Bull Environ Contam Toxicol (2009) 83:19–22
DOI 10.1007/s00128-009-9721-9

Quantitative Changes in Digestive Gland Cells and Oocytes of *Helix aspersa*, as Biomarkers of Copper Oxychloride Exposure Under Field Conditions

R. G. Snyman · A. J. Reinecke · S. A. Reinecke

Received: 3 April 2008 / Accepted: 7 April 2009 / Published online: 12 May 2009

Abstract This study investigated the effects of accumulated copper, on digestive epithelium height and percentage area, and on oocyte numbers of the snail *Helix aspersa*, in a vineyard where copper oxychloride is sprayed. The ultimate aim was to determine the usefulness of these cellular responses as biomarkers. Results showed that snails collected 2 months after fungicide application, had a significantly lower mean digestive epithelium height and percentage area, as well as significantly fewer oocytes per 1 mm^2 of ovotestis, compared to snails collected only 1 week after fungicide application and those from a control vineyard. It was concluded that these cellular responses are clear, measurable responses to copper oxychloride exposure and copper accumulation. However, they do not provide an early warning of copper exposure, which impacts on their usefulness as biomarkers.

Keywords Digestive cells · Oocytes · Copper · Snail

Broad-spectrum fungicides, such as copper oxychloride ($Cu_2Cl(OH)_3$), are extensively used in South African agriculture. Copper oxychloride is sprayed at a rate of 1.25–7.5 kg ha^{-1} with up to nine applications per season and affords crops protection against a wide range of diseases, such as downy mildew, anthracnose and leaf spot (Krause et al. 1996).

This fungicide not only affects the target fungus but nontarget organisms as well. A number of recent South African studies (Helling et al. 2000; Snyman et al. 2000, 2002, 2004, 2005; Maboeta et al. 2003; Reinecke et al. 2002) have illustrated the negative effects of copper oxychloride exposure on cell structure and functioning, in soil organisms such as snails and earthworms.

The digestive gland of mollusks has been shown to be a major organ involved in accumulation and storage of metals (e.g. Blasco and Puppo 1999; Snyman et al. 2005). Changes in digestive cell structure and function, as a result of metal accumulation, have been studied extensively (e.g. Lowe and Clarke 1989; Marigomez et al. 1998; Snyman et al. 2005; Guerlet et al. 2006). The reproductive organs of molluscs are also known to be targets for metal toxicity, affecting the structure and numbers of gametes particularly (e.g. Russell et al. 1981; Gould et al. 1988; Snyman et al. 2004). In two previous studies (Snyman et al. 2004, 2005), we found that increased copper loads in the digestive gland and ovotestis of *Helix aspersa* resulted in decreased oocyte numbers and decreased digestive epithelium height and area, respectively. We suggested that these responses might possibly be used as biomarkers of copper oxychloride exposure and concluded that a field validation study is necessary, to investigate this possibility.

The present study investigated the effects of accumulated copper, on digestive epithelium and oocytes of the snail *Helix aspersa*, under field conditions, in a vineyard where copper oxychloride is sprayed. The ultimate aim was to determine the usefulness of these cellular responses as biomarkers of copper oxychloride exposure and to validate our laboratory findings, reported on in Snyman et al. (2004, 2005).

R. G. Snyman (✉)
Department of Biodiversity and Conservation, Cape Peninsula University of Technology, PO Box 652, Cape Town 8000, South Africa
e-mail: snymanr@cput.ac.za

A. J. Reinecke · S. A. Reinecke
Department of Botany and Zoology, University of Stellenbosch, Private Bag X1, Matieland 7602, South Africa

Materials and Methods

Snails of similar weight (6.4 ± 0.4 g) were collected from two vineyards in the Western Cape, South Africa. One of these sites represented a vineyard where copper oxychloride had been applied, and one site served as control, i.e. a vineyard with no history of pesticide use. Animals from the treated site were collected 1 week after copper oxychloride application and 2 months thereafter. No fungicide was applied during the period between the sampling dates. A total of twenty snails were collected from each vineyard and on each sampling date.

