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Changes in Oocyte Numbers in the Ovotestis of *Helix aspersa*, After Experimental Exposure to the Fungicide Copper Oxychloride

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In agriculture throughout the world, great emphasis is placed on the protection of crops through the control of plant diseases. The broad-spectrum fungicides, such as copper oxychloride ($Cu_2Cl(OH)_3$), are the most popular chemicals used for this purpose in South African agriculture, on various crops, orchards and vineyards. Copper oxychloride is sprayed intensively 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, including downy mildew, anthracnose and leaf spot (Krause et al. 1996).

Despite the extensive use of this fungicide, very little is known about its effects on non-target organisms. However, a number of recent South African studies (e.g. Helling et al. 2000; Snyman et al. 2000; 2002; Maboeta et al. 2002; 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. These authors have identified a number of cellular responses that may be used as biomarkers of exposure to this fungicide.

Mollusks are widely used as models in studies on biomarkers of exposure to heavy metals and pesticides (e.g. Russell et al. 1981; Vega et al. 1989; Berger et al. 1995; Marigomez et al. 1990; 1998; Svendsen and Weeks 1995; Snyman et al. 2000; 2002). It is well known that exposure to xenobiotics negatively affects the reproductive organs of mollusks (e.g. Russell et al. 1981; Myint and Tyler 1982; Lowe and Pipe 1986; Gould et al. 1988; Cajaraville et al. 1991; Bauer et al. 1995; Gomot-de Vaufleury and Kerhoas 2000). A number of these authors (Myint and Tyler 1982; Russell et al. 1981; Lowe and Pipe 1986; Gould et al. 1988) illustrated the effects of accumulated heavy metals (also Cu) and hydrocarbons on, specifically, the size, structure and numbers of gametes in the molluscan gonad. Such cellular responses may have potential as biomarkers of exposure to xenobiotics.

The garden snail *Helix aspersa* was selected for the present study, since it occurs commonly in South African agricultural fields and can therefore be used as sentinel of prevailing environmental conditions, resulting from the use of agrochemicals. The aim of the study was, firstly, to investigate the accumulation of copper in the ovotestis of *Helix aspersa*, after exposure to copper oxychloride

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and, secondly, to investigate the possible relationship between accumulated copper in the ovotestis and changes in oocyte numbers, in order to ultimately determine the usefulness of such cellular responses as biomarkers of copper oxychloride exposure.

MATERIALS AND METHODS

Helix aspersa were reared in the laboratory in aerated plastic containers, on a soil substrate. Animals were moistened every day and fresh water was also provided. A total of 45 adult individuals that hatched from eggs produced by this stock culture were used for the experiment. The test animals, all of similar age and body size (7.2 ± 0.5 g), were starved for two days before the experiment commenced, in order to clear the digestive tract. They were then divided into three treatment groups of 15 animals each. Each treatment group was subdivided into three replicates of five snails each. The test animals were kept in a temperature-controlled chamber, at 16 – 18 °C, 70 ± 2 % humidity and a constant photoperiod of 14 hours light:10 hours dark, for a period of six weeks. Two of the test groups were exposed to sublethal concentrations of copper oxychloride, namely 80 $\mu g\ g^{-1}$ and 240 $\mu g\ g^{-1}$ respectively. These concentrations were determined by means of an LC_{50} test. The third group served as control and received uncontaminated food. Food was offered in Petri dishes, in the form of a mixture of Agar, distilled water and a commercial fruit and vegetable juice mixture. This was prepared according to the method described by Berger et al. (1993). Fresh food was given each week and the actual concentrations of copper in the food were determined at the end of the experiment. The food offered to the 80 and 240 $\mu g\ g^{-1}$ copper oxychloride exposure groups contained 40.11 ± 4.03 and 129.0 ± 15.64 $\mu g\ g^{-1}$ copper respectively, which corresponded to the prescribed percentage of copper in this fungicide. The copper concentrations in the food of the control group were below detectable limits.

At the end of the six-week experimental period, six snails from each treatment group were then killed by decapitation. The ovotestis of each animal was rapidly excised and fixated in Bouin's fluid (Preece 1972) for 20-23 hours. The organs were then placed in stainless steel embedding cassettes and rinsed in 50% alcohol for 1 hour, 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 ovotestis were studied under a Nikon compound microscope at 100x and 400x magnification. Within each ovotestis, at least three randomly selected $1mm^2$ blocks were studied and the total number of mature oocytes counted therein, using the Leica QWin computer software package.

The remaining animals in each treatment group were weighed and killed by freezing, after which they were thawed and dissected, in order to remove the ovotestis. The excised ovotestis of each snail was weighed and dried for 24 hours at 60 °C. Dried ovotestes were then prepared and analysed for copper content by atomic absorption spectrophotometry, according to the method described by Snyman et al. (2000).

Kruskal-Wallis One-Way ANOVA on Ranks and multiple pairwise comparisons (Dunn's test) were used to test for statistical differences in oocyte numbers per $1mm^2$ ovotestis, as well as copper concentrations in the ovotestis, among the three *H. aspersa* treatment groups. All calculations were done with the Jandel Scientific Sigmastat 2.0 computer program.

