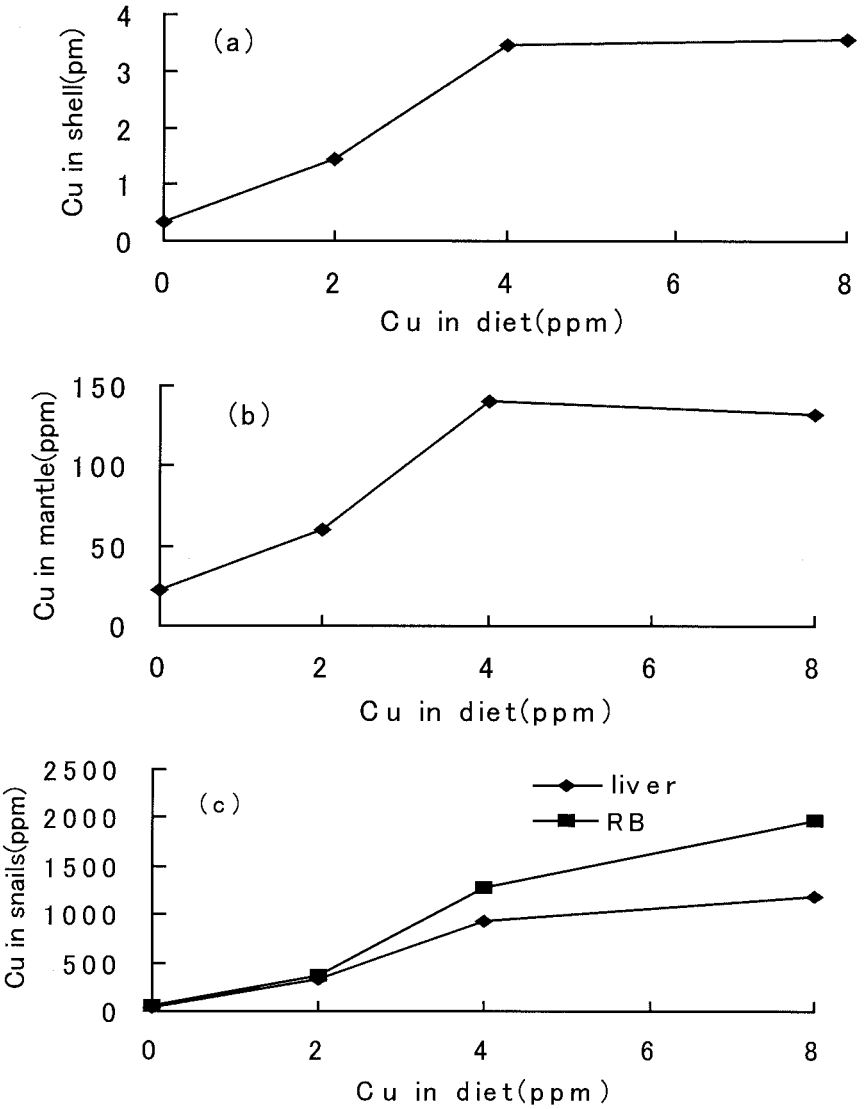

Figure 1. Cu concentration in each part of the body (dry wt.) against the concentration added to diet (wet wt.): (a) shell, (b) mantle (c) liver and RB (Control 0ppm, n=16; 2ppm, n=14; 4ppm, n=15; 8ppm, n=15)

