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The Influence of Pesticides on the Biology and Physiology of the Land Snail *Bulimulus tenuissimus* (Orbigny, 1935)

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***Abstract*—** *Some terrestrial gastropods are agricultural pests and are exposed to toxic products capable of causing physiological and biological alterations. The action of some herbicides and molluscicides has been studied on freshwater snails, but there are few studies of terrestrial species. This study analyzed the biological and physiological responses of Bulimulus tenuissimus exposed to the natural molluscicidal latex of Euphorbia milii var. hislopii and the synthetic herbicide Roundup® Original. A total of 210 snails were divided and exposed for 24 hours to the products (latex or herbicide in concentrations of 1,000 ppm and 100,000 ppm) and type 2 water. Of this total, 150 exposed animals were analyzed biochemically after 24 hours, and the others were observed during 30 days for analysis of the mortality rate. After 24 hours, some snails presented escape mechanisms such as epiphragm formation, cephalopedal mass retraction and burial. The latex did not cause metabolic alterations but the herbicide altered the carbohydrate metabolism of B. tenuissimus, reducing energy substrates in the sites analyzed (hemolymph, digestive gland and cephalopedal mass). Regarding the protein metabolism, there was no alteration after exposure to both analyzed substances. After 30 days of exposure, higher mortality was recorded for the groups exposed to the herbicide. These findings suggest that B. tenuissimus is resistant to the molluscicidal latex of Euphorbia milii var. hislopii more than the herbicide Roundup® Original.*

Keywords*— *Bulimulus tenuissimus, carbohydrate metabolism, Euphorbia milii var. hislopii, Roundup® Original.

I. INTRODUCTION

Agrotoxics are substances used to increase agricultural productivity through pest and disease control [1]. However, their indiscriminate use can cause serious damage to the environment and to human health, as well as putting selective pressure for development of resistance by pests [2].

Brazil has been the largest consumer of agrotoxics in the world since 2008 according to data from the Brazilian Institute of Geography and Statistics (IBGE) [3], with numerous active principles and commercial formulations, which can be classified according the action on the target organism and the chemical group to which they belong [2]. Of the agrotoxics used in Brazil, 56% are herbicides [4]. Among the commonly used Roundup® stands out, which has as its active ingredient Glyphosate (N-(phosphonomethyl) glycine) combined with a surfactant that facilitates transport by the plant. It is a non-selective

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herbicide, effective in the total destruction of weeds [5], and in the control of plants in reforestation, gardens and aquatic plants in fish farming tanks and in lakes [6]. From the Roundup® herbicide several formulations were commercialized by the Monsanto Company, one of them is Roundup® Original, which presents polyoxyethylene amine surfactant (POEA), which helps the herbicide to penetrate the leaves of plants [7]. Some herbicides have deleterious effects on limnic mollusks, interfering at the cellular and physiological, molecular level to reduce the survival rate and reproductive activity [8, 9, 10].

Molluscicide is the classification of agrotoxic for control of mollusks present in gardens, fields and greenhouses, including those that are intermediate hosts of parasites in aquatic environments, such as snails that transmit schistosomiasis. Poisoning by such substances can cause osmotic imbalance, and as a consequence two mechanisms that cause mortality may occur: retraction of the cephalopedal mass with the release of hemolymph and/or abnormal projection out of the shell [11], called "distress syndrome".

Molluscicides can be synthetic or natural. Chemical control of terrestrial species occurs mainly through synthetic molluscicides. Although effective, these compounds also act on non-target organisms, causing significant changes in ecosystems [12]. As a consequence, there is increasing demand for readily biodegradable substances as an alternative to synthetic molluscicides [13].

The latex of *Euphorbia milii* var. *hislopii* (N. E. B.) (Euphorbiaceae), popularly known as crown of thorns, Christ plant, or Christ thorn, is considered one of the most promising Brazilian molluscicides. In laboratory and field conditions it complies with the recommendations of the World Health Organization (WHO) for use as a natural molluscicide, in addition to being biodegradable and less hazardous to non-target organisms [14], with effective results on limnic mollusks and amphibians [15].

The land snail *Bulimulus tenuissimus* (Orbigny, 1935) is present in many Brazilian regions [16], where it is considered an agricultural pest. It is also the only species in the family Bulimulidae cited as an intermediate host of some parasites of poultry [17]. Recently, studies showed that this mollusk is host of the nematode *Angiostrongylus cantonensis* (Chen, 1935) [18,19], a rodent lung parasite capable of infecting humans and causing meningitis, characterized by eosinophilic inflammation [20].

