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# The Effects of the Pre-Emergence Herbicides Chloramben, Atrazine, and Alachlor on Growth and Development of Leopard Frog, Rana Pipiens (Schreber), Larvae

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Groups of 20 Rana pipiens larvae were exposed to Atrazine, Chloramben, or Alachlor in a 96-hour, static bioassay. Threshold lethal concentrations of 8.5 ppm and 250 ppm were determined for Atrazine and Chloramben, respectively, for larvae at Taylor-Kollros Stage I. The threshold lethal concentration of Alachlor was 1.5 ppm for larvae at Taylor-Kollros Stage III.

Determinations of growth inhibition, group variability, and examinations for external abnormalities were made 40 days after the termination of the 96-hour exposures at the lethal thresholds of Atrazine and Chloramben.

Atrazine and Chloramben did not significantly inhibit growth under those conditions, but did induce a greater degree of variability in weights, lengths, and developmental stages of the larvae. The larvae exposed to Chloramben showed no physical abnormalities. Thirty-two percent of the Atrazine exposure group showed a skin abnormality resembling warts.

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# CHAPTER I

## INTRODUCTION

During the 1960's several workers suggested that pesticides may have contributed to the decline of the Common Frog, Rana temporaria, and the European Toad, Bufo bufo, in Britain (Cooke, 1972). Although these studies indicate that the decline was associated with high human populations, certain other demographic data indicated a relationship between pesticide usage and the amphibian population declines.

Gibbs, et al (1971) estimated that frog populations in the United States have declined 50% in the past decade. Nace, of the University of Michigan Amphibian Facility (personal correspondence), stated that there was clear evidence that frog populations were still declining in many parts of the country. Nace further indicated that pesticides, chemical fertilizers, and industrial run-off had all been blamed for the decline.

Betty Les, of the Wisconsin Department of Natural Resources (personal correspondence), indicated that Rana pipiens has undergone mass mortality in past autumns, and that they had undertaken a study to determine the cause. She stated that frog populations in Wisconsin had so declined that only one population of reproducing adults was found in

that state. Herbicides were among the suspected causes, and their study included a residue analysis of frog mortalities.

Pesticides, including herbicides, insecticides, and other pest control chemicals, have been known to affect many non-target species. Stickel (1968) noted that on numerous occasions non-target species have died in conspicuous numbers following pesticide treatments. She also stated that wildlife mortality had been a common result of pesticide usage, even though the recommended application procedures and rates were used.

Several workers have noted the specific effects of some pesticides on non-target species. These effects include tumors, teratologies, and mutations, and appear to be characteristic of certain classes of pesticides (Epstein and Legator, 1971; Hilton, 1974; Ho and Gibson, 1972; Koudela, 1972; Nishmura, 1973; Nose, 1972; Robens, 1969; Sen, 1957; Waldbott, 1973; Warner, et al, 1966; Weil, et al, 1972). Hilton (1974) listed most of the major herbicides and their effects on non-target species including; fish, rodents, and other laboratory test animals.

Sanders (1970) noted that amphibians were not usually affected by the concentrations of pesticides that kill fish and invertebrates. However, Cope (1966) said that amphibians were susceptible to some of the commonly used organic pesticides, and Mulla (1962) reported the use of insecticides in the control of frog and toad populations. Other workers have demonstrated the lethal effects of various pesticides on

amphibian larvae (Cooke, 1972; Cooke, 1973; Mulla, 1963; Pravda, 1973; Sanders, 1970; Wojcik, 1971).

Certain amphibian populations have been often exposed to applications of pesticides. Cooke (1972) noted those sites most likely to be contaminated with harmful levels of pesticide as: those bordering agricultural lands that had been recently treated; those in which empty pesticide containers had been recently dumped or rinsed; and those being treated for the control of mosquito larvae.

R. pipiens larvae may be exposed to direct applications, and run-off from pre-emergence herbicides, as the tadpole stage corresponds to the seasonal applications of these agents. There is a lack of information on the toxicities and sub-lethal effects of some of the major pre-emergence herbicides on amphibians.

For these reasons, this study examined the effects of three common pre-emergence herbicides. The lethal threshold, which was that concentration that killed one more organism in the exposure group than in the control group, was determined for each herbicide. The effects on growth and development were then examined at that concentration.

