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The Effect of Acute Exposure to Glyphosate on Reproductive Potential and Embryo Development of Pseudosuccinea Columella Snail

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THE EFECT OF ACUTE EXPOSURE TO GLYPHOSATE ON REPRODUCTIVE POTENTIAL AND EMBRYO DEVELOPMETN OF PSEUDOSUCCINEA COLUMELLA SNAIL

A THESIS

Presented to the

Honors College at Southern University
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In Partial Fulfillment of the Requirements for the

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by

Ambika N. Osborn

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ABSTRACT

Glyphosate (Roundup) is one of the most commonly used broad spectrum herbicides used with little to no toxicity to higher animals or the environment (Smith and Ochme, 1992). Due to its widespread use, there is a continuous contamination of the environment in both soil and water, with this herbicide. This study was developed to determine the effects of sublethal concentrations of glyphosate on development and survivability of Pseudosuccinea columella snails (intermediate host of Fasciola hepatica). This was assessed by acutely exposing third generation snails to varying concentrations (0.01-1ppm) of glyphosate. The snails were kept on artificial spring water to which doses of glyphosate were added and the laboratory in which the water temp (22-25°C) and photoperiod (approx. 12 hours of light) were unregulated. Abnormalities and polyembryony were observed in snails exposed to 0.01 and 0.1 ppm glyphosate. These results did not indicate that glyphosate affects the snails reproductivity and embryo development. The difference in generations used may play a factor in the fluctuation with these results. Hopefully in further experimentation, it may be proven that glyphosate affects the egg-laying capacity of Pseudosuccinea columella snails. This in turn, may influence the rate of infection of Fasciola hepatica in higher animals (mammals).

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CHAPTER I
BACKGROUND OF THE STUDY

Introduction

Facioliasis is a disease caused by the liver flukes, Fasciola hepatica. Fasciola hepatica is cosmopolitan in distribution and occurs in sheep and cattle raising countries of the world. It parasitizes these animals and other herbivores on almost every continent and also on several islands. Human fascioliasis has also been reported from various part of the world and is believed to be more prevalent than commonly considered.

Pseudosuccinea columella belong to the molluscan class of lymnaeids. The tentacles are broad and triangular, with black eyes placed on small swellings at their inner base. Being hermaphrodites, the Pseudosuccinea columella snails have a reproductive system similar to that of other pulmonates. The male and female tracts are separated for the most part; the male genital pore opens behind the right tentacle, whereas the female pore opens beneath the mantle collar near the respiratory cavity (5). These snails are aquatic, with a tendency to be amphibious (7).

Pseudosuccinea columella lives in stagnant habitats such as small ponds, but may also be found in large lakes, swamps, and occasionally quiet microhabitats of running streams. The surface of the water in the small ponds is usually covered with pond scum or floating or rooted vegetation. Cattails and water lilies are among the common vegetation associated with these snails. The snails are found on leaves and stems at or near the waters' surface; they are also sometimes found on moist mud at the waters' edge or on any flotsam (3).

Pseudosuccinea columella's shell has a low spire, much shorter than the high and elongated aperture, with a large and expanded body whorl; the surface is sculptured with microscopic raised spiral periostracal threads. Lateral teeth of the radula are tricuspid. The penis sheath is short, less than half the length of the prepatium; the prostate is long and narrowly, roundly cylindrical, with the proximal end somewhat enlarged.

Pseudosuccinea columella serves as an intermediate host for *Fasciola hepatica*, the sheep liver fluke. The survival of this snail species is important for the liver fluke infection.

Glyphosate

Glyphosate is a weak organic acid composed of glycine and a phosphonomethyl moiety (7). The empirical formula $C_3H_8NO_5P$. Glyphosate is customarily expressed as a salt of the deprotonated acid of glyphosate and a cation (12). The purity of technical grade glyphosate is generally above 90% (1). Technical grade glyphosate is an odorless white crystalline powder with a specific gravity of 1.704, a very low vapor pressure, and a high different ionic species, dependent on the actual pH (4).

Glyphosate is a post-emergent, systemic and non-selective herbicide that is used in both agricultural and non-agricultural areas all over the world. Glyphosate is applied to many crops and in various commercial formulation (6). The major formulation is Roundup in which glyphosate is formulated as the isopropylamine salt. Recommended application rates do not exceed 5.8 kg a.i./ha and are dependent on the type of use. Environmental exposure may occur because of deposition due to drift and accidental releases (4).

