Bull. Environ. Contam. Toxicol. (2000) 64:434-442

DOI: 10.1007/s001280000019

Bulletin of Environmental Contamination and Toxicology

Effects of Cadmium on the Reproductive System of the Land Snail *Helix aspersa*

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Received: 8 July 1999/Accepted: 6 January 2000

Snails are good bioindicators of environmental metal pollution because of their ability to accumulate certain metals such as Cd, Cu, Pb and Zn in their tissues (Coughtrey & Martin 1976, 1977 ; Dallinger & Wieser 1984). Even if environmental factors (season, type of soil, etc.) and biological parameters of snails (age, species, etc.) influence metal accumulation in tissues, the analysis of metal concentrations in several common snail species has given quantitative information on metal contamination in the vicinity of roads (Williamson 1980; Beeby & Eaves 1983), on industrial and mining sites (Hopkin 1989; Rabitsch 1998) and in towns (Dallinger & Berger 1992). On the basis of the snails' metal burden, Berger & Dallinger (1993) distinguished three pollution levels: no pollution (class 1: reference sites), moderate (class 2: traffic and other human activities in urban areas) and high pollution (class 3: mining and heavy industry).

Cytological and biological studies have shown that snails are able to sequester metals in their digestive gland, either stored in granules (Hopkin 1989) or bound to metallothioneines (Dallinger 1993).These mechanisms of retaining and detoxifying toxic elements within their bodies are probably at the origin of the tolerance of these animals with respect to metal pollution of their environment and to experimental contamination of their food (Russel et al. 1981; Laskowski & Hopkin 1996; Gomot 1997). However, in highly contaminated areas, Jones (1991) reported that *Helix aspersa* is absent around the mines of Avonmouth (U.K.). Mechanisms other than acute toxicity have been proposed to explain the absence of snails from contaminated sites (Laskowski & Hopkin 1996). Factors that may be involved are avoidance behavior and reduction of food intake, and also the inhibition of growth and/or fecundity. The importance accorded to these factors varies from author to author and, as mentioned by Laskowski & Hopkin (1996) there is no consensus of opinion regarding metal toxicity in snails. Russel et al. (198 1) did not note any mortality over four weeks during experiments with *Helix aspersa* involving food contamination by Cd concentrations of 10 to 1000 µg g^{-1}. The most obvious effect of the Cd treatment consisted in regression of feeding rates and growth rates; reproductive activity (mating) declined from 25 µg g^{-1} but was still observed in a few cases at 1000 µg g^{-1}. During longer experiments (120 days) Laskowski & Hopkin (1996) studied the effects on *Helix aspersa* reproduction of food contamination by a single metal (Cd, Cu, Pb or Zn) and by

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mixtures of the four metals at various concentrations. Significant negative exponential regressions of food consumption and fecundity with respect to concentration were found for all treatments and EC20 and EC50 (fecundity) values for Cd estimated at 120 and 183 µg g^{-1} respectively. According to calculated scenarios of population dynamics under the stress of a mixture of the four metals, the estivation of snails may be the main cause of delayed reproduction and population decline at high metal concentrations in the food.

In the present work, we investigate how Cd, a toxic non-essential metal, acts on reproduction (egg-laying) in *Helix aspersa* adults and on the development of the genital apparatus organs. The Cd concentrations we used inhibited growth but not food intake (Gomot 1997). They were of the order of those encountered in surface soils in mining areas (vicinity of Pb and Zn mines: 0.6-468 µg g^{-1}), the metal-processing industry (3.2 - 1781 µg g^{-1}), sewage sludge (up to 1500 µg g^{-1}) and with some plants that show a high affinity for Cd, absorbing it from the substrate (Kabata- Pendias & Pendias 1992).

