THE UNIVERSITY OF MANITOBA

ECOLOGY OF SLUGS IN MANITOBA, AND ACCUMULATION, STORAGE AND EXCRETION OF DDT IN THEIR BODIES

by

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ABSTRACT

The purpose of this study was to observe different aspects of slug ecology in Manitoba, and examine the accumulation, storage and excretion of DDT by slugs. Reference was made to the culture of slugs for use in long-term laboratory experimentation.

A field survey was done in August 1972 with samples of slugs being collected from 19 rural communities and urban areas in the southern part of the province. Three species of slugs are found in Manitoba at present. Agriolimax laeve occurred in nearly all areas sampled, whereas Lehmannia valentiana was found only in 2 greenhouses. Agriolimax reticulatus was found in all but 9 of the sampled areas. This species is responsible for much of economic and asthetic damage done by slugs in Manitoba.

Severe climatic conditions during the summer and winter prevent slugs such as Lehmannia valentiana from becoming established in the field. Severe winter conditions likewise limit Agriolimax reticulatus to winter survival in the egg stage.

Particular reference was made with respect to accumulation, storage and excretion of DDT. ^{14}C-pp-DDT was fed to slugs at 4, 16, 40 and 80 ppm. dry weight in the diet. A variety of food sources were tested as vehicles for adminis-

tering the DDT to the slugs and were exposed to the slugs for up to 104 days.

For both Agriolimax reticulatus and Lehmannia valentiana, rate of accumulation was proportional to time of exposure and level of intake. Agriolimax reticulatus showed a greater rate of accumulation of DDT from both leaf litter and oatmeal pellets than did Lehmannia valentiana. Both species concentrated the majority of the DDT in the hepatopancreas. The rate of excretion was fairly slow when the slugs were feeding on DDT-free food. Of the 20% retained after feeding on DDT for 20 days, 12% was still present in the slug tissue after 79 days. Mucous secretion and production of eggs resulted in little loss of DDT from the tissue.

The possibility of these ubiquitous invertebrates acting as indicators of pesticides in the soil environment was also considered. It is hoped that this study will act as an introduction to the study of terrestrial molluscs in Manitoba.

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INTRODUCTION

Terrestrial slugs are an important component of the ecosystem. In many parts of Europe slugs form a large part of the biomass of a given biotic community. Most species of slugs are herbivorous and detritis feeders, but a few are carnivorous. The amount of food consumed by slugs is considerable, thus in many areas the herbivorous slugs are important primary consumers. Although slugs are apparently utilized as food by predatory organisms, their importance in food chains is undetermined. Climatic conditions and pathogenic organisms are apparently the key factors determining the distribution and abundance of slugs in a given area.

DDT usage increased greatly from 1947 to 1960. Much of the DDT now in the environment was introduced by spraying for agricultural pests, forest pests and mosquito-fly control. Much of the DDT reached the top soil layers directly by spray application, or indirectly by being washed off of plants by precipitation or by being adsorbed to plant material which eventually dies and falls to the ground. The DDT has become bound to the humus and may remain there for several years.

Slugs may be exposed to pesticides by direct contact with the spray or indirectly through the food or from contact with a contaminated substrate. As the highest

densities of slugs tend to be found on the types of cultivated land where much of the DDT application has taken place, DDT and other pesticides are now a integral part of the slug's environment.

Because slugs utilize a wide variety of habitats, are ubiquitous and sedentary, it is quite conceivable that slugs could be used for monitoring the biological availability of pesticides in the lower trophic levels.

DDT was chosen as the study chemical since its accumulation in the higher trophic levels has been well documented and thereby stirred up considerable controversy. The use of DDT labelled with the ^{14}C isotope eliminated contamination errors due to polychlorinated biphenyls and facilitated the analytical measurement of DDT residues.

The objectives of this thesis were:

1) To initiate the study of slug ecology in Manitoba and determine what species are present;
2) To investigate the accumulation, storage and excretion of DDT by slugs.

Chapter 1

LITERATURE REVIEW

Ecology of Slugs

There has been little research published on the ecology and distribution of slugs in Canada. Thus this review will be primarily of research done in the United States and Europe.