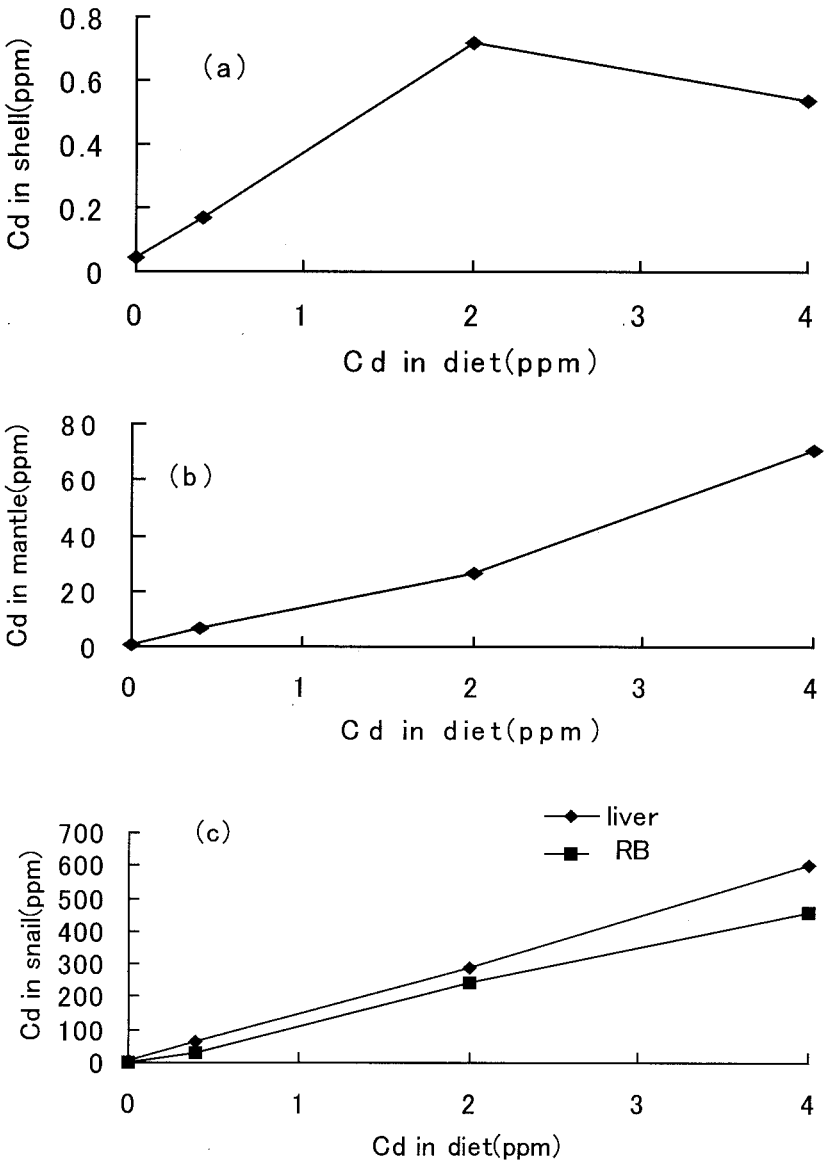

Figure 2. Cd concentration in each part of the body (dry wt.) against the concentration added to diet: (wet wt.) (a) shell, (b) mantle (c) liver and RB (Control 0ppm, n=16; 0.4ppm, n=17; 2ppm, n=10; 4ppm, n=12)

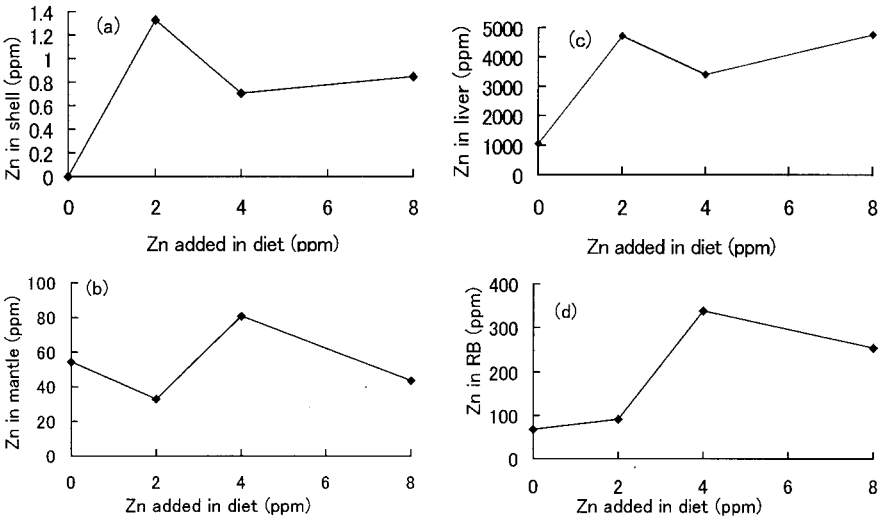

Figure 3. Zn concentration in each part of the body (dry wt.) against the concentration added to diet: (a) shell, (b) mantle (c) liver and (d) RB. (Control 0ppm, n=16; 2ppm, n=16; 4ppm, n=11; 8ppm, n=11)

The shells accumulated Cu and Cd by nearly 10^{-2} times the livers, and Zn and Cd by approximately 10^{-3} times. The uptake of Cu by the mantle and the shell exhibited a plateau over the range of 4ppm Cu diet, though the mantle concentrated the element about 40 times more than the shell (Fig. 1a and 1b). There was a significant correlation ($p<0.05$) between Cu concentration in each body part and the shell (Table 1a). However, as presented in Fig. 2a, the Cd concentration in the shells ceased increasing in snails fed with >2ppm Cd diet. The shells showed a slight correlation with the body parts (Table 1b). For Zn, only the metal accumulated in the liver and in the shell correlated to each other ($r=0.93$) (Table 1c). It is noteworthy that considering the amount of Zn accumulated in the liver, Zn was considerably less accumulated than the other two metals into the shell.