MATERIALS AND METHODS

Helix aspersa were reared in our laboratory from stock originally collected in the area of Cavaillon (France). The breeding conditions were: 20°C with a long-day photoperiod (18h light, 6h dark) and the food was special meal for snails Helixal® with two formulae: one for very young snails "first age" (nursery: 1 to 4 weeks) and another, "second age", for growth and reproduction. The meal was supplied by Ets Lépine, Clairvaux-les-Lacs, 39 France. At the age of three months (end of growth, start of sexual maturation), snails were placed in hibernation at 6 ± 1°C for seven months: this corresponds to the optimum duration of rest and enables the snails to start reproducing 6 to 7 weeks after being brought back to activity (Gomot & Deray 1987). To study the action of cadmium, snails were placed in groups of four individuals in transparent polystyrene containers (2 IFFA-CREDO mouse boxes ref. 08.0001, one of which was turned up-side-down and used as a lid) with a volume of 3200 cm^3. Relative humidity was maintained with a layer of wet absorbent paper placed on the floor of the containers. Fresh second-age feed in the form of meal was put in plastic Petri-dishes three times a week; at the same time, the containers were washed and the damp paper replaced. Four weeks after snails emerged from hibernation, period required for the maturation of the genital tract, we placed a glass pot full of peat in each plastic cage in which the snails could lay eggs.

Cadmium (as anhydrous CdCl2 powder, Aldrich was added to dry "Helixal" feed at nominal concentrations of Cd of 200 µg g^{-1} and 400 µg g^{-1} dry feed for 4 weeks. Actual concentrations of Cd in contaminated feed, measured by flame atomic absorption spectrophotometry, were respectively 182 ± 133 and 359 ± 207 µg g^{-1} dry weight. Three replicate containers of snails were used for each treatment.

During the experiment, the number of eggs produced was counted as were the

number of young that hatched from them. Each week, the weight of animals was recorded as was the mortality so as to determine the LD50 time for each concentration applied. When the LD50 was reached, the remaining snails of the treatment were killed after anaesthesia with succinyl choline (200 µl of a 1% solution) and the genital apparatus removed to examine its state of development and to weigh its main organs.

Table 1. Mean weight (± SD) of snails in control and Cd-exposed groups after exposure to 7 or 10 weeks.

Group	Mean total fresh weight (g)		
	T0	T 7 weeks	T 10 weeks
Controls	10.67 ± 2.24 a (12)	12.66 ± 2.24 a (10)	12.40 ± 2.42 a (6)
200 µg Cd g^{-1} food	9.45 ± 1.58 a (12)	9.72 ± 1.23 a (9)	8.68 ± 1.09 b (6)
400 µg Cd g^{-1} food	10.24 ± 0.85 a (12)	8.20 ± 0.56 b (5)	All dead

For each group, means with different letters are significantly different ($P < 0.05$); ( ) = number of snail.

The non-parametric Kruskal-Wallis l-way ANOVA test ($p<0.05$) followed by a multiple comparison test ($p<0.05$) (Sokal & Rohlf 1997) was used to evaluate differences between the mean total fresh weight of the 3 batches at the start of the experiment and after 7 weeks of experiment; after 10 weeks, only 6 snails survived in 2 batches and they were compared with the Mann-Whitney U-test which is very powerful for small sample sizes. This test was also used to compare the mean weights of the different organs of the genital tract ($p<0.05$).

RESULTS AND DISCUSSION

Mortality of snails fed 400 µg Cd g-l meal resulted in half the animals dying (LD50) after seven weeks whereas snails that received 200 µg g^{-1} reached the LD 50 after 10 weeks. Over the same period, none of the control snails died. This experiment demonstrate the importance of time in assessing Cd toxicity. Concentrations of 10 to 1000 µg g^{-1} (Russel et al. 1981) or 50 to 800 µg g^{-1} (Gomot 1997) cadmium inhibits the growth of *Helix aspersa* at 20°C without causing mortality for a month, but it can induce high mortality in young adults when exposed for longer exposure at concentrations of 200 and 400 µg g^{-1}. The weakest mortality observed by Laskowski & Hopkin (1996) in adults of the same species reared at 15°C under 16 hours light - 8 hours dark for 120 days is probably due to the lower concentration of cadmium (under the form of nitrate 125 µg g^{-1}) and lower temperature in their experiment reducing the metabolic rate of the animals.