Before the settlement of North America there were few native species of slugs present. However, in the last 200 years there has been a rapid introduction of slugs from Europe (Chichester and Getz, 1968). The early introduction of slugs from Europe was reported by Binney (1842). *Agriolimax reticulatus* is found in many of the cultivated areas of North America and *Lehmannia valentiana* is found in greenhouses and similar protected places (Chichester and Getz, 1968).

The ecology of *Agriolimax sp.* has been well documented in Europe (Carrick, 1942; Gould, 1961; South, 1965; and Hunter, 1967, 1968a, b, c). *Agriolimax sp.* breeds throughout the year except in very dry or cold weather, and under laboratory conditions can lay 500 eggs in a season (Carrick, 1942). Many of the slug eggs laid in the field in the latter part of the fall fail to hatch in the spring

(Carrick, 1938). Many species of slugs, including Agriolimax, complete their life cycles in one year. Hunter (1971) reports that Agriolimax reticulatus has two generations a year in England, and that those slugs hatched in the fall will overwinter to produce eggs the following year.

The incubation period of Agriolimax reticulatus eggs varies from 15 days at 20°C to 105 days at 5°C (Carrick, 1942). The eggs of Agriolimax appear to have no structural provisions for resistance to desiccation (Bayne, 1968). Carrick (1942) and Arias and Crowell (1963) determined that turgidity of eggs depends on contact with a moist surface. During development the embryoes become increasingly resistant to extremes of temperature (Carrick, 1942) and weight loss due to desiccation (Bayne, 1969). Bayne (1969) reported that the embryos of Agriolimax reticulatus were able to survive a dehydration weight loss of 60-80%. Fully embryonated eggs that had developed at 15°C and were frozen for 2 hours at -2°C hatched within 3 hours when returned to 15°C (Stephenson, 1968).

Dainton (1954) mentions that adult Agriolimax can survive a weight loss due to desiccation and rapidly regain this moisture when in contact with damp surroundings. Mellanby (1961) found that Agriolimax reticulatus is not completely immobilized even at 0°C and at 0.8°C moves normally. Pinder (1969) found that there were no Agriolimax mortalities at -2.5°C, at -3.5°C they could survive for 35 hours but were killed in 28 hours at -4.5°C.

Kalmus (1942) and Dainton (1954) found that air currents will initiate orthokinetic and klinotactic responses in slugs. Crozier and Pilz (1923) showed that the speed of locomotion in Limax varied directly with temperature, movement being greatest at high temperature. Dainton (1953) concluded that the diurnal activity of slugs was controlled by fluctuations in temperature rather than humidity, which was previously assumed to be the controlling factor. Furthermore, Dainton (1953) concluded that light initially stimulates activity in dark-adapted animals, but the response is short lived.

Most animals are not randomly distributed, but tend to aggregate (Andrewartha, 1961). Agriolimax was found to be aggregated in grassland habitats, but the evidence for aggregation on arable land was not conclusive (South, 1967). Thomas (1948) determined that slug populations could range from 26,000 to 275,000 per acre on cultivated land. Stringer and Pickard (1964) estimated that the population of Agriolimax reticulatus in orchards could exceed one million per acre. However, it is difficult to get an accurate estimate of a slug population, as many of the slugs may be underground (South, 1965).

Bruel and Moens (1958) and Gould (1961) found that slugs were most common in heavy soils where moisture is retained at fairly high levels and that cover crops providing shelter favor high population. Runham and Hunter (1970) reported that with most slug populations there is a vast

surplus of food, and the number of animals feeding can have little affect on the food supply. However, speed of development and initiation of egg production tend to be dependant on the quality of food.

Collinge (1921); Blezard (1967) and Holyoak (1968) reported that several species of birds such as rooks and gulls feed on slugs. Stephenson (1964) found that carabid beetles preyed on Agriolimax. Knutson, Stephenson and Berg (1965) found that the fly Tetanocera plepeia, which is widespread in Canada and the United States, parasitizes Agriolimax sp. Hunter (1967) found that several species of slugs were parasitized by a nematode of the genus Cosmocercoides. Arias and Crowell (1963) found nematodes of the genera (Rhabiditus, Panogrolamus and Diplogaster in Agriolimax. Arias and Crowell (1963) and Brooks (1967) reported that ciliates of the genus Tetrahymena may be responsible for high mortality in slug populations.

