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Magnesium and the regulation of lead in three populations of the garden snail *Cantareus aspersus*

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Lead assimilation by Cantareus aspersus provides no indication of an evolved regulatory mechanism for Pb or for any interaction with dietary Mg.

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ABSTRACT

Helicid snails appear to regulate Pb more closely than other toxic metals, though it is reported as the least toxic. No regulatory mechanism has been described in animals, and the possible role of Mg in limiting Pb assimilation is examined here for the first time. Three populations of *Cantareus aspersus* were fed Pb and Ca with three levels of Mg for up to 64 days. Metal assimilation and production efficiency was calculated for each of 108 snails. Populations differed in their pattern of uptake but soft tissue Pb was unaffected by dietary Mg. The proportion of Pb assimilated did not change as soft tissue concentrations increased, indicating no specific regulatory mechanism. The daily addition of Pb to the soft tissues increases with growth rate suggesting uptake is instead some function of growth or cell turnover. Bioconcentration factors varied with time and are unreliable indicators of an evolved regulatory mechanism for Pb.

1. Introduction

Of the major toxic metals, lead is commonly found to be the least toxic for most invertebrates. Compared to Cd, Cu and Zn, Pb was 5–6 times less toxic across a variety of measures in the snails *Cantareus aspersus* (Laskowski and Hopkin, 1996a,b) and *Helix engaddennsis* (Swaileh and Ezzughayyar, 2001). Because it had the lowest bioconcentration factor, Laskowski and Hopkin (1996a) concluded that Pb was also the most closely regulated, with concentrations in *C. aspersus* always less than half those of its diet. It appears that Helicid snails have the most effective regulatory mechanism for the least toxic of these four metals.

Lead, like Cd, has no biological role but, unlike Cd, Cu and Zn, does not induce a specific metallothionein (Dallinger et al., 2001). Other than these binding proteins, the regulation and excretion of several toxic metals appears to be linked with Ca metabolism and especially the Ca-rich intracellular granules by which they are isolated and excreted (Simkiss et al., 1982). An association between Ca and Pb has been known for some time, for a range of animals (Beeby, 1978; Bondgaard and Bjerregaard, 2005; Dallinger et al., 2001; Eeva and Lehikoinen, 2004; Gillis and Wood, 2008; Ireland, 1975; Rogers et al., 2003; Schanne et al., 1989; Snoeijs et al., 2005), but the mechanisms of Pb assimilation are not fully described, nor its partitioning between tissues (Cunha et al., 2008; Dallinger and Wieser, 1984). Lead can act as a chemical analogue for Ca in some enzymes and regulatory proteins (Behra, 1993; Schanne et al., 1989), and it may inhibit the mineralization of the shell in juvenile snails (Beeby et al., 2002). However, Pb does not simply follow metabolic pathways for Ca since some Ca-rich tissues, such as the dart sac or eggs, have little detectable Pb (Beeby and Richmond, 1998).

Given the close association of Ca and Mg metabolism (Burton, 1972; Vormann, 2003), perhaps the affiliation of Pb with Ca is mediated by Mg. Because of its role as an intracellular messenger, Mg concentrations in the cell are kept within a very small range (Vormann, 2003). Magnesium is crucial to several aspects of metabolism, including energy release and storage, and these may be perturbed by Pb (Dowd et al., 1990). Competition between Mg and Pb for the binding sites in a range of phosphodiesterases may contribute to the neurological effects associated with Pb poisoning (Srivastava et al., 1995). However, any possible role that Mg regulation plays in limiting Pb assimilation does not appear to have been examined before in any animal group.

Part of the problem is deciding whether soft tissue concentrations are actually regulated or if assimilation is reduced by a toxic effect of Pb, following some functional impairment of snail metabolism or activity. Extremely high dietary levels of Pb will reduce food consumption (Laskowski and Hopkin, 1996a; Swaileh and Ezzughayyar, 2001) though concentrations closer to those

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encountered in most polluted habitats induce no feeding aversion (Beeby, 1985; Marigomez et al., 1986). In the absence of a specific inducible protein, several authors suggest the low bioconcentration factors for Pb indicate regulation, though these have typically been measured following a short feeding trial with no quantification of Pb retention.