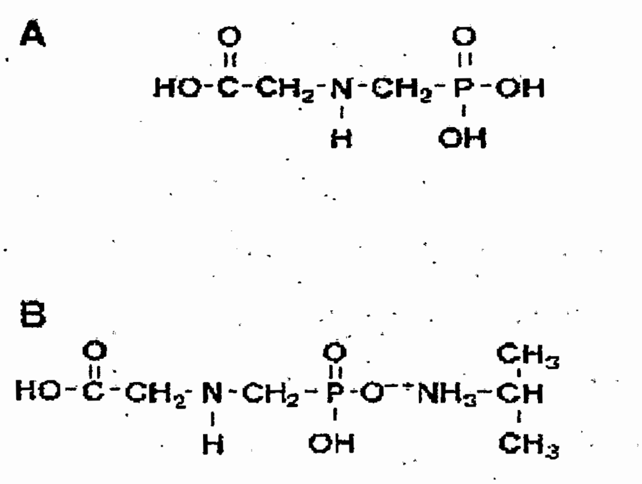

Figure 1

A) Structural formula of glyphosate
B) Isopropylamine salt (glyphosate)

Hypothesis

The presence of glyphosate at sublethal concentrations in the aquatic environment of Pseudosuccinea columella snails will affect the reproductive maintenance of these snails, which serves as an intermediate hosts of Fasciola hepatica.

Statement of the Problem

Fascioliasis is of economic importance and this is especially true when considering the reduced milk yield and growth rate of the infected animals, their chronic low-grade anemia, the emaciated carcasses, the loss of condemned livers when the animals are slaughtered, and the reduction, in some cases of up to 39, in sheep-wool production. Fasciola hepatica parasitizes cattle and sheep. Fascioliasis is caused by the ingestion of the infective metacercariae on vegetation or through the intake of free metacercariae in water (9).

There is a paucity of long-term exposure studies with sublethal concentrations of glyphosate on aquatic snails. This study was developed to determine the effects of sublethal concentrations of glyphosate on development and survival of Pseudosuccinea columella (intermediate snail host of Fasciola hepatica). Previous reports indicate that low concentrations of some herbicides have a significant influence on the mortality of the intermediate snail hosts (Pseudosuccinea columella) of Fasciola hepatica (2). In general, the incidence of Fasciola *hepatica* is directly related to the incidence of the susceptible lymnaeid snails, such as *Pseudosuccinea columella*. The exposure of nontarget organisms such as *Pseudosuccinea columella* to these herbicides is enhanced by their water solubility and extensive usage in the environment. These chemicals may directly affect these nontarget organisms by causing developmental, morphological, physiological, immunological, and biochemical changes.

CHAPTER II
LITERATURE REVIEW

USE OF THE HERBICIDE

A review of literature reveals that the isopropylamine salt of glyphosate is the active ingredient in Roundup and is marketed as a non-selective, non-residual, broad spectrum, foliar applied, post-emergence herbicide (15,20). It is used primarily to control weeds in fruit and vegetables crops, forest and aquatic areas (14,21), and along railway and/or highway rights-of-way (13). Due to its non-selectivity it cannot be used during the active growth season of crops (22). The product's inert ingredients include water and a surfactant or surface active agent, polyoxyethyleneamine (POEA), to aid in adsorption of the herbicide on to the plant (23).

After application, glyphosate is absorbed by the foliage and translocated throughout stems, leaves and roots of the entire plant. It is effective on deep-rooted perennial species, annual and biannual species of grasses, sedges, rushes, broad-leaf weeds and woody plants (13). Excellent control of most annual species has been obtained at application rates of 0.34-1.12 kg acid equivalent (ae)/ hectare (ha), and 1.68-2.24 kg/ha for most perennial species (15). The best weed control is obtained if applications are made at later stages of plant maturity. Wiping devices instead of sprayers are recommended when height differences of the plants allow for selective removal of weeds from crops (15).

PHOSPHATE REVERSAL OF GLYPHOSATE BINDING

Inorganic phosphate excludes glyphosate from adsorption sites on clay and organic matter through the phosphoric acid moiety and decreases the inactivation of glyphosate in soil (18,16). Experiments with plants grown in culture solutions in soil is not due simply to its high soil adsorption, but rather is the result of moderate adsorption and the low intrinsic activity of glyphosate when applied to the root system (16). However, it is through the phosphoric acid moiety's reversible binding to clay soils and organic matter that the initial inactivation of glyphosate is produced (18).

ABSORPTION INTO PLANTS

Glyphosate enters the plant primarily through the foliage (13). Less than 0.1% of glyphosate is absorbed through the roots of the plant due to tight adsorption of glyphosate to soil (17). Glyphosate crosses the cuticle of the plant by diffusion via a hydrophilic pathway and is not affected by pH (19). Polar, water-soluble compounds such as glyphosate use cutin and carbohydrate fibers for a polar route among the non-polar, waxy portions of the cuticle. In general glyphosate penetrates most plants to the extent of 34% within the first 4 h after application then reduces to a slower rate for the next 2 d

(24,27).