Slug Culture

Most research on slugs has been conducted with field collected specimens. Reynolds (1936), Kozcloff (1956) and Arias and Crowell (1963) reported that they had difficulty in maintaining slugs under laboratory conditions. Mortality was blamed on fungus infections, nematodes and protozoa. The standard procedure for slug culture in the past has been to maintain slugs in a container with soil, leafmold or other bulk substance as a substrate (Sivik, 1954; Stephenson,

1961; and Arias and Crowell, 1963). Various foods have been tried with carrot and lettuce considered the best (Sivik, 1954; Stephenson, 1961).

Arias and Crowell (1963) attempted to eliminate fungus and parasites from their cultures by maintaining slugs on a special nutrient agar media impregnated with antibiotics. They were successful in eliminating nematodes but mortality still continued due to the protozoan parasites.

Review of DDT

The organochlorine chemical DDT has been widely used as an agricultural insecticide since 1946. This pesticide has proven invaluable in controlling insect pests and disease-carrying arthropods. During the 1950's and early 1960's, there were reports of large residues of DDT in the soil (Fleming and Maines, 1953; Ginsburg and Reed, 1954; Lichtenstein, 1958; and Woodwell and Martin, 1964). Orchard soils had the highest DDT residue levels, often over 100 ppm. dry weight (Lichtenstein, 1958; Harris et al, 1966). Many fields used for cereals and vegetables had DDT residue levels greater than 5 ppm. (Harris et al, 1966, Duffy and Wong, 1967).

DDT movement in the soil is limited by a tendency to form bonds with inorganic and organic components of soil and a very low water solubility (Ballard, 1971). Therefore, most DDT in the soil is relatively immobile (Lichtenstein, 1958; Woodwell, 1961; Peterson et al, 1971).

The persistence of DDT in the soil is variable with a half-life of 3 to 10 years (Menzie, 1972), depending in part on soil type and climatic conditions (Edwards, 1966).

Davis and Harrison (1966); Wheatly and Hardman (1968) and Davis (1966-68) reported DDT residues in various soil invertebrates, including slugs. Gish (1970) found that snails also contain DDT residues. Gish (1970) reported that terrestrial gastropods are able to accumulate DDT with no noticeable toxic effects.

Little work has been done concerning the metabolic dynamics of DDT and similar compounds in molluscs and other invertebrates. Nimmo et al (1970) found that shrimp were able to concentrate polychlorinated biphenyls from their surroundings. PCB's have similar properties to DDT (Riseborough et al, 1968; Gustafson, 1970). The main site of concentration in the shrimp was the hepatopancreas. Similar results were reported by Dindal and Wurzinger (1971) who found that the terrestrial snail, Cepaea hortensis had higher DDT concentrations in the hepatopancreas than in any other body tissue.

DDT and other pesticides are readily available to snails and slugs, not only in the soil organic matter in which they live, but also on the food which they eat (Waites and van Middelm, 1958). The DDT that slugs and other invertebrates concentrate in their tissues may be readily available to higher trophic levels, by transfer up the food chain (Stickel et al, 1965; Jeffries and Davis, 1966; and Vernon,

1970).

Most organochlorine pesticides, including DDT, are lipophilic and therefore stored in adipose tissue. The lipid content of Agriolimax columbianus is 1.12% of the wet body weight (Thompson and Hanan, 1963).

Chapter 2

MATERIALS AND METHODS

Taxonomy

The slugs described in this thesis were classified using the keys described by Barnes and Weil (1943) and Pilsbury (1948). To verify the classification of two species of slugs collected, samples were sent to Dr. L. Chichester, University of Connecticut, who likewise identified them as Agriolimax reticulatus (Fig. 1) and Lehmannia valentiana (Fig. 2). A third species, Agriolimax laeve (Fig. 3) was identified using the key described by Pilsbury (1948) and a key devised by Dr. Chichester.

Location of Sampling and Collecting Sites

A laboratory culture of Lehmannia valentiana was established from slugs collected in a campus greenhouse. Specimens of Agriolimax reticulatus were collected at the edge of the Faculty of Agriculture experimental plots, University of Manitoba, Winnipeg, in the fall of 1970 and the spring of 1971.