Studies of synthetic or natural substances for the control of terrestrial species are scarce, as well as the physiological changes induced by these substances. For this reason, this study analyzed the biological and physiological responses of *B. tenuissimus* exposed to the natural molluscicide latex of *E. milii* var. *hislopii* and the synthetic herbicide Roundup® Original.

II. MATERIAL AND METHODS

2.1. Determining concentrations of *E. milii* var. *hislopii* latex and Roundup® Original

The crude latex extract was collected in the Ilha do Governador district (22°48´09´´S/ 43°12´35´´W) of the city of Rio de Janeiro, Brazil. Determination of the concentrations of the two substances was based on the procedures described by Vasconcellos and Amorim [21], and based on pre-tests, concentrations of 1,000 ppm (parts per million) (0.1%) and 100,000 ppm (10%) for both substances were chosen (mg/L for latex and ml/L for herbicide).

2.2. Formation of groups

The parents of the snails used were obtained in a garden located in the municipality of Seropédica, Rio de Janeiro, Brazil (about: 22º 44' 38" S; 43º 42' 27" W). A total of 210 hatched mollusks were kept in the Laboratory for Evaluation and Promotion of Environmental Health of Fiocruz in Rio de Janeiro, where they were monitored until reaching sexual maturity (110 days).

For the 24-hour and 30-day follow-up, the snails were divided into five groups, with 30 and 12 specimens each, respectively: two groups exposed to latex (L) at concentrations of 1,000 ppm and 100,000 ppm (L1,000 and L100,000, respectively); two groups exposed to Roundup® Original (R) (R1,000 and R100,000, respectively); and one control group sprayed with type 2 water.

2.3. Exposure

For the 24-h follow-up, 150 mollusks were exposed individually in disposable flasks with a capacity of 150 ml containing 50 g of sterile soil moistened with water. Addition of the compounds was done by spraying the concentrations prepared for each product using 5 ml glass hand sprayers. Each mollusk received 2 ml of the product corresponding to its group, under a fume hood. Twenty-four hours after exposure, the specimens were observed and dissected for the collection of biological samples.

For the 30-day follow-up, the exposure occurred plastic terrariums with 12 cm in diameter and 8 cm in depth, containing 3 cm of moistened sterilized soil as substrate. Each terrarium received 24 ml of the substance corresponding to its group through glass sprayers: 2 ml of the corresponding substance was sprayed onto each mollusk, under a fume hood. They were observed every

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other day for 30 days (four weeks) after exposure to determine the mortality rate.

In both cases, the control groups were sprayed with 2 ml of type 2 water.

2.4. Collection of hemolymph and dissection after 24 hours

After 24 hours of exposure, 150 snails (30 specimens from each group) were dissected. The hemolymph was collected by extravasation from the apex rupture of the shell, collected in microtubes, kept in an ice bath at 10 °C and then stored at -20 °C in a freezer until the biochemical analyses [18].

The specimens were dissected in Petri dishes and the cephalopedal mass and digestive gland tissues were kept separately in an ice bath at 10 °C during collection to avoid enzymatic degradation of carbohydrates. Subsequently, the samples were weighed and stored at -20 ºC in a freezer until use.

2.5. Biochemical analysis

The hemolymph was analyzed with a BioSystems A15 automatic biochemical analyzer, with reading through the use of reagent kits for biochemical analysis prepared for glucose, total protein and lactate dehydrogenase (LDH E.C. 1.1.1.27).

Glycogen extraction from the digestive gland and cephalopedal mass was performed as described by Pinheiro and Gomes [22] and quantified through the 3,5 dinitrosalicylate (DNS) technique [23], with results expressed as mg of glucose/g tissue, fresh weight.

2.6. Maintenance of the terrariums

The feed consisted of 3 g ± 1 g, of chayote (*Sechium edule*) and carrot (*Daucus carota*), plus lettuce leaves (*Lactuca sativa*) *ad libitum*. *In natura*. The feed was supplemented with bird feed enriched for growth with calcium carbonate in proportion of 3:1 [24], served in plastic containers (3 cm in diameter and 0.5 cm in depth [25]. All feed was changed every two days. The soil sprinkled with the substances was kept in the terrariums until the end of the experiment (30 days).

2.7. Mortality records

The terrariums were observed every two days, for 30 days (four weeks), for quantification and removal of dead snails. The mortality rate was expressed as a percentage (%).