The three herbicides used in the study were; Atrazine (Aatrex 80W), a chlorinated triazine, Chloramben (Amiben Granular), an aminodichloro benzoic acid, and Alachlor (Lasso Liquid), a chlorinated acetanilide. Each herbicide represents a major class of herbicides, and together these groups accounted for over 95% of the herbicides used on the non-irrigated croplands of the Mid-west in 1971 (Reese, 1972).

There is only limited information on the effects of these herbicides on any wildlife, and no information on their effects on R. pipiens larvae (Hilton, 1974). Information on the toxicities and the threshold lethal effects of these herbicides may lead to a better understanding of the decline of this organism in all stages of development. Nace (1968) noted that post-metamorphic success was dependent upon the larvae obtaining maximum size, and this implied that a larval study was appropriate. Also, a study of growth and development might lead to a better understanding of the generalized effects of these chemical types of herbicides on non-target species.

## CHAPTER II

## MATERIALS AND METHODS

The Rana pipiens larvae used in the study were obtained from the University of Michigan Amphibian Facility, Ann Arbor, Michigan. They were at Shumway Stage 25, which is just past the onset of feeding, but prior to limb-bud development. The larvae were progeny of laboratory-reared adults, whose genetic background was known, and who exhibited no known growth anomalies. Previous pesticide exposure was limited by use of animals reared in the laboratory.

The larvae were shipped in groups of 200, and arrived at four-week intervals. Upon arrival the larvae were placed in 8" finger bowls that had been washed and rinsed twice in distilled water, as was all the hardware used in the study. Dead larvae were removed when the shipments arrived, and at every subsequent observation. The larvae were fed pulverized Glencoe trout food suspended in agar and unflavored gelatin (Appendix A).

The larvae were maintained in an artificial culture medium consisting of modified Marking and Dawson's solution (Appendix B). This was used in preference to Steinberg's Solution (Nace, 1974), because of a flocculence which developed when the pH of Steinberg's Solution was adjusted.

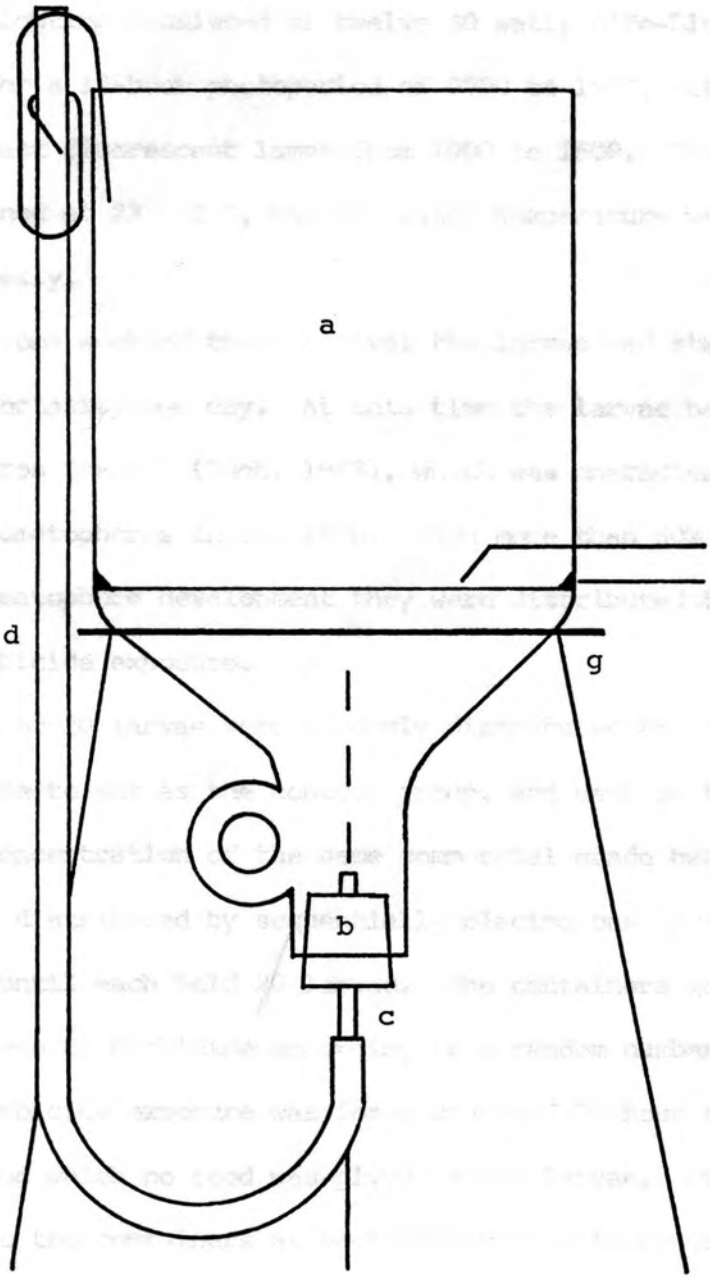
The larvae were acclimated to the culture medium by removal of 10% of their shipping water, and addition of an equal volume of culture medium at one hour intervals over a two day period. After acclimation, the larvae were transferred to the culture containers in which they were maintained.