Test Chambers

Four different containers were used for maintaining the slugs:

(a) Large plastic containers 25.5 cm. in diameter by 8.75 cm. high

(b) Small plastic containers 15.24 cm. in diameter by 2.5 cm. high

(c) Large glass jars - 16 oz.

(d) Small glass jars - 8 oz.

Because of the danger of the plastic retaining some of the pesticide, the plastic dishes were disposed of after use. The glass containers were reused after being cleaned in a solution of potassium dichromate and sulfuric acid.

The large plastic containers were used primarily for maintaining slugs prior to experimentation and for culture purposes.

Small plastic containers were used for incubating eggs, maintaining immature slugs and for pesticide experiments. These containers were ideal because of ease of handling and they were large enough to accommodate 10 adult slugs.

The large glass jars were used in DDT accumulation experiments involving leaf litter. Nylon net was used to cover the top of jars. This tended to keep the slugs in the leaf litter instead of on the sides of the container, by allowing for water evaporation from the surface of the substrate.

The small glass jars were used for maintaining slugs individually for a short period of time.

Experimental Conditions

Unless otherwise stated, all slug culturing and experiments were carried out in incubators at 15°C and 100% relative humidity. The slug containers were kept in total darkness except for a short period of daily maintenance, when the containers were opened to ensure adequate oxygen. The humidity was maintained by the addition of distilled water whenever necessary.

All slugs used in a given experiment were of the same age and were normally the immature stages, so that egg laying was not occurring during the experiment. For the 6 days prior to initiation of an experiment the slugs were conditioned to the climatic conditions and food supply to be used in the experiments. Slugs that were collected at the end of the various experiments were maintained on a lettuce diet for a period of time to purge the gastro-intestinal tract. The slugs were weighed and then frozen at -20°C to kill them. The slugs were stored at this temperature until extracted for analytical purposes.

DDT Stock Solution

The DDT used in all experiments was the pp-isomer. Randomly ring-labelled pp-^{14}C-DDT (Specific activity 50 µc./ 2.8 mg.) was obtained from New England Nuclear. Unlabelled pp-DDT was obtained from the Aldrich Chemical Company. Several solvents were tested as DDT carriers for uniformly adding the DDT to leaf litter and oatmeal. Acetone, benzene

and ethanol (95%) were tested. Only ethanol was found to be suitable as it did not solubilize organic components of the leaf litter to as great an extent as did the other solvents.

Unlabelled pp-DDT and pp-^{14}C-DDT were individually dissolved in 95% ethanol. 0.025 millicuries of the ^{14}C-DDT solution was then added to the unlabelled solution. The resulting DDT-ethanol stock solution contained 532,000 dpm. per mg. DDT. When 10 mls. of the stock solution was added to 25 gms. of dry substrate, a DDT level of 80 ppm. dry weight and a ^{14}C specific activity of 42,000 dpm. per gram of dry matter was obtained.

Media and Foods

The feeding habits of slugs vary with the seasonal availability of different foods. To simulate the feeding conditions encountered by slugs in the spring and late fall, samples from the top 1/2 inch of a litter layer were collected in the spring and late fall of 1971. These samples were taken at the Glenlea Research Station from a mixed hardwood biome surrounded by fields on three sides and bordered on the fourth by the Red River. Mature oak, maple, elm and ash trees formed the canopy layer. The ground vegetation consisted primarily of grass, nettles and burdock.

Upon collection the litter samples were air dried at 80°C for 48 hours to eliminate soil invertebrates and moisture. These dried samples were then stored in sealed plastic bags.

Twenty five gm. samples of the leaf litter were placed in the large glass jars and aliquots of the DDT ethanol solution were added. Control containers were treated with ethanol only. The ethanol was allowed to evaporate from the leaf litter for 72 hours, and then mechanically mixed. Twenty mls. of distilled water were pipetted onto each 25 gm. sample of leaf litter to ensure adequate moisture for the slugs.

As slugs tend to feed on fresh green material during much of the summer, several different foods were tested for use as carriers in the pesticide experiments. Bran, oatmeal, dried lettuce and dried carrot were tested, with oatmeal being the most palatable; thus the slugs consumed this preferred food before the other foods offered. Fresh foods such as lettuce, as used by Dindal and Wurzinger (1971) were found entirely unsatisfactory due to the difficulty in adding a known amount of DDT and the perishable nature of the foodstuff.