2.8. Statistical analyses

The results were expressed as mean ± standard error of the mean, and were submitted one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test (α= 5%) to compare the means (GraphPad InStat and GraphPad Prism, v. 6., Prism Inc.).

III. RESULTS

After 24 hours of exposure, exhaust mechanisms, characterized by increased mucus release and presence of epiphragm, were observed in the snails of all groups exposed to both toxic products. Also, in the L1,000 group, burial behavior occurred, and in the R1,000 group, cephalopedal mass retraction was observed.

Significant reductions of 29.99%, 24.21% and 12.21% in the glycogen content of the digestive gland were observed in the groups L100,000 (17.09 ± 0.29), R1,000 (18.5 ± 0.66) and R100,000 (21.43 ± 0.77), respectively, in relation to the control group (24.41 ± 0.37). However, a non-significant increase was observed of 7.57% in the group L1,000 (26.26 ± 0.66) in relation to the control group (Fig. 1A).

The pattern was repeated for glycogen content in the cephalopedal mass, with significant reductions of 37.9%, 60.63%, 28.66% in groups L100,000 (18.96 ± 0.02), R1,000 (12.02 ± 0.14) and R100,000 (21.78 ± 0.03), respectively, in relation to the control group (30.53 ± 0.08). And group L1,000 (35.83 ± 0.14) presented a significant increase of 17.36% compared to the control group (Fig. 1B).

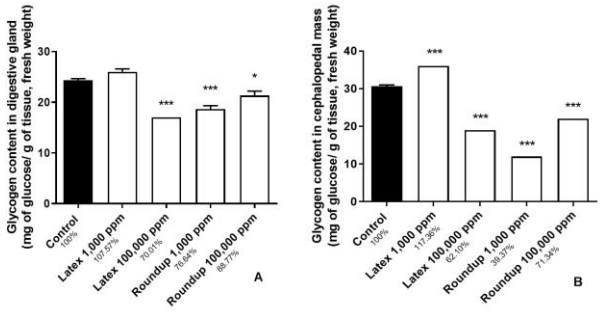

*Fig. 1. Changes in the glycogen content (mg of glucose/g tissue, fresh weight) in Bulimulus tenuissimus tissues exposed to two concentrations of natural molluscicide latex of E. milii var. hislopii and the synthetic herbicide Roundup® Original. **A** Digestive gland. **B** Cephalopedal mass (p value<0.0001, strongly significant).*

(P<0.001***, strongly significant/ P<0.01**, significant / P<0.05*, weakly significant difference/ P>0.05 no significant difference in relation to the control group).

The glucose concentration in the hemolymph showed significant reductions of 72.08% and 74.99%, respectively, in the L1,000 (6.33 ± 0.88) and R100,000 (5.67 ± 1.2) groups in relation to the control (22.67 ± 1.2). The decrease of 29.42% observed in group R1,000 (16 ± 0.0) was not significantly different than the control. In addition, a non-significant increase of 27.92% in the L100,000 group (29 ± 3.05) was observed compared to the control group (Fig. 2A).

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The analysis of lactate dehydrogenase (LDH) activity in hemolymph showed increases in activity of 48.43% and 65.85% in L100,000 (142 ± 43.15) and R1,000 (158.67 ± 2.73), respectively, after 24 hours of exposure. Moreover, reductions of 46.69% and 38.68% of this activity were observed in L1,000 (51 ± 6.03) and R100,000 (58 ± 12.44), respectively. However, no significant differences were observed in relation to the control group (95.67 ± 12.55) (Fig. 2B).

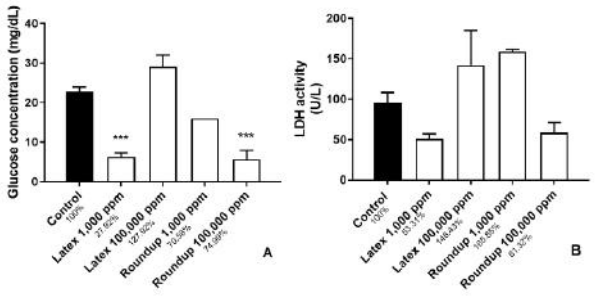

Fig. 2. ***A*** *Glucose concentration expressed in mg/L (P<0.0001, strongly significant) and* ***B*** *Lactate dehydrogenase (LDH) activity expressed in U/L (P= 0.0153, significant) in Bulimulus tenuissimus hemolymph after 24 hours of exposure to Euphorbia milii var. hislopii latex and the synthetic herbicide Roundup® Original.* (P<0.001***, strongly significant/ P<0.01**, significant / P<0.05*, weakly significant difference/ P>0.05 no significant difference in relation to the control group).