The present experiment measures the consumption and proportion of Pb retained by *Cantareus aspersus* over 64 days, on diets with three levels of Mg. It compares three populations from habitats with a wide range of soil Ca and Mg levels, to which the snails might be locally adapted. All snails were sub-adults about to embark on new season growth of shell and soft tissues, so their assimilation was assessed at a time of high Ca and Mg demand. By recording the food consumption of individual snails at each feeding interval, Pb assimilation was measured over time to assess soft tissue regulation and the effect of dietary Mg in each population.

2. Method

Hibernating *Cantareus aspersus* were collected from three locations in March and April 2008. These were sub-adults (lacking an operculum lip), with fresh weights ranging between 1 and 3 g (overall mean = 2.46, $s = 0.76$), which would add a shell extension (and perhaps become adult) with their new season growth.

2.1. Snail populations and culturing

Two populations came from Ca-rich habitats – the chalkdowns of southern England (LE) and a limestone plateau in the Corbières on the Mediterranean coast of France (RF). The third population came from a site with a Ca-poor soil (SB) in southern England (Table 1). These habitat differences may have promoted ecotypes with essential metal metabolisms adapted to the local Ca and Mg availability. Some authors suggest that *C. aspersus* from the Mediterranean Basin represent a distinct sub-species (*maxima*) based on their larger adult size (Madec and Daguzan, 1993). Certainly RF snails produce adults that are much larger than SB or LE in laboratory cultures (Beeby and Richmond, 2007).

On collection all snails were washed in running tap water, and allowed to feed on commercially-grown lettuce for 3–4 weeks, with frequent further washings. After this time, four individuals from each population were sacrificed and analysed for their metal content (day 0; Table 2). For each population 36 snails were randomly allocated to an artificial diet with one of three levels of Mg but with similar Pb and Ca levels (Table 3a), to measure the effect of dietary Mg concentrations on Pb assimilation.

Each snail was kept in a 250 ml plastic container with deionised water supplied from a cotton wool wick. At each feeding interval a snail was provided with a glass slide coated with food for 2 days, after which the slide was removed, the container washed and the snail starved for 2–3 days. After washing again, the snail was presented with the next coated slide. The food consumed at all feeding intervals were summed to give the total for each snail, and from this, its consumption of Ca, Mg and Pb.

Following 24 h depuration, four individuals from each population on each diet were sacrificed at three intervals – 16, 32 and 64 days. The metal content of their soft tissues and shells were measured and the assimilation rate of a metal calculated from the increase in mass compared to the day 0 snails:

$$\text{Percentage assimilation} = \frac{\text{Soft tissue metal content} - \text{soft tissue metal content at start } (\mu g)}{\text{Weight of metal consumed } (\mu g)} \times 100$$

Food assimilation efficiency (or production efficiency – PE) was measured as the increase in total dry weight of the snail as a percentage of the weight of food consumed.

Using the same protocol, a subsequent experiment measured the effect of dietary Pb on feeding rates and soft tissue Mg concentrations in RF juveniles. Twenty-four 80 day old juveniles, raised in the laboratory from a single brood, were fed a diet with Mg levels close to those of the Mg 2 diet above, but with one of three concentrations of Pb (Table 3b) for 16 days. Six replicates were fed on each diet, and 6 unfed juveniles were sacrificed at day 0.

There were no mortalities during any of the experiments.

Table 1
The mean concentrations (and SE) of Mg, Ca and Pb in the native soils of the three populations of *Cantareus aspersus* ($n = 4$).