The culture containers consisted of one gallon glass jugs with the bottoms removed. They were inverted and suspended from three-legged ring stands. A one-hole rubber stopper, with attached rubber tubing was placed in the neck of each container. This arrangement allowed the culture medium to be drained with a minimal disturbance of the larvae (Fig. 1).

Sections of stainless steel wire (26 gauge, 16 mesh) were fitted to the shoulders of the containers with paraffin collars. The mesh gave the larvae a substrate to rest upon, and allowed the wastes to settle through. The paraffin collar prevented the larvae from entering the neck of the container.

Two liters of culture medium were placed in each container, and the two liter level was marked on the outside wall of the glass. This level was restored with distilled water to compensate for evaporation when the larvae were observed twice daily. At each observation 400 ml was drawn from each container and 400 ml of fresh medium was added. Once each week the culture containers were drained and fresh medium was added to limit algal growth and to remove wastes.

Figure 1. Culture container consisting of: (a) one gallon jug with the bottom removed, (b) rubber stopper with (c) glass tubing inserted, (d) rubber tubing, (e) wire mesh with a (f) paraffin collar, and (g) supported by a three-legged ring stand.


a
b
c
d
e
f
g

The study was conducted in a Honeywell Model CEL 511-38 growth chamber. Lighting consisted of twelve 40 watt, Life-Line, fluorescent lamps set for a 12-hour photoperiod of 0700 to 1900, with an additional twelve 40 watt fluorescent lamps from 1000 to 1600. The air temperature was maintained at 23 $\pm$ 1 C, and the water temperature was 20 $\pm$ 1 C when measured weekly.

Within one week of their arrival the larvae had stabilized at less than 0.5% mortality per day. At this time the larvae had reached Taylor-Kollros Stage I (Rugh, 1963), which was characterized by development of chromatophores in the skin. When more than 50% of the larvae showed chromatophore development they were distributed to containers for the herbicide exposure.

Groups of 20 larvae were randomly distributed to each of five containers: one to act as the control group, and each of the others at a different concentration of the same commercial grade herbicide. The larvae were distributed by sequentially placing one in each of the five containers until each held 20 larvae. The containers were then assigned concentrations of herbicide according to a random numbers table.

The herbicide exposure was for a standard 96 hour test (Taras, 1971), during which no food was given to the larvae. Distilled water was added to the containers at each observation to replace evaporative loss. At that time any mortalities were noted and removed from the containers.

In the preliminary exposures the concentrations of herbicide used were 90, 75, 50, and 25% of the maximum solubility (Hilton, 1974) of each active ingredient in water. If those tests did not yield the threshold lethal concentration, gradations of the appropriate range were made until that value was obtained.

When the threshold lethal concentration had been determined, groups of 20 unexposed larvae were distributed to containers; one to act as the control group, and one for the threshold lethal concentration of each herbicide. A 96 hour exposure was made, and at the end of that time the larvae were removed to new containers with fresh medium. After 24 hours that medium was drained and replaced with fresh medium to remove any herbicide residues.

The larvae were maintained for 40 days after the termination of exposure. According to Rugh (1963) this should have allowed for a five-fold increase in length, and the passage of several Taylor-Kollros Stages involving observeable limb-bud development.

After 40 days the larvae were killed with 5% formalin, and allowed to harden one day in that solution. The larvae were then individually weighed to the nearest milligram on a Torbal balance, and measured to the nearest millimeter on a ruler. The larvae were examined under a dissecting scope to determine the Taylor-Kollros Stage, and to note any external anomalies.

Mean lengths and weights were compared to the control group. The median Taylor-Kollros Stage of each group was used to determine

developmental retardation, and a Chi-square analysis applied to the distribution of the staging to determine variability.