Table 2. Mean weights (± SD) of the genital tract organs of control snails and snails fed a Cd contaminated diet.

Group	Duration	Mean weight genital tract organs (fresh tissues in g)			
		Ovotestis	Albumen gland	- Ovispermiduct - Penis - Dart sac - Multifid glands	Total genital tract
Controls	7 weeks	0.061 ± 0.016 a	0.810 ± 0.22 a	0.917 ± 0.13 a	1.787 ± 0.29 a
	10 weeks	0.053 ± 0.007 a, b	0.892 ± 0.406 a	0.889 ± 0.11 a	1.834 ± 0.51 a
200 µg Cd.g^{-1} food	10 weeks	0.041 ± 0.024 a, b (- 23 %)	0.169 ± 0.26 b (-81%)	0.431± 0.19 b (-51%)	0.641 ± 0.45 b (-65%)
400 µg Cd.g^{-1} food	7 weeks	0.030 ± 0.014 b (-50%)	0.031 ± 0.016 c (-96%)	0.257 ± 0.12 c (-72%)	0.319 ± 0.15 c (-82%)

For each organ, means that share different letters are significantly different ($P < 0.05$) whereas means with the same letter are not significantly different from each others at $P < 0.05$. ( ) = percentage weight reduction with respect to the controls at the same time.

Comparing the mean weights of the snails (Table 1) during the experiment indicated a dose-dependent effect of Cd. Control snails increased their weight substantially during the first 7 weeks, the snails consuming food contaminated with 200 µg g^{-1} Cd increased in weight slightly over the same period and then their mean weight decreased. Those receiving 400 µg g^{-1} lost weight significantly during the first 7 weeks during which time half died.

Reproduction proved to be very sensitive to contamination of food by Cd. The control snails started to lay normally from the 7th week : during the 7th week, we collected three layings corresponding to 332 eggs giving rise to 272 young from 12 snails. From the 7th to the 10th weeks, the six control snails which had not been killed for genital tract measurements gave 5 layings, *i.e.* 711 eggs giving rise to 580 young. In the groups treated with 200 or 400 µg Cd g^{-1} food, no eggs were produced, whereas Russel et al.(1981), who used the total number of individuals mating or with spermatophores in place as criterion of reproductive activity, observed a decrease of this activity from 25 ppm but noted that mating did occur a few times at 300 and 1000 µg g^{-1}. The EC50 estimated by Laskowski & Hopkin (1996) from the number of eggs collected and of young hatched was 183 µg g^{-1} over the duration of their experiment (120 days) at 15°C. This value, extrapolated from the number of hatchlings per snail exposed to dietary Cd contamination at 1, 5, 25 and 125 µg g^{-1} is comparable to our observations (EC100 < 200 µg g^{-1}) but seems to be a little overestimated. Further experiments at intermediate concentrations (100, 150, 200 µg g^{-1}) and at different temperatures (15 °C and

