EFFECTS OF SELECTED CONCENTRATIONS OF DIETHYLSTILBESTROL UPON THE GUPPY, *LEBISTES RETICULATUS* (PETERS)

By

ROBERT JOE BOLES

Bachelor of Science
Southwestern College
Winfield, Kansas
1938

Master of Science
Kansas State University
Manhattan, Kansas
1949

Submitted to the Faculty of the Graduate School of
The Oklahoma State University
in partial fulfillment of the requirements
for the degree of
DOCTOR OF PHILOSOPHY
May, 1960

EFFECTS OF SELECTED CONCENTRATIONS OF
DIETHYLSTILBESTROL UPON THE GUPPY,
LEBISTES RETICULATUS (PETERS)

Thesis Approved:

W. H. Irwin
Thesis Adviser

Geo. A. Moore

Roy W. Jones

H. J. Featherly

DeHomell

Robert Kamm
Dean of the Graduate School

PREFACE

The tendency for fishes to overpopulate bodies of water, especially in small impoundments, is one of the major problems in fishery management. The great increase in the number of individuals results in the fish population becoming stunted and undesirable for food or sport. This paper discusses laboratory experiments conducted to determine the effects of selected concentrations of the synthetic estrogenic hormone, diethylstilbestrol, upon the guppy, and the possible use of the hormone to control fish populations in small impoundments.

I wish to thank my adviser, Professor W. H. Irwin, who supervised and directed the research. I am also indebted to committee members Professors Roy W. Jones, George A. Moore, Henry I. Featherly, and Dariel E. Howell for their suggestions.

Dr. Stanley Wearden, Department of Statistics, Kansas State University, made the statistical analysis of the data.

Space and equipment used in the study were provided by the Zoology Department of Oklahoma State University.

S. B. Pennick and Company and Eli Lilly and Company generously furnished the diethylstilbestrol used in the tests.

Finally, I wish to thank my wife, Louise, for without her assistance and encouragement the problem would not have been undertaken.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

CHAPTER I

INTRODUCTION

The data presented herein concern the effects of selected concentrations of the synthetic hormone, diethylstilbestrol, upon the growth, behavior, and reproduction of the guppy, Lebistes reticulatus (Peters). The toxic effects which were observed are also reported. The observations were restricted to the external effects. The cytological and histological changes resulting from treatment with androgenic and estrogenic substances have been rather extensively explored by Berkowitz (1938, 1941), Eversole (1939, 1941), Cohen (1946), and others.

The writer chose this particular problem because he felt that it might show, or at least suggest, some possible uses of diethylstilbestrol as a fishery management tool, especially in small impoundments. Impoundments, particularly small ones, tend to develop large populations of the fishes which have gained entrance into them, and create a food demand that prevents full and rapid growth of the individuals. This results in great numbers of fish too small to be of interest either as food or sport. Any factor which would reduce the number of fish present in the impoundment would increase the amount of food available to those that remained. The writer was interested (1) in finding whether diethylstilbestrol would have an effect upon the reproduction without killing an excessive number of the fish treated, and (2) at what concentrations reproduction could be suppressed with a minimum of toxic effects.

Restrictions placed upon the use of diethylstilbestrol as a food additive, due to its carcinogenic effect, will definitely limit the use of the chemical except for experimental purposes. The restrictions were applied by the Food and Drug Administration, under the limitations placed upon the use of cancer-producing substances by the so-called Delaney clause. These restrictions were announced after the study reported in this paper had been completed.

The following terms as used in the manuscript are defined.

1. Hormone is used to designate the chemical, diethylstilbestrol. The American Illustrated Medical Dictionary defines "hormone" as "a chemical substance which is produced in some organ and then when transported to some other organ produces there a specific effect". This definition provides that the substance must be produced within some organ of the body. Eli Lilly and Company, producers of diethylstilbestrol, have adopted the following terminology concerning this chemical: "a synthetic organic chemical which has hormonal activity". The writer has followed the common usage of the term "hormone" as applied to diethylstilbestrol in the literature. The terms diethylstilbestrol and stilbestrol are used synonymously.

2. Parts per million of diethylstilbestrol is written as ppm. Under the discussion of the toxic effects of the chemical when mixed with aquarium water, ppm refers to the concentration by weight of diethylstilbestrol in the water. Throughout the remainder of the paper, ppm is used to refer to the concentration by weight of diethylstilbestrol in the food.