## CHAPTER III

## RESULTS AND DISCUSSION

The lowest concentrations of active ingredient which caused 100% mortality were: 370 ppm of Chloramben, 16 ppm of Atrazine, and 5 ppm of Alachlor. All of the herbicides tested were 100% lethal at concentrations below their maximum solubility in water. The threshold lethal concentration for larvae at Taylor-Kollros Stage I in Chloramben was 250 $\pm$ 12 ppm, and in Atrazine it was 8.5 $\pm$ 0.5 ppm. Difficulties in coordinating the availability of Alachlor with the larvae at the proper stage made it impossible to determine the lethal threshold at Stage I. A threshold lethal concentration of 1.5 $\pm$ 0.75 ppm was determined for larvae at stage III. Due to the late stage of development at that point, determinations of growth and developmental inhibition were not made for Alachlor.

The groups exposed to Chloramben and Atrazine appeared to be growth inhibited seven days after the terminations of the exposure. That inhibition became most pronounced ten days after the termination of the exposure, after which the groups appeared to resume growth at a more normal rate.

The larvae were weighed and measured forty days after the termination of the exposures, and these data were used to determine growth

inhibition. The mean lengths and weights are smaller for the exposure groups than for the control group (Table 1). However, the differences were not significant at the 95% confidence level.

The plot of length vs. weight was made for each group to illustrate variability in the relation of these parameters. The control group showed a linear relationship (Fig. 2). The Chloramben and Atrazine exposure groups showed a curvilinear relationship (Figs. 3 and 4).

Table 1. Mean weights and lengths of larvae in two exposure groups and one control group with standard deviations of the means

| Group | Mean weight (g) | $S_d$ | Mean length (cm) | $S_d$ |
|---|---|---|---|---|
| Control | .645 ± .373 | .179 | 3.62 ± .751 | .360 |
| 8.5 ppm Atrazine | .544 ± .410 | .284 | 3.31 ± 1.08 | .518 |
| 250 ppm Chloramben | .549 ± .467 | .223 | 3.31 ± 1.03 | .493 |

A one-way analysis of variance showed that there was greater variability in the relation of the weights and lengths of both exposure groups than in the control group (Table 1). This indicated that although the herbicides did not significantly inhibit the growth of the larvae under the test conditions they did induce a greater degree of variability in growth patterns.

Figure 2. Length-weight relationship of the control group of tadpoles after a 40 day study period.