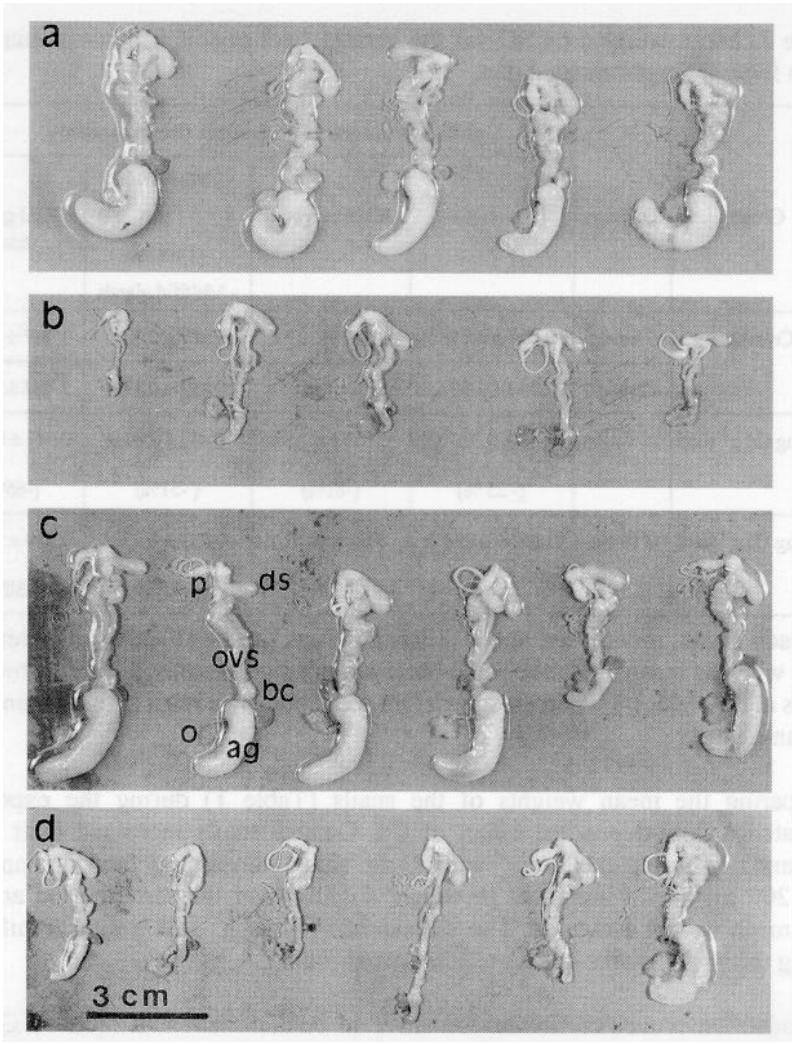

Figure 1. Macrophotographs of genital tracts of adult snails. Uncontaminated: **a:** 7 weeks of activity, **c:** 10 weeks of activity. Orally contaminated: **b,** 7 weeks of contamination at 400 µg Cd µg g-1 dry food, **d:** 10 weeks at 200 µg Cd g-l dry food. ag: albumen gland, bc: bursa copulatrix, ds: dart sac, o: ovotestis, ovs: ovispermiduct, p: penis.

20°C) over a whole reproductive cycle of 12 to 16 weeks should enable the EC50 and LD50 for Cd in this species to be established more accurately. Moreover, in the event of contamination, it should enable clearer evaluation of the relative influence of temperature and photoperiod on this species (Gomot et al. 1989).

Morphological observation and the weights of the genital tract organs of the snails surviving at 7 weeks and at 10 weeks after receiving 400 µg Cd g^{-1} food and 200 µg Cd g^{-1} food respectively, showed inhibitory action of Cd on development of the genital tract. In control snails, seven weeks after recovery of activity, the

genital apparatus showed normal composition of animals beginning to lay (Fig. la). There was normal development of the albumen gland and of the ovispermiduct, which showed increased volumes at 10 weeks (Fig 1c). Part of the secretions of these organs contributed towards making up the perivitellin fluid and the envelope of eggs laid between the 7th and the 10th week. Among the snails consuming food contaminated with 200 µg Cd g^{-1}, one had a more or less normal genital apparatus, whereas in all the others, it was rudimentary (Fig 1d). It can be noted that it was especially the albumen gland (which remains small) and the ovispermiduct (long and narrow) did not develop. The sheath of the dart and the penis of exposed snails were of a size little different from that of the controls (Fig la, 1d). At 400 µg g^{-1}, Cd did not greatly modify the appearance of the dart sheath or penis, but did cause atrophy of the albumen gland and ovispermiduct (Fig. lb).

Comparison of the weight of the genital tract or its organs between the different groups of snails supported anatomical observations (Table 2). The various organs of the genital tract did not have the same sensitivity towards cadmium. The albumen gland was affected most (Table 2), both for contamination at 200 µg Cd g^{-1} (-81%) and 400 µg Cd g^{-1} food (-96%) and the ovispermiduct lost the festooned appearance that the development of the glands of the oviduct usually give it after Cd exposure.