The laboratory procedures followed were similar to those described by us in Snyman et al. (2004, 2005). Snails were starved for 2 days, after which ten snails from each vineyard and sampling date were killed by decapitation. The ovotestis and digestive gland of each animal were rapidly excised and fixated in Bouin's fluid (Preece 1972) for 20–23 h. The organs were then placed in stainless steel embedding cassettes and rinsed in 50% alcohol for 1 h, after which they were taken through various steps of dehydration in alcohol and clearing in xylene (room temperature), as well as a number of impregnation steps with Paraplast wax, at 58°C. Finally, the organs were embedded in fresh Paraplast wax and cooled overnight at 5°C. Sections of each organ were made with a Leica Rotary Microtome, at a thickness of 6–8 µm, mounted on microscope slides, and stained with Erlich Hematoxylin and alcohol dissolvable Eosin according to the recipes and method described by Presnell et al. (1997). At least three slides of each organ were studied under a Nikon compound microscope at 100× and 400× magnification. Measurements were made randomly across the slides, using the Leica QWin computer software package. The following measurements were taken within each digestive gland: total areas of the individual digestive tubules, total areas of the tubule lumina, and heights of the tubule epithelium cells. The former two measurements were used to determine the total areas of tubule epithelia. This was expressed as a percentage of the total area of the tubule. Within each ovotestis slide, at least three randomly selected 1 mm^2 blocks were studied and the total number of mature oocytes counted therein.

The remaining animals from each sampling site and sampling occasion were weighed and killed by freezing, after which they were thawed and dissected, in order to remove the ovotestis and digestive gland. The excised organs of each snail, as well as the rest of the snail body, were weighed and dried for 24 h at 60°C. Dried samples were then prepared and analysed for copper content by atomic absorption spectrophotometry.

Kruskal-Wallis One-Way ANOVA on Ranks and multiple pairwise comparisons (Dunn's test) were used to test for statistical differences between the sampling sites and sampling occasions. The following parameters were compared: digestive epithelium height (µm), digestive epithelium area (%), oocyte numbers per 1 mm^2 ovotestis, and copper concentrations in the digestive gland, ovotestis, and rest of the body. All calculations were done with the Jandel Scientific Sigmastat 3.1 computer program.

Results and Discussion

The mean (±SD) copper concentrations (µg g^{-1} dry mass) in the digestive glands, ovotestes and rest of the bodies of snails from the two vineyards and different sampling dates, are shown in Fig. 1. Copper was only detected in ovotestes of snails collected 2 months after fungicide application, therefore statistical comparisons for this organ were not done. The results showed that copper concentrations in the digestive glands of animals from the treated vineyard were significantly higher ($p < 0.05$) than in the rest of their bodies. No such differences in copper distribution were found for snails from the control vineyard ($p > 0.05$). Snails collected 2 months after fungicide application had the highest ($p < 0.05$) concentrations of copper in their digestive glands and rest of their bodies (453.4 ± 123.68 and 254.33 ± 112.76 µg g^{-1}, respectively), and snails from the control vineyard the lowest (44.75 ± 19.43 and 47.23 ± 14.58 µg g^{-1}, respectively).

Table 1 shows the mean (±SD) height (µm) of the digestive gland epithelium, the mean digestive gland epithelium area, expressed as a percentage of the total area of each digestive tubule, as well as the mean number of mature oocytes per 1 mm^2 of ovotestis, of snails collected from the two vineyards and different sampling dates. Results showed that snails collected 2 months after fungicide application, had a significantly ($p < 0.001$) lower

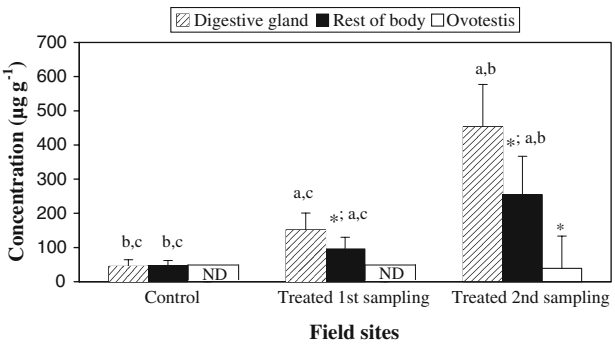

Fig. 1 Mean (±SD) copper concentrations (µg g^{-1} dry mass) in the digestive gland, ovotestis and rest of the body, of *Helix aspersa*. Control = untreated vineyard. Treated 1st sampling and Treated 2nd sampling = 1 week and 2 months after copper oxychloride application, respectively. ND = not detectable. *Asterisks* indicate significant difference from digestive gland. *Letters* indicate significant difference between sites and sampling dates. n = 10 snails