MODE OF HERBICIDAL ACTION

Since glyphosate is rapidly absorbed and translocated to various parts of the plant, it comes in contact with and alters a wide range of plant functions. Glyphosate affects photosynthesis, plant respiration, and synthesis of plant nucleic acids. However, the primary mode of herbicidal action is the blocking of aromatic amino acid synthesis and the metabolism of phenolic compounds (13,25). These effects are especially important as they influence the formation of plant protein and tissue. The fact that shikimate, a precursor for phenylalanine, tryptophan or tyrosine, is not metabolized in the presence of glyphosate suggests that glyphosate may inhibit any of the three enzymes that convert shikimate to chorismate--shikimate kinase, 5-enolpyruvyl shikimate-3-phosphate synthetase, or shikimate synthetase, or shikimate synthetase. The conversion of shikimate to anthranilate was inhibited by 50% in 5-7 uM glyphosate and resulted in the accumulation of an acidic polar product. Shikimate was released from this polar product by alkaline phosphatase treatment. This indicated the product was shikimate-3-phosphate and that the enzyme inhibited was 5-enolpyruvyl shikimate-3-phosphate synthetase (26). The addition of chorismate and o-succinylbenzoate alleviated the inhibition of this reaction by glyphosate (25). In another study, root-fed aromatic amino acids at concentrations less than growth-retarding reversed the root growth inhibition caused by glyphosate to a small but significant extent; pretreatment with the aromatic amino acids did not enhance the reversal (28). The inhibition of the aromatic amino acid metabolism and the accumulation of phenolic compounds eventually led to a reduction of protein synthesis, resulting in cessation of growth, cellular disruption and plant death.

Glyphosate also reduces plant chlorophyll (29-33) causing an increase in respiration (31). This results from glyphosate inhibiting the formation of delta-aminolevulinic acid, thus blocking the synthesis of chlorophyll. The site of glyphosate action in blocking the formation of delta-aminolevulinic acid may involve two enzyme pathways --one controlling the conversion of alpha-ketoglutarate to delta-aminolevulinic acid, and the other controlling the condensation of glyphosatecine with succinyl co-enzyme A to form delta-aminolevulinic acid and carbon dioxide (34). The ultrastructural changes observed from the blocking of these reactions include: formation of granular bodies; deterioration of oil bodies, endoplasmic reticulum and ribosomes; and vacuolation of the cytoplasm (30). In Euglena gracilis strain, this inhibition occurs at concentrations below 0.12 muM, but above this concentration the glyphosate may cause photosynthesis stimulation (31,33). The mode of glyphosate action in producing this change is complex, causing changes in both catabolic and anabolic plant cell reactions (30).

TOXIC EFFECTS ON PLANTS

The response of plants to glyphosate application is fairly slow. Depending on the plant species treated, herbicidal effects may not be visible for 2-4 d in annuals or for up to 7 d or more in perennials (13,35). Visible effects seen are the gradual wilting and yellowing of the treated plant, followed by complete browning, deterioration of underground root or vegetative propagules (13).

Glyphosate primarily accumulates in the apical meristem and roots. Extremely low levels of glyphosate were detected in the mother tuber regardless of the plant's age when it was treated with between 0.28-2.24 kg/ha. Phytotoxic effects were seen on the eyes of the mother tuber, the degree of which increased in severity with increasing glyphosate application rates; this was evidenced by abnormal sprouting in the form of severely suppressed sprouts on those eyes that resprouted (35).

Depending on the moisture level of the grain at the time of application, glyphosate reduces seed weight and the emergence, vigor, and weight of progeny seedling. Glyphosate applied 2.5 w or more before maturity of some crops also caused reduced seed weight, seed discoloration, and reduced emergence, vigor and weight of the progeny seedling (36).

EFFECTS IN ANIMALS

When rats and mice were given glyphosate by mouth (po) or intraperitoneal (ip), severe stress, increased respiration, elevated rectal temperatures, and occasional asphyxial convulsions were noted. Median lethal doses of 4704 mg/kg to the rat and 1581 mg/kg to the mouse po were significantly higher than the 235 and 130 mg/kg, respectively, median lethal doses obtained when the herbicide was given ip. Lung hyperemia was the major lesion noted in the glyphosate-poisoned animals (38-40).

SKIN AND EYE IRRITATION

Technical glyphosate (98% pure plus the isopropylamine salt of glyphosate) and the undiluted isopropylamine salt of glyphosate applied to intact or abraded rabbit skin for 24 h produced no dermal irritation. The undiluted commercial formulation (41% isopropylamine salt of glyphosate) under the same conditions produced moderate to severe skin irritation, suggesting that other components cause the irritation. The majority of animals recovered by day 9 (38-40).