Concerning the causes of the regression of the two glandular structures (albumen gland and ovispermiduct), which synthesise the perioocytic components of the eggs, they probably result from the indirect action of Cd because this metal does not accumulate in the albumen gland unlike in the digestive gland in snail in contaminated areas (Coughtrey & Martin 1976). Cd probably exerts its action by disturbing the nervous and neuroendocrine factors which regulate the secretory functions of the genital apparatus since in the basommatophore pulmonate *Lymnaea stagnalis* Cd modulates the effects of neurotransmitters (S-Rózsa et al. 1988) and modifies the structure of the Ca channels in the neurons of the nerve collar (Szücs et al. 1994). Such modifications of the cerebral ganglia and dorsal bodies that control the function of the albumen gland (Goudsmid 1975; Gomot & Courtot 1979; Wijdenes et al. 1983) may be responsible for this inhibition. But it is also possible that the endocrine stimulation of the gonad (Berset de Vaufleury et al.1986) is affected. The same type of regulation also exists for the oviduct (Bride & Gomot 1988). The anatomic alterations observed indicate the absence of secretion of polysaccharide by these glands, galactogen being the main component of the albumen gland during the phase of reproduction (Nieland & Goudsmid 1969). In addition to interfering with neuroendocrine control of female organ secretion in the genital tract, Cd can affect the transfer of metabolites of dietary origin (required for the synthesis of the polysaccharides) towards the haemolymph and the main organs of the snails. The dart sheath and the penis (male organs), kept a normal appearance, suggesting that Cd acts essentially on the control of the female organs of the genital tract. The fact that the male organs are affected little probably explains the occurrence of mating in conditions of very strong contamination (Russel et al. 1981). The ovotestis only diminished in weight after 7 weeks of 400 µg Cd g^{-1} food (Table 2). However, Russel et al.

(1981) observed after 30 days, the disruption of the sperm bundles and fragmentation of the flagella at 300 µg Cd g^{-1} food whereas the ova did not appear to be affected at any level of Cd.

These experiments show how the study of various aspects of reproduction (mating, laying, weight of the reproductive organs) can bring complementary elements of information on the modes of action of environment contaminants. So, although very strong contamination for a month (up to 1000 µg Cd g^{-1} food) did not completely suppress behaviour leading up to mating (implantation of the dart into the partner) (Russel et al. 1981), we observed that prolonged contamination (7 to 10 weeks) at lower levels (200 and 400 µg Cd g^{-1} food) completely inhibited the development of the albumen gland preventing the formation of eggs and thus laying. In addition to the easily measurable criterion of the number of eggs laid and the hatching success, it is possible to use the development of the genital tract and mainly of the albumen gland, as the marker of endocrine disturbances. This macroscopic observation of the albumen gland could be backed up by the cytological examination of the granules in the galactogen-secreting cells or the microvilli of the centrolobular cells of this gland. Assaying the galactogen in the albumen glands could also be used to detect endocrine disturbances. Knowledge of the factors involved in the determinism of reproduction in snails and other stylommatophoran gastropods (Griffond & Gomot 1989; Gomot & Gomot 1994; Gomot & Griffond 1993) make it possible to identify the organs or cell groups affected by endocrine disrupters. They also provide information on the disorders induced in the neuroendocrine targets (neurosecretory cells of the nervous ganglia of the perioesophageal collar, dorsal bodies, optic tentacles, gonad). Snails represent a good model to study the effects of "hormone disrupting substances" in reproductive processes of terrestrial invertebrates. As bioindicators of Cd toxicity, snails are most sensitive (total inhibition of reproduction at concentrations of 200 and 400 µg g^{-1}) than earthworms which can continue reproducing in soils containing 600 and even 1200 µg g^{-1} Cd (Reinecke et al. 1999).

Acknowledgments. This work was financially supported by the ADEME (Agence de l'Environnement et de la Matrîse de l'Energie), contract n°9693019 and is dedicated to the memory of Isabelle. We thanks also the reviewers for comments that improved the manuscript.

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