Exposure to the toxic substances caused non-significant reductions in circulating concentration of protein in the hemolymph of 33.35%, 1.27% and 28.19% in the L1,000 (17.33 ± 2.85) L100,000 (25.67 ± 3.48) and R100,000 (18.67 ± 4.70) groups, respectively, compared to the control group (26 ± 3.46). In R1,000 there was a 33.34% increase in this parameter (34.67 ± 0.3) (Fig. 3).

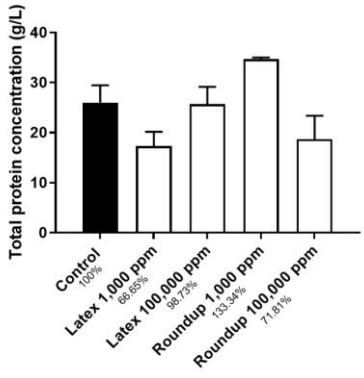

Fig. 3. Circulating protein concentration, expressed in g/L of Bulimulus tenuissimus hemolymph after 24 hours of exposure to Euphorbia milii var. hislopii latex and the synthetic herbicide Roundup® Original.

(P<0.001***, strongly significant/ P<0.01**, significant / P<0.05*, weakly significant difference/ P>0.05 no significant difference in relation to the control group).

Dead mollusks were found in terrariums after 24 hours in the R100,000 group, after 48 hours in the R1,000 group, 96 hours in the L100,000 group and 168 hours in the L1,000 group.

For mortality of *B. tenuissimus* after 30 days (720 hours), the exposure to the synthetic herbicide in the groups R1,000 and R100,000 caused mortality rate of 91.67%. In the L1,000 and L100,000 groups, these rates were 75% and 83.33%, respectively, for the exposed snails. The control group had a 75% mortality rate (Table 1).

Table 1. Mortality rate of Bulimulus tenuissimus accompanied for 30 days after exposure to Euphorbia milii var. hislopii (L) latex and the synthetic herbicide Roundup® Original (R).

Hours of exposure (h)	Exposed groups				
	Control	L1,000	L100,000	R1,000	R100,000
24	-	-	-	-	1
48	-	-	-	1	-
72	-	-	-	-	-
96	-	-	1	1	2
168	2	3	4	3	2
336	-	2	2	3	3
504	3	1	2	1	2
720	4	3	1	2	1
Total no. of dead snails	9 (75%)	9 (75%)	10 (83.33%)	11 (91.67%)	11 (91.67%)
N- Survival percentage (%)	3- 25%	3- 25%	2- 16.63%	1- 8.33%	1- 8.33%

IV. DISCUSSION

Land mollusks can present physiological and behavioral strategies to ensure their survival under unfavorable conditions, such as estivation, retraction of the cephalopedal mass, and burial [26, 27], all of which can favor survival after exposure to molluscicides [28].

In this study, the first reaction to the toxic substances, observed after 24 hours of exposure, was increased release of mucus in all terrariums containing exposed mollusks. According to the literature, increased mucus production and secretion is one of the first reactions of gastropods to stress, among them chemical irritation caused by molluscicidal products [29]. Other strategies, such as cephalopedal mass retraction and epiphragm formation, were also observed in the present study after stress, corroborating the finding of D'ávila and collaborators, who examined *Bradybaena similaris* (Férussac, 1821), *Leptinaria unilamellata* (d'Orbigny, 1837) and *Subulina octona* (Brugüière, 1789) at a constant temperature of 35 °C for 48 hours and observed cephalopedal mass retraction after 12 h of exposure and the presence of epiphragm in four replicates of *B. similaris* with 24 hours of exposure [30].

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Exposure to toxic substances can alter the physiological homeostasis of an organism [31,32,33]. Silva et al. [34]., exposing *B. similaris* to the LC_{50} of the aqueous extract of *Solanum peniculatum* L. (Solanaceae) for 72 h, observed after 24 h an increase in the carbohydrate content of the digestive gland and the concentration of glucose in the hemolymph, and a significant reduction of carbohydrates in the cephalopedal mass. According to the authors, this profile demonstrates the existence of metabolic compensation for the regulation of homeostasis in the organism [34].