In *B. similaris*, no apparent correlation was seen for Zn between the diet, shells, or body, except the liver. The reason for this may be that the snail is capable of accumulating Zn easily (Puente et al. 1996). The concentration of Zn in the diet in this experiment was perhaps too high for *B. similaris* to observe a more sensitive, multifunctional activity of this element in the snail. Diet groups in lower Zn concentrations may be required. Zn accumulated in the *B. similaris* shell could be a possible elimination route for detoxification; however, only a small proportion absorbed by the body is eliminated by this route. Puente et al. (1996) also reported similar phenomena about Zn in the *Mytilus galloprovincialis* shell. It must be noted that Zn in plant and forest litter is present ca. 100 times greater in concentration,while it is ca. 13-24 times less toxic than Cd (Laskowski and Hopkin 1996). In the case of our study, when *B. similaris* given 4ppm Zn diet and 4ppm Cd diet was compared, a similar amount was accumulated into the shell, although Zn was accumulated ca. 8 times higher in the liver than Cd. This suggests that there could be a mechanism other than the level of toxicity of each metal for the incorporation of these metals into the shell.

The growth rates of snails under different diet regimes differed significantly. There were variations in snail weights between control diet and highly concentrated diet

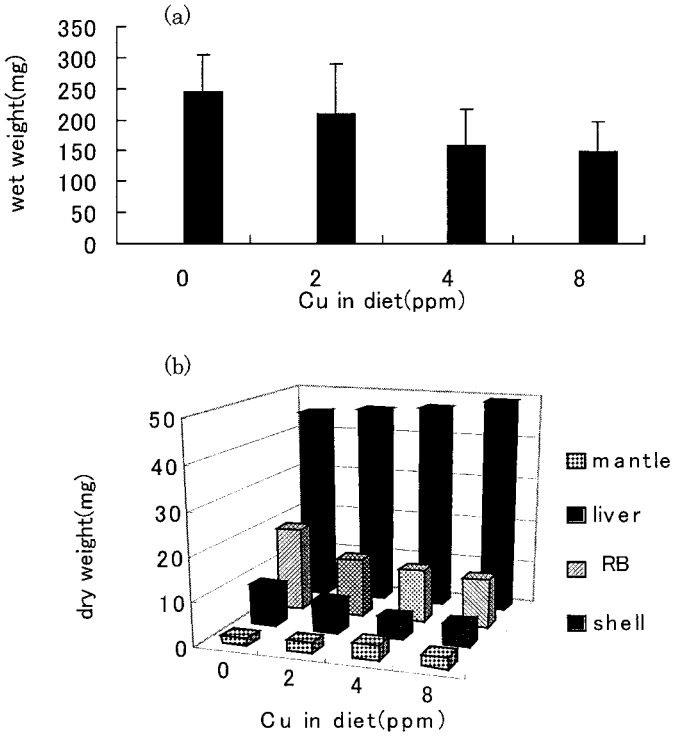

Figure 4. Growth of snails after the 12 weeks fed on Cu diet : (a) whole snail weight and (b) dried weight of each part. (Control 0ppm, n=16; 2ppm, n=14; 4ppm, n=15; 8ppm, n=15)

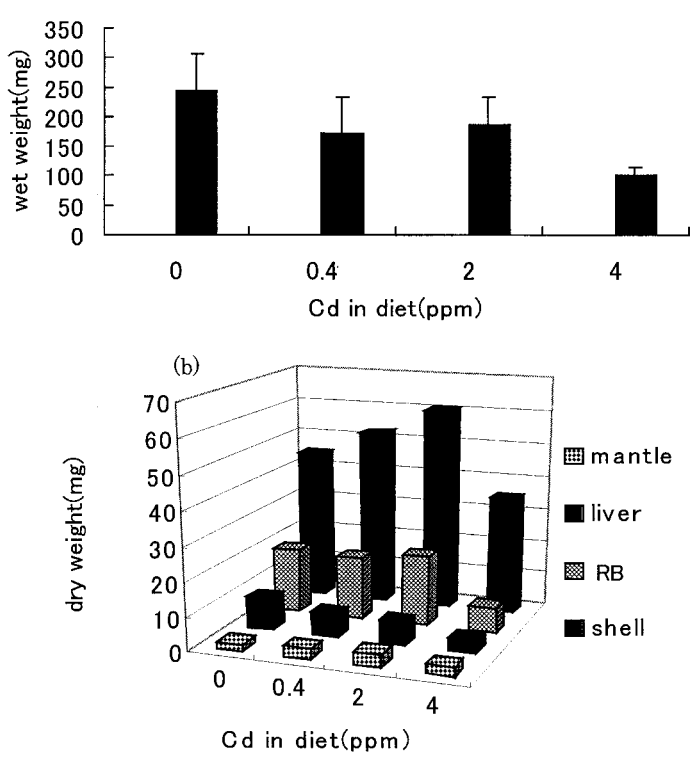

Figure 5. Growth of snails after the 12 weeks fed on Cd diet: (a) whole snail weight and (b) dried weight of each part. (Control 0ppm, n=16; 0.4ppm, n=17; 2ppm, n=10; 4ppm, n=12)