RESULTS AND DISCUSSION

The mean (± SD) copper concentrations ($\mu g\ g^{-1}$ dry mass) in the ovotestes of snails from the three treatment groups, after the six-week experimental period, are shown in Figure 1. The results showed that, at the end of the experiment, mean copper concentrations in the ovotestes of animals from the 80 and 240 $\mu g\ g^{-1}$ groups were 263.99 ± 260.47 and 328.09 ± 362.27 $\mu g\ g^{-1}$ dry mass respectively, whereas for the control group, copper concentrations were below detectable limits. There was no significant difference ($p>0.05$) in ovotestis copper concentrations between the two groups exposed to the fungicide.

Figure 1 also shows the mean number of mature oocytes per $1mm^2$ of ovotestis, measured for the three treatment groups of *Helix aspersa* at the end of the six-week experimental period. Significant differences ($p<0.001$) in oocyte numbers were found between all three treatment groups. The control exhibited the highest mean number of oocytes (5.09 ± 3.44; n = 125 x $1mm^2$ blocks) per $1mm^2$ ovotestis and the 240 $\mu g\ g^{-1}$ exposure group the lowest (2.33 ± 1.64; n = 60 x $1mm^2$ blocks).

The results showed that copper was generally strongly accumulated in the ovotestes of snails exposed to copper oxychloride. The individual variation in copper accumulation within each group was to be expected, since Snyman et al. (in press) also found large variation in the accumulation capacity of the most important copper storage organ of *Helix aspersa*, namely the digestive gland. Individuals with a lower digestive gland capacity could therefore have higher circulating copper concentrations, resulting in higher concentrations in other organs, such as the ovotestis. The opposite would then be true for individuals with a higher digestive gland capacity.

The results of the present study also showed that, due to the accumulated copper in the ovotestes of snails from the exposure treatment groups, these organs contained significantly fewer mature oocytes than those of the control group. This response was clearly dose-related.

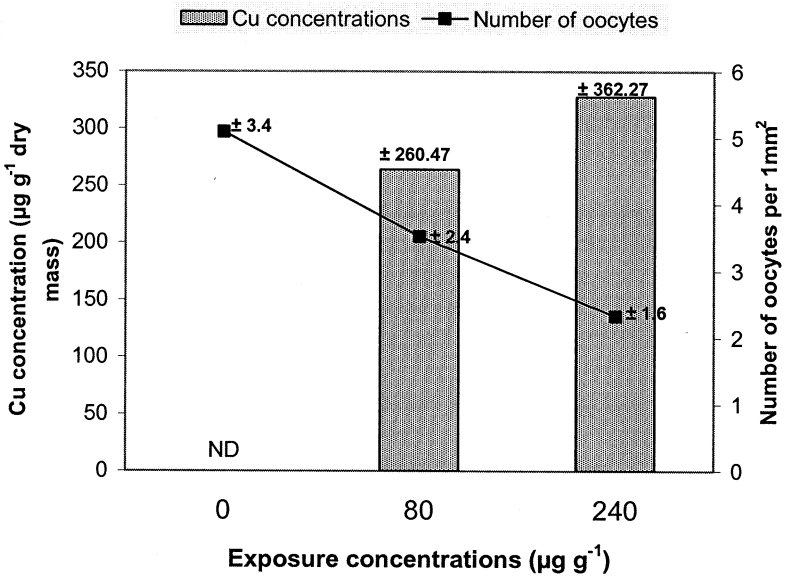

Figure 1. Mean (±SD) copper concentrations (µg g^{-1} dry mass) and mature oocyte numbers (per $1mm^2$) in the ovotestis, measured for three treatment groups of *Helix aspersa*, after six weeks of exposure to copper oxychloride. ND = not detectable.

It is not known whether the above-mentioned results were due to an inhibitory effect of copper on final oocyte maturation, or atresia of mature oocytes, or a combination of both. Certainly, the negative effects of copper on oocyte maturation are well documented for marine mollusks (e.g. Gould et al. 1988; Myint and Tyler 1982) but no information exists for terrestrial mollusks. The exact mechanisms by which copper inhibits oocyte maturation and causes oocyte damage are not clear but it seems that lysosomes may possibly play an important role. It is well known that copper affects the integrity of lysosomal membranes of molluscan haemocytes and digestive cells (e.g. Svendsen and Weeks 1995; Ringwood et al. 1998; Snyman et al. 2000; 2002), causing the lysosomal contents to leak into the cytosol and leading to cellular damage (Moore 1990). It is possible that the lysosomal compartment of oocytes could also be a target for the toxic action of copper. Alternatively, the lysosomal membranes of yolk granules, the latter of which increase dramatically in numbers during the final oocyte maturation in mollusks (De Jong-Brink and Geraerts 1982), could also possibly be affected by copper, thereby inhibiting the final maturation step. This theory has also been considered by Lowe and Pipe (1986), for the toxic action of hydrocarbons.

Irrespective of the exact mechanism of cellular damage, a reduction in oocytes, as observed in the present study, holds serious implications for the survival of a population in agricultural fields where copper oxychloride is sprayed. It may lead

to lowered fecundity and fitness and, ultimately, lowered abundance. This may therefore also render it a useful way of controlling unwanted snails.

It is concluded that changes in mature oocyte numbers in the ovotestes of *Helix aspersa*, can be considered a quantifiable response to experimental copper oxychloride exposure. This response may possibly serve as a general biomarker in studies to confirm exposure to this fungicide. It may however be less useful as a practical biomarker in the field due to the complexity of the analysis. Final conclusions about its usefulness in this regard can only be drawn once its reliability under field conditions has been investigated.

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