In the present study, the L1,000 group showed an increase in glycogen content in the digestive gland and cephalopedal mass, and a significant reduction of glucose concentration in the hemolymph. In the L100,000 group, there was a significant reduction of the glycogen content in the tissues and an increase in the glucose concentration in the hemolymph. In this respect, according to Pinheiro et al. [35], mollusks mainly use glycogen stores in the digestive gland and muscle tissue to maintain normoglycemia in the hemolymph. This explains our observation that the snails in group L100,000 had tendency to stabilize the glucose in the organism through gluconeogenesis to maintain homeostasis. In the L1,000 group, the energy metabolism was maintained through the consumption of free glucose in the hemolymph, causing accumulation of glycogen in the tissues, to guarantee the regulation of glycemia.

Unlike latex, the exposure to the herbicide Roundup® Original caused reductions in glucose concentration in the hemolymph and glycogen content in the digestive gland and cephalopedal mass of *B. tenuissimus*. Barky et al. [36], when exposing *Biomphalaria alexandrina* to the LC_{10} of the herbicides Atrazine (0.33 ppm) and Roundup® (0.84 ppm) for four weeks, observed an increase in glucose concentration in the hemolymph and reduction of glycogen content in the tissues of these animals, a different pattern than the present study.

LDH is an enzyme involved in the activation of anaerobic metabolism, and its measurement can be used to understand energy production by mollusks, which can occur either aerobically or anaerobically. Silva et al. found a significant increase in LDH activity in *B. similaris* after 24 h exposure to the LC_{50} of the aqueous extract of *S. paniculatum*. According to the authors, acceleration of anaerobic metabolism occurred, a possible consequence of intoxication [34]. In the current study, the results obtained show that the different concentrations of the molluscicide latex and herbicide did not significantly alter LDH activity, so that aerobic metabolism was maintained.

Faced with a deficiency of energy substrates first consumed in the body, mollusks can search for proteins as an alternative means of obtaining energy [37]. In this study, the total protein concentration in the exposed groups did not differ significantly from the control group, showing that it was not necessary to use this source for homeostatic maintenance.

Regarding the mortality of *B. tenuissimus* during 30 days of exposure, the first deaths occurred in 24 and 48 hours for the groups exposed to the herbicide. Mortality after exposure to the latex was slower, with the first death occurring after exposure for 96 h. Mortality was recorded until the end of 720 h at both concentrations. Afonso-Neto et al. [38], submitting a terrestrial gastropod to the latex of three Euphorbiaceae species, verified that *E. milii* var. *hislopii* was able to kill 100% of the specimens within 24 hours after exposure to the different dilutions, and highlighted the decrease of the latex effect revealed by 25% mortality after exposure to the concentration of 1: 1,000 ml/ml of the product.

The WHO determines that the concentrations used for natural molluscicides in aquatic mollusks are 20 ppm for plant extracts and 100 ppm for the raw plant) [39], and the literature does not find dosage recommendations for land mollusks species [40]. In this study, the concentrations used for the molluscicide exceeded the limit determined by the WHO. In our work, the concentrations of herbicides were equal or higher than those used by other authors, using the profile mollusk vs. herbicide [8, 9, 10, 36]. The specimens of *B. tenuissimus* was able quickly recovery of homeostasis.

The use of agricultural chemicals in plantations is common [41], in Brazil. The applications of these substances are justified by the fact that about 10% of the harvest is lost due to the presence of different agricultural pests in the cultivation system [42]. Because of this, the resistance or resilience to pesticides found in this work in *B. tenuissimus* can be explained in two ways: the first related to the habit of mollusks to feed on vegetations with pesticides and the other through the generation of phenotypes of resistance induced by selective pressure imposed by the frequent use of pesticides. The resilience and persistence of invertebrates is explained in the literature [43, 44] and according to Brigante et al. [45] are characteristics of biondicator species such as land mollusks.

V. CONCLUSION

In our study, *B. tenuissimus* showed resistance to high concentrations of pesticides. The snails were able to survive exposure to Roundup® Original at both concentrations used in this work, but the energy metabolism was altered. New studies on the behavioral, physiological and epigenetic effects of pesticides on these

land snails are already underway. These studies are necessary because these mollusks have wide geographical distribution, are considered agricultural pests and are intermediate hosts of *Angiostrongylus cantonensis*, etiological agent of eosinophilic meningoencephalitis.

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