Oatmeal proved to be an ideal carrier for DDT, especially when made into pellet form. Both L. valentiana and A. reticulatus could be maintained on a diet of uncontaminated oatmeal for 60 days with no apparent adverse effects.

Oatmeal was ground to a powder in a Sorval blender at 6000 rpm. for 2 minutes. Twenty five gm. samples of this powder were then placed in small glass jars and aliquots of the DDT-ethanol solution were added, or in the case of the

controls, ethanol only. The oatmeal was then allowed to dry for 24 hours, at which time it was again placed in the Sorval blender and mixed at 4000 rpm. for 2 minutes. Fifteen grams of this powder was then placed in an 8 inch x 8 inch flat bottomed glass dish and distilled water was added until all the powder was dampened. The oatmeal was then allowed to dry at room temperature, forming a solid pad. This was broken down to form small pellets which were stored in sealed containers.

These oatmeal pellets became fairly soft when placed in the experimental containers, and the slugs did not aggregate about or on the pellet as much as they did with fresh lettuce. There was no problem with the pellets being scattered about by the slugs as the powdered oatmeal was. Thus the accumulation of DDT could be accurately determined.

Homogeneity of ^{14}C-DDT distribution in the leaf litter and pellets was checked by ^{14}C determination of randomly collected 0.5 gm. samples. The ^{14}C-DDT distribution was found to be very homogeneous in the media.

Analytical Techniques

The extraction procedure used was basically that described by Bligh and Dyer (1959) for the extraction of lipid from tissue. Several modifications had to be made to this procedure due to difficulties in filtration. Frozen slugs (pooled sample) were placed in the blender cup containing chloroform and methanol. This mixture was blended

for 5 minutes at 6000 rpm. in a Sorval blender. The mixture was then filtered through one layer of filter paper (Whatman #2) in a Buchner funnel using a water aspirator. Filtration was rapid provided the water was added directly to the collection flask rather than to the mixture being blended. The filter paper was rinsed with chloroform and then placed in the blender cup with chloroform and homogenized at 4000 rpm. for one minute. This mixture was then suction filtered and rinsed with chloroform before being added to the collection flask. Distilled water was then added to the suction flask and the contents poured into a 250 ml. separatory funnel. The suction flask was then rinsed with methanol which was in turn added to the separatory funnel. This slug extract was allowed to settle for 24 hours before the lower chloroform layer was drained into a 100 ml. volumetric flask. The volume was brought to 100 ml. with chloroform. Subsequently the methanol-water layer was drained into a volumetric flask and the volume brought to 100 mls. by adding methanol.

Scintillation Counting

Ten mls. of the chloroform extract was placed in each of three scintillation vials. The chloroform was evaporated by warming the vials in a Temp-Blok module heater and using a stream of nitrogen gas. When drying was complete, 15 mls. of toluene scintillator solution (see Appendix 1) was added and the samples counted in a liquid

scintillation counter (Nuclear Chicago).

Five ml. samples of the methanol-water layer were likewise counted using a p-dioxane scintillator solution (see Appendix 1). The ^{14}C-counts for both layers were added together to give a total ^{14}C-count for the slug sample. In the scintillation counter two background samples were placed in front of the unknown and single background samples were placed after every 10th unknown sample. All samples including backgrounds were counted for 4000 total counts or 100 minutes. As the machine counting efficiency varied with time, and the efficiency of the scintillation solutions differed, correction factors were used as necessary. The count value for the pooled control samples were subtracted from the test samples to acquire the final DDT levels.

Culture of Agriolimax reticulatus

Initially an attempt was made to maintain slugs at 15°C in 1 gallon jars containing damp leaf litter. Because of the high mortality rate, often reaching 90% in 3 weeks, other alternatives were tested. It was found that mortality was greatly reduced by lowering the temperature from 15°C to 4°C. At this temperature mortality of newly collected slugs was 23% in 3 weeks. As it would have been impossible to run long term experiments at even this mortality, slugs relatively free of disease and parasites were reared in the laboratory.