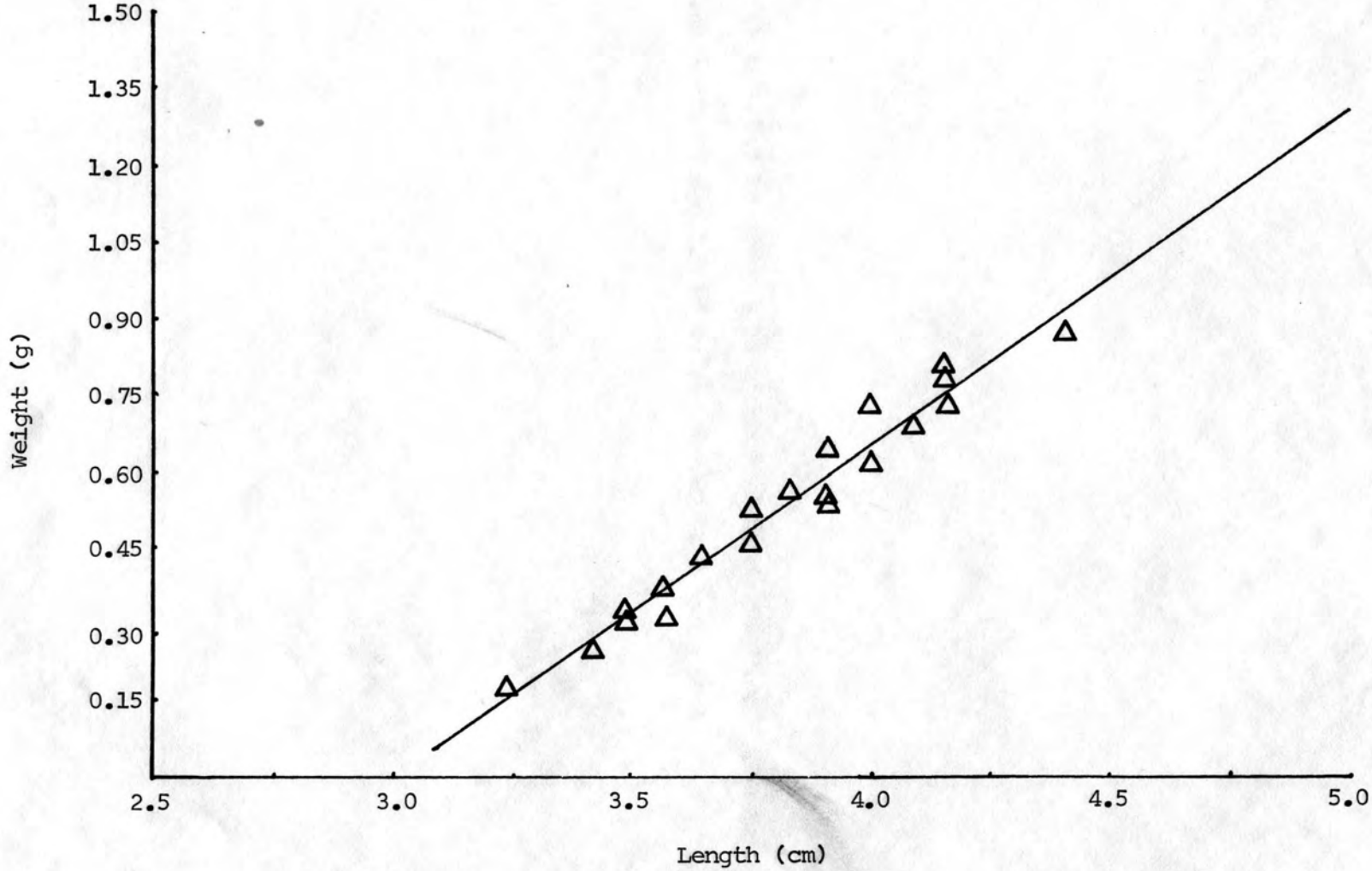

1.50
1.35
1.20
1.05
0.90
0.75
0.60
0.45
0.30
0.15
Weight (g)
2.5
3.0
3.5
4.0
4.5
5.0
Length (cm)

Figure 3. Length-weight relationship of a group of tadpoles 40 days after a 96-hour exposure to 8.5 ppm of Atrazine.