Soil	Mg (μg g^{-1})	Ca (mg g^{-1})	Pb (μg g^{-1})
LE	1692 (47)	108.3 (0.5)	86.5 (0.8)
SB	958 (51)	1.1 (0.03)	154 (7.9)
RF	950 (46)	595.1 (38)	3.3 (0.5)

LE = Ca-rich habitat, S. England; SB = Ca-poor habitat, S. England; RF = Ca-rich habitat, S. France.

2.2. Diets and the measurement of food consumption

To assess the effect of Mg on Pb assimilation, three diets were provided with different concentrations of Mg, as $MgCO_3$ (Table 3a) covering the range found in the three native soils (Table 1).

The base diet consisted of a 1:1:1 mixture of dried egg albumin, rice flour and dried lettuce to which were added single supplements of $CaCO_3$ and $PbSO_4$. Lead sulfate represents a common form of lead occurring both naturally and as a pollutant (Harrison and Laxen, 1981). The constituents were ground to a fine powder in a laboratory mill and presented to the snails as a uniform covering on one side of a glass slide smeared with sunflower margarine. The weight presented to each snail was measured as the increase in weight of the greased slide after powdering. Because the food was evenly distributed across the slide the amount consumed was directly proportional to the area of slide cleared (details of the measurement technique are given in Beeby and Richmond, 2007).

2.3. Metal analyses

After 24 h depuration the snail was sacrificed by freezing. The thawed soft tissues were separated from the shell and both dried for 12 h at 80 °C. Soft tissues were then digested in 10 ml of concentrated Analar-grade HNO_3, boiling gently for 1 h before filtering (Whatman 541 paper) and making up to 25 ml with distilled deionised water. Shells were digested in 10 ml of 50% HNO_3 for the same period, but not filtered. For each digestion run 4 glassware and 4 filter paper blanks were used to measure any background contamination. Average recovery rates for Pb from four replicates of a reference material (Lobster hepatopancreas TORT-2 – Environment Canada) were within the certified range. Certified values for Ca and Mg are not provided for TORT-2 but the uniformity of their results between digestion runs was taken to indicate consistent recoveries rates.

Four prepared slides for each diet were digested in 10 ml of concentrated HNO_3, prior to making up to 25 ml. Table 3 gives the overall mean concentration of metals for the two batches used in the first trial and for the diets of the juvenile trial.

Soil was collected from the top 4 cm of each location and oven-dried at 80 °C. Four replicate 0.5 g samples of sieved and milled soil were each boiled in 10 ml of concentrated HNO_3 for 1 h. After cooling and filtering these were made up to 100 ml. All metal determinations were made using a Thermo Tracescan ICP-AES against standards in equivalent nitric acid concentrations.

2.4. Statistical analysis

Data were analysed principally by analysis of variance and regression analysis using Minitab 15.1 (Minitab Inc.). Data were checked for normality using the Anderson-Darling statistic and for homogeneity of variances with Bartlett's test, either as populations when these were analysed singly or collectively as a combined dataset. Assimilation data, based on percentages, were transformed using the angular (arcsine) transformation (Zar, 1996) before analysis.

3. Results

3.1. Snail growth and feeding behaviour

Only one of the 108 sub-adult snails failed to produce a shell extension, whilst 10 had developed a lip by day 64. Irrespective of diet, most of the increase in snail total dry weight was achieved by day 16, when growth rates were 3–4 times those of day 64. Because shell ratios fell below 1.0 in all snails (Table 4), growth was principally in the soft tissues, unmatched by a proportionate increase in shell weight. Presumably subsequent shell mineralization would have restored these ratios, but this must be a longer term process, limited by the rate at which Ca and Mg can be precipitated at the mantle wall. It was not complete in any snail by the end of this experiment.

Feeding rates were relatively uniform between populations on all diets, and there was no consistent preference or aversion to the Mg-rich diets (Fig. 1). The feeding trial with juveniles from the RF brood demonstrated no affect of dietary Pb on feeding rates, with concentrations up to 650 μg g^{-1}. Daily food consumption declined in the sub-adults of all three populations after day 16 and thereafter differences between populations, times and diets were small.