Table 1 Mean (±SD) digestive gland epithelium height (μm), epithelium area (%), and number of oocytes (per 1 mm^2 ovotestis), measured for *Helix aspersa*

Histological parameter	Control	Treated 1st sampling	Treated 2nd sampling
Digestive epithelium height (μm)	33.99 ± 7.15 (n = 420)	33.67 ± 8.89 (n = 436)	28.9 ± 7.29* (n = 456)
Digestive epithelium area (%)	80.24 ± 9.19 (n = 122)	79.07 ± 9.24 (n = 126)	70.58 ± 10.5* (n = 105)
Number of oocytes (per 1 mm^2 ovotestis)	5.33 ± 3.58 (n = 91)	5.02 ± 3.3 (n = 97)	3.87 ± 2.8* (n = 68)

Control, untreated vineyard; Treated 1st sampling and Treated 2nd sampling, 1 week and 2 months after copper oxychloride application, respectively; n, number of measurements

Asterisks indicate significant difference from control

mean digestive gland epithelium height, a significantly ($p < 0.001$) lower percentage epithelium area, and significantly fewer ($p < 0.001$) oocytes per 1 mm^2 of ovotestis, compared to snails collected 1 week after fungicide application and snails from the control vineyard.

The results of the present study confirmed our laboratory findings (Snyman et al. 2005), that the digestive gland of *Helix aspersa* is the most important organ in copper accumulation (Fig. 1). Measurable changes in epithelium height and area in this organ were demonstrated (Table 1). Previously we related the cellular responses observed in our laboratory study, to the copper accumulation in the digestive gland. We concluded that the responses are probably due to a combination of copper-induced factors such as the release of calcium cells, losses of apical cytoplasm and autophagy of digestive cells, the latter due to reduced lysosomal membrane integrity. These conclusions are supported by the work of, for example, Viarengo et al. (1981), Vega et al. (1989) and Marigomez et al. (1998).

In the present study copper was not accumulated in the ovotestis (Fig. 1) but did rise above detectable levels in snails collected 2 months after fungicide application. A significant decrease in oocyte numbers per 1 mm^2 ovotestis was observed in these snails. In our laboratory study (Snyman et al. 2004), we also found significantly fewer oocytes in ovotestes of snails exposed to copper oxychloride. We concluded that the accumulated copper probably affects lysosomal membrane integrity of the oocytes themselves or of the yolk granules, thereby causing autophagy or influencing maturation. The effects of copper on molluscan lysosomal membranes, in general, are well documented (e.g. Svendsen and Weeks 1995; Ringwood et al. 1998; Snyman et al. 2000; Bigas et al. 2006).

Since the present study was a field study, it is of course possible that other factors, apart from copper accumulation, may have induced the observed cellular responses. For example, various stressors, especially related to diet, as well as the digestive stage of the animal's digestive gland tubules, are known to affect digestive gland epithelial thickness (Langton 1975; Ireland and Marigomez 1992). However, it is accepted that copper was a main contributing factor in the present study, particularly when considering the strong relationship between copper and cellular responses of *Helix aspersa* illustrated with our laboratory studies (Snyman et al. 2004, 2005).

The results of the present study revealed the importance of exposure time, as factor determining the degree of cellular change. We did not investigate exposure time in our laboratory experiments (Snyman et al. 2004, 2005). In the present study the decreases in epithelial height and percentage area, and in oocyte numbers, were only observed in snails collected 2 months after fungicide application, therefore exposed for 2 months to copper in the vineyards. Snails collected 1 week after application did not show significant cellular responses, even though, in the case of the digestive gland, copper concentrations had already risen markedly in this organ.

Considering the results of our field study, in conjunction with our previous laboratory findings, we conclude that changes in digestive epithelium height and area, and in oocyte numbers in the ovotestis of *Helix aspersa,* can indeed be considered clearly measurable responses to copper oxychloride exposure and copper accumulation. Used in conjunction with other cellular and physiological parameters and toxicological endpoints they could improve the reliability and accuracy of interpretations regarding cause and effect. However, it is evident from the results of this field study that these responses do not provide an early warning of contaminant-induced stress, since they can only be measured after several weeks of exposure. Also, since the length of the period after copper oxychloride application is clearly an important factor affecting these parameters, knowledge of specific spray programs used in the vineyards is crucial, in order to improve the usefulness of these measurements as possible biomarkers.

Acknowledgments We thank the National Research Foundation for the financial support, as well as A. Alblas for her assistance in the laboratory.

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