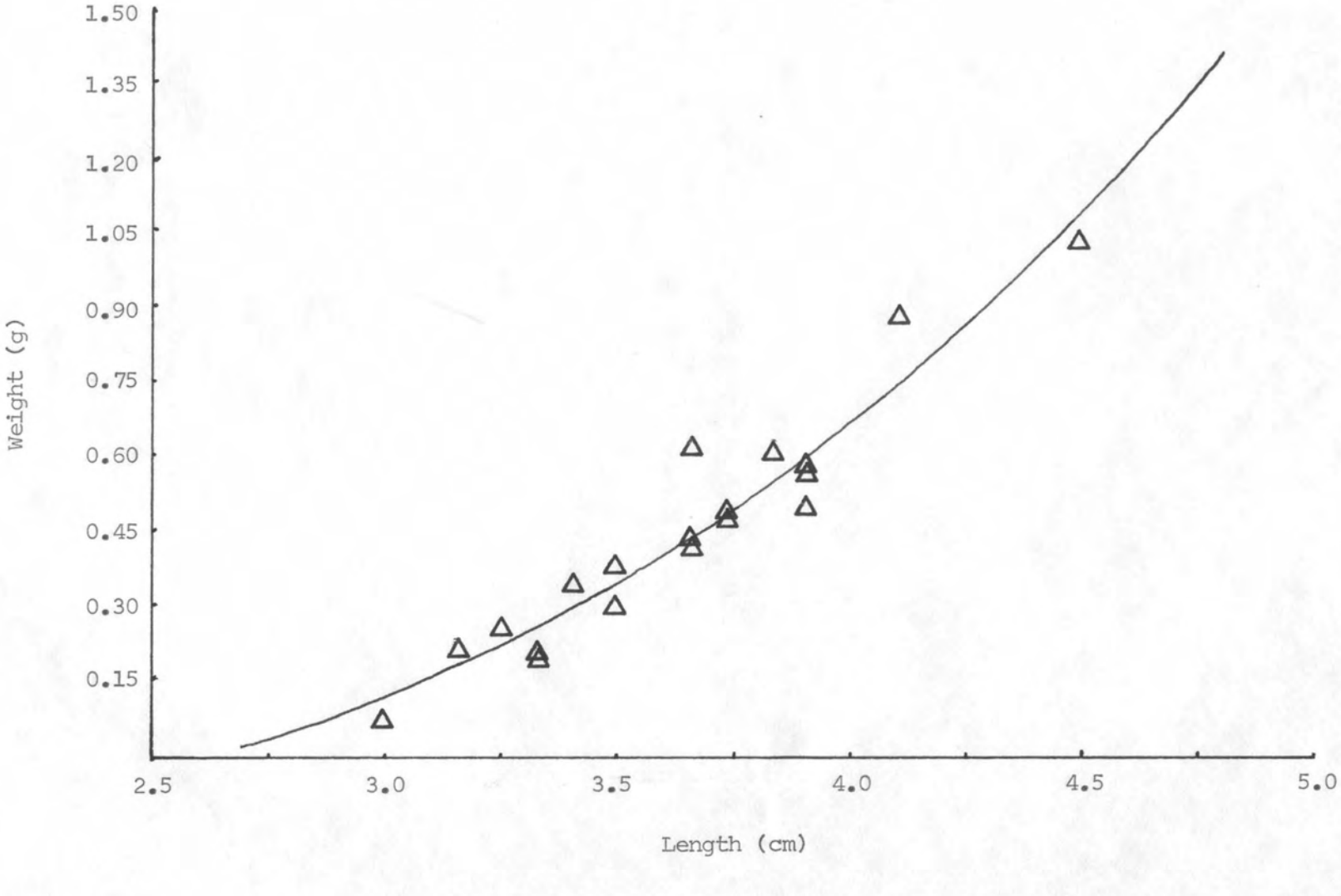
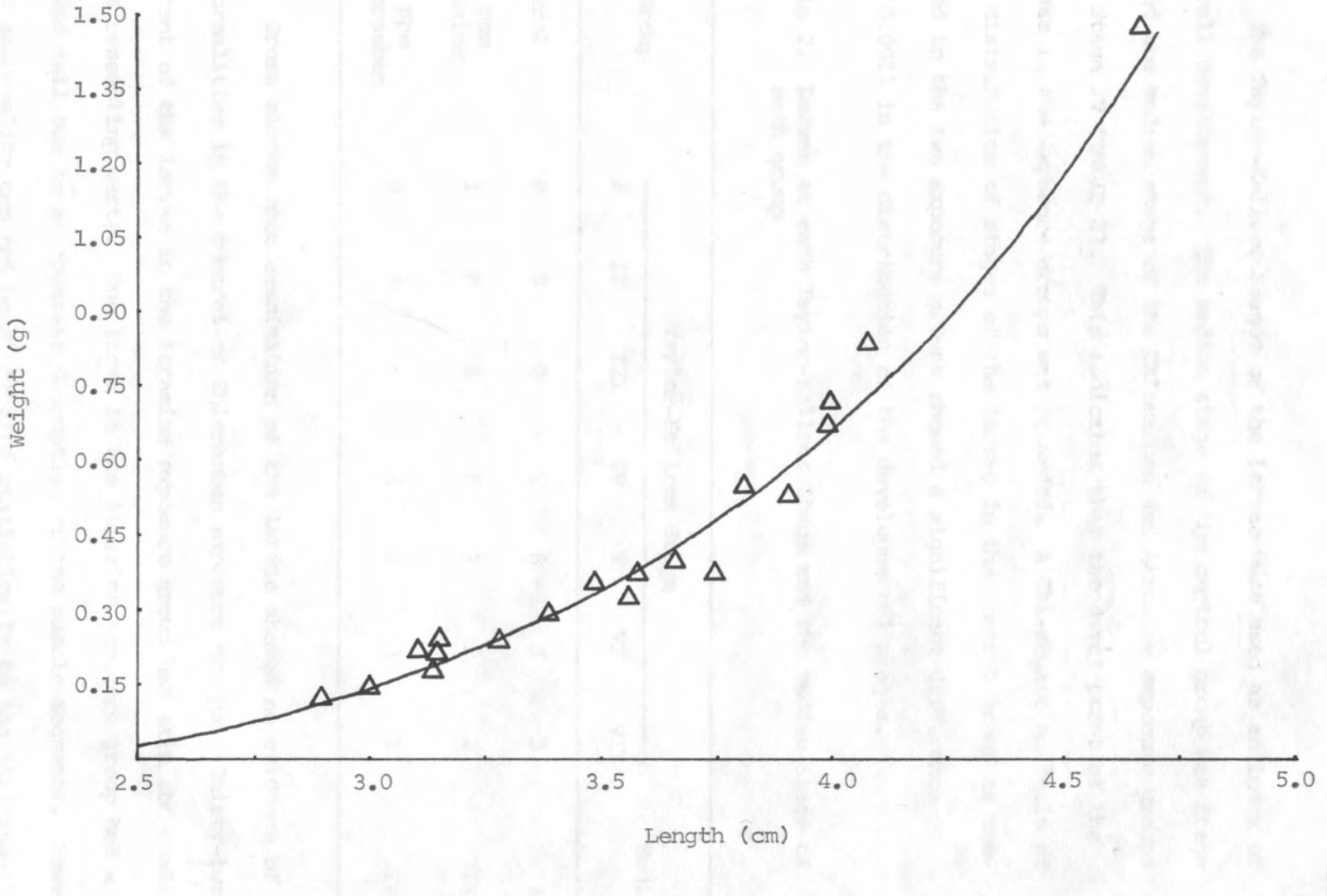