groups, especially for Cu and Cd. The whole snail weight decreased with increased Cu in the diet by 30% (Fig. 4a). There was a significant difference ($p<0.05$) in average snail weight between the snails given 0ppm Cu and 8ppm Cu diet, after 12 weeks of cultivation. However, as the Cu concentration in the diet increased, the shell weights increased while the weights of body parts decreased, except for the mantle (Fig. 4b). For Cd, the average snail weight of the 4ppm diet group showed a significant difference ($p<0.01$) relative to the control diet after the 12th week (Fig. 5a). For Zn, the variation was not so consistent. Yet, it is suspected that the variation in whole snail weights is largely due to the decreasing weights of soft tissues with increasing metal concentration in diet. These variations in snail weights and shell weights indicate a large natural intrapopulation variability in shell metal concentration even in animals grown in the same environment (Watson et al. 1995).

The above finding suggests that growth rate varies among snails of different environments. It is not in accordance with some studies on mollusks from natural environments, where some authors proposed that it is appropriate to apply mollusks of similar size for analysis (e.g., Coughtrey and Martin 1977). Our study indicates that for *B. similaris*, the age and size of snails are not necessarily the same when they are fed on diets in different metal concentrations implying different environments. Thus, caution must be paid when studying snails from the field, since if the above is true, the snails of the same size from different environments do not necessarily indicate that they are of the same age.

In order to solve the problem of size differences of snails, Watson et al. (1995) have normalized the metal content in shells by shell weight. However, as presented in our study, the whole snail weight differed significantly ($p<0.05$) between snails given

Table 1 Correlation coefficient (r) between each body part: (a)Cu, (b)Cd, and (c)Zn. Bolded values are those that are significant ($p<0.05$).

(a) r=	shell	mantle	liver	RB
shell	1			
mantle	**0.996**	1		
liver	**0.984**	**0.968**	1	
RB	**0.948**	**0.924**	**0.989**	1

(b) r=	shell	mantle	liver	RB
shell	1			
mantle	0.669	1		
liver	**0.746**	**0.994**	1	
RB	0.783	**0.985**	**0.997**	1

(c) r=	shell	mantle	liver	RB
shell	1			
mantle	-0.443	1		
liver	**0.930**	-0.404	1	
RB	0.158	0.661	0.358	1

metal containing diet, yet the shell weights did not differ significantly. Thus, if live snails could be sampled in the field, it may be best to normalize the metal content in snails by employing parameters containing both the shell weights and the whole snail weights. For an example, we have tried normalizing the metal content in shells by multiplying it by shell weight/ whole snail weight.

$$\frac{[metal\ \ concentration] \times shell\ \ weight}{snail\ \ weight} \cong adjusted\ \ \ concentration$$

In the case of our study, the above methods could only be applied to the Cu fed snails. Good correlation between the shell and whole snail weights were observed only among these snails but not among snails given Cd and Zn. For the Cd fed snails, the snails given the most contaminated diet, 4 ppm, showed an abrupt change in its growth rate, the weight of each body part dropped considerably (Fig.5b). A similar drop in growth rate was observed for *Helix aspersa* (Gomot 1997). This may have been caused due to the acute toxicity of Cd at this concentration level. When the data for 4ppm diet group were eliminated, these methods fit well for Cd as well. As mentioned earlier in the discussion, the concentration of Zn in the diet in the designed experiment was perhaps too high. Thus, no correlation between shell and whole and snail weights was observed. The measured and normalized metal content in shell versus the added metal content in diet are presented in Table 2, along with values obtained by Watson's method.

Table 2. Correlation coefficient between metal content in shells and metal added in diet (*=results eliminating the 4ppm diet group)

	r_{Cu}	r_{Cd}	r_{Cd}*
Watson's	0.8903	0.8790	0.9999
measured	0.8914	0.7629	0.9997
normalized	0.9276	0.7997	0.9999

We have confirmed that *B. similaris*, a terrestrial snail, shows a linear relationship between some trace metals (Cu and Cd) in diet and their uptake into its tissue. The shell incorporates metal during its formation and this is a slow process compared to the accumulation and liberation of metals from the tissues. Considering these conditions, the shells may be considered advantageous over the soft tissues for environmental monitoring, as long as the same aged animal is sampled for comparison. In addition, the shells are more consistent in weight than the body parts, or the soft tissues, and thus are easily handled during the sample preparation for metal analysis. Thus, the shells of *B. similaris* are more convenient and effective than its soft tissues for monitoring heavy metals in terrestrial environments, especially in Japan, because *B. similaris* is widely spread on the islands of Japan and are a good species to study as a representative pollution-inflicted animal.

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