Technical glyphosate (98% pure plus the isopropylamine salt of glyphosate) instilled into rabbit eyes produced conjunctival redness, chemosis, and corneal opacity/ ulceration. All eyes were normal within 7 d. Washing the eyes with warm water 20 s after glyphosate application did not prevent the irritation (38,40).

CHAPTER III
MATERIALS AND METHODS

Third generation Pseudosuccinea columella snails were reared in the laboratory in 2-gallon plastic aquaria. Two snails were placed into each aquarium. Each aquaria contained 1000mL of artificial spring water. This solution was prepared by mixing the following stock solutions: 1mL ferric chloride; 5mL calcium chloride; 5mL magnesium chloride and 2.5mL phosphate buffer solution. The solution was made by using 1% stock solution. Aquaria #1 is the control. Aquaria #2 is the 1 ppm of Glyphosate. Aquaria #3 is the 0.1 ppm of Glyphosate. Aquaria #4 is the 0.01 ppm of Glyphosate. Water hardness was maintained in each aquarium at 80-120 mg/L measured as $CaCO_3$, DO= 6-8 mg/L, pH= 6.5-8.5, and ammonia nitrate levels were maintained at 2 mg/L. Snails were exposed to sublethal concentrations of glyphosate ranging from 1 ppm-0.01 ppm (pH= 6.8-7.2). All snails were fed endive lettuce leaves *ad libitum* and each aquarium was aerated (2,11).

Glyphosate (97%), was obtained from Chem Service, West Chester, Pennsylvania. The aquariums were kept between 19-24°C. Each concentration tested (1 ppm, 0.1 ppm, and 0.01 ppm) was measured and placed in flasks (1000 ml). Third generation Pseudosuccinea columella laboratory-reared snails were placed into 0.01 ppm, 0.1 ppm, and 1 ppm of glyphosate and maintained. Sublethal concentrations of glyphosate were determined initially by exposing egg masses to varying concentrations of glyphosate and determining toxicity. Fourth generation egg masses were selected according to similarities in size and/or number of embryos for exposure and were maintained in the laboratory in small glass containers containing 100 ml of each concentration in duplicate with one to two egg masses each. To determine changes in development, egg masses were observed under a Nikon dissecting microscope equipped with a video recording camera. Photographs were taken of each egg mass at day 0 and every three days thereafter until the eggs hatched (usually 12-13 days). Each embryo within the masses was measured to determine growth and observed for abnormalities. Each container was covered with parafilm, and punctured with holes for circulation of oxygen. Growth, development, hatching rate, egg-laying capacity, and mortality were observed.

CHAPTER IV
RESULTS AND DISCUSSION

RESULTS

The control snails (Table 1) laid a total of twenty-four egg masses. Nineteen of these eggs within the egg masses were uninucleated. The five remaining eggs were binucleated. The snails placed in the concentration of 0.01 ppm laid a total of eighteen egg masses. Ten of the eggs were uninucleated, five were binucleated, two were trinucleated, and one egg was tetranucleated. The snails placed in the concentration of 0.1 ppm laid a total of ten egg masses. Six of the eggs were uninucleated, one was binucleated, two were trinucleated, and one was tetranucleated. The snails placed in the concentration of 1.0 ppm laid a total of five egg masses. All five egg masses were uninucleated. The fourth generation egg masses began hatching within 19-22 days. Some of the embryos did not develop or developed much smaller than the other embryos.

Table 1
Effect of glyphosate on egg laying capacity and embryo development of third generation Pseusosuccinea columella snails

	Treatment (ppm)			
Egg laying capacity and embryo development	Control	0.01	0.1	1
Total number of egg masses laid	24	18	10	5
Number of egg masses with single embryo	19	10	6	5
Number of egg masses with two embryos (twins)	5	5	1	-
Number of egg masses with three embryos (triplets)	-	2	2	-
Number of egg masses with four embryos (quadruplets)	-	1	1	-

DISCUSSION

The results of this experiment do not support the hypothesis. The results do not show that glyphosate applied in sublethal concentrations have an affect on the egg laying capacity and development of Pseudosuccinea columella snails. Compared to other papers written about this experiment, different generations of snails were used. The snails mentioned in other papers were chronically exposed and seventh and eighth generation Pseudosuccinea columella snails were used. The snails used in this experiment were acutely exposed third generation Pseudosuccinea columella snails. Therefore, the difference in the generation of snail used may be the reason for the differences in the results. The difference in the egg masses laid between the control group and the exposed group is not enough to show an effect caused by glyphosate. Further studies may be conducted on future generations to determine if results of the effect of glyphosate on Pseudosuccinea columella snails are the same. These future experiments will hopefully provide information on the egg-laying capacity of Pseudosuccinea columella snails which may influence the rate of infection of Fasciola hepatica in animals and man.

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