1.50
1.35
1.20
1.05
0.90
0.75
0.60
0.45
0.30
0.15
Weight (g)
2.5
3.0
3.5
4.0
4.5
5.0
Length (cm)

Figure 4. Length-weight relationship of a group of tadpoles 40 days after a 96-hour exposure to 250 ppm of Chloramben.


1.50
1.35
1.20
1.05
0.90
0.75
0.60
0.45
0.30
0.15
Weight (g)
2.5
3.0
3.5
4.0
4.5
5.0
Length (cm)

The Taylor-Kollros Stages of the larvae were used as an index of overall development. The median stage of the control group was Stage V and the median stage of the Chloramben and Atrazine exposure groups was Stage IV (Table 2). This indicates that the development of the larvae in the exposure groups was retarded. A Chi-square analysis of the distribution of stages of the larvae in the control group as compared to the two exposure groups showed a significant difference ($P < 0.001$) in the distribution of the developmental stages.

Table 2. Larvae at each Taylor-Kollros Stage and the median Stage of each group

| Group | Taylor-Kollros Stage | | | | | | | Median |
|---|---|---|---|---|---|---|---|---|
| | I | II | III | IV | V | VI | VII | |
| Control | 0 | 0 | 0 | 6 | 8 | 3 | 3 | V |
| 8.5 ppm Atrazine | 1 | 0 | 5 | 8 | 2 | 2 | 2 | IV |
| 250 ppm Chloramben | 0 | 3 | 5 | 4 | 3 | 3 | 1 | IV |

Gross microscopic examination of the larvae showed no evidence of abnormalities in the control or Chloramben exposure groups. Thirty-two percent of the larvae in the Atrazine exposure group had skin abnormalities resembling warts. One larva in the Atrazine exposure group had a kinked tail due to an apparent disruption of the muscle segments. However this abnormality can not be attributed statistically to the Atrazine exposure.

# CHAPTER IV

## SUMMARY

The threshold lethal concentrations of Chloramben and Atrazine for Rana pipiens larvae at Taylor-Kollros Stage I are 250 and 8.5 ppm respectively. The lethal threshold for larvae at Stage III in Alachlor was 1.5 ppm.

Larval growth was not significantly inhibited forty days after a 96 hour exposure to either Chloramben or Atrazine, but developmental variability was introduced by these agents.

Chloramben did not cause any anomalies in the larvae, but the Atrazine group exhibited a skin condition resembling warts in thirty-two percent of the larvae. One larva in the Atrazine group had a kinked tail but this cannot be statistically attributed to the Atrazine.

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# APPENDIXES

# APPENDIX A

## LARVAL FROG FOOD

## FROM HIRSHFELD, RICHARDS AND NACE, 1970

250 g. Glencoe trout food

20 g. granular agar

14 g. unflavored gelatin

1 l. distilled water

The agar and gelatin were dissolved in the distilled water. The trout food was pulverized and slowly mixed into the agar solution. When the trout food had been completely mixed it was allowed to boil one minute before being poured into petri dishes to a depth of one cm.

The food was stored in a freezer. When needed, it was thawed and cut into cubes of less than one cm.