The guppy was chosen as the experimental fish because, first, it is small, and considerable numbers could be used in each test aquarium; second, the fish displays conspicuous differences between the sexes; third,

it readily continues breeding behavior and reproduction throughout the year under laboratory conditions; and fourth, large numbers of fish of the various ages and sizes to be used in testing could be raised and kept available in brood tanks in the laboratory.

The study was started in September, 1958, and continued until September, 1959. A National Science Foundation Graduate Assistant Grant enabled the writer to devote full time to the problem during June and July, 1959.

CHAPTER II

REVIEW OF THE LITERATURE

Diethylstilbestrol, or 4, 4-dihydroxy-α-β-diethylstilbene, was discovered by Dodds in 1938. Clinically, it acts like natural estrogens, but has some unpleasant side effects. It is not a steroid, and is easily and cheaply prepared from common chemical agents. (Neverla, 1944).

The hormone has been shown to have a definite effect upon reproductive processes. Adams (1946) showed that the administration of diethylstilbestrol to the salamander, Triturus viridescens, caused atrophy of the testes and Wolffian ducts in the male, and hypertrophy of the oviducts in the female. Black and Booth (1946) and Moreng, et al. (1956) found that the hormone caused decreased testes size in chickens. Westermarch and Aaltonen (1950) found it suppressed libido and secondary sexual characteristics such as the size of comb and wattles. Bouvier, et al. (1949) secured the same results while working with White Leghorn cockerels. Fraps, et al. (1956) found that diethylstilbestrol delayed the onset of semen production in male chickens, and caused a reduction in volume when semen was produced. Higher dosages were required to impair hatchability of the eggs. Semen production was apparently more adversely affected than was egg production.

Sharof and Hafez (1958) reported that stilbestrol prevented pregnancy in rabbits when it was administered at any time from mating up to six days later, and pregnancy was interrupted when stilbestrol was given to does

20 days after coitus.

Mulligan (1947) showed that the testes, penile sheath and mammary glands of male dogs apparently returned to normal following cessation of orally administered stilbestrol.

Roberts (1950) reported that mink, fed chicken heads in which diethylstilbestrol pellets had been inserted, bred willingly but did not produce kits.

Peterson and Boyd (1944) mentioned that a number of sterile heifers and non-breeder cows were brought into satisfactory milk production by the use of diethylstilbestrol, but that others did not respond to the hormone. They also stated that the hormone caused a growth of the udders in bulls and steers, and that diethylstilbestrol produced heat with ovulation in cows. However, spontaneous estrus and ovulation usually occurred in one or two weeks following the cessation of treatment.

Green (1958) discussed the production of gynecomastia in a 3-year old boy and pseudoprecocious puberty in his 23-month old sister upon exposure to diethylstilbestrol pellets used in "caponizing" poultry. He stated that the mother also developed an abnormal menstrual pattern from contact during washing with the hormone-spattered clothes. Dunn (1941) said that stilbestrol induced testicular degeneration in hypersexual human males, with a consequent loss of sexual interest. However, behavior problems returned following the discontinuance of treatment.

Diethylstilbestrol has also been claimed to have a measurable effect upon growth. Black and Booth (1946), Westermarch and Aaltonen (1950), and Kelly and Roberts (1950) all reported that treated chickens outgained their controls. Fraps, et al. (1956) stated that in battery caged New Hampshire chickens the weight was not affected appreciably at any dosage.

Lorenz (1945) found that diethylstilbestrol caused a marked increase in fat content of treated chickens.

Perry, et al. (1951) said that hormone-treated lambs gained faster than non-treated lambs in the same field.

Cohen (1946) found that alpha estradiol benzoate suppressed spermatogenesis in genetic, immature male platyfish, Platypoecilus maculatus. Young males treated 8 to 12 weeks showed ova in the ovotestes; males treated 20 weeks showed no ova, suggesting a decrease of the inhibiting effects of the estrogen. Hamon (1946) reported estradiol benzoate caused the appearance of female characteristics in the male, and hastened it in the female of Gambusia holbrooki. Testosterone propionate inhibited growth in the young of both sexes, and caused the appearance of male sexual characteristics in the female. Hildemann (1954) stated that female sexual characteristics may be developed in sexually undifferentiated guppies, but not in mature males, by injecting or feeding estrogens. Scott (1944) claimed that all estrogen-treated guppies of either sex developed normal female-type skeletons, and all pregneninolone-treated animals developed normal male-type skeletal structures.

Berkowitz (1938) found that young male guppies injected with various estrogens (estrone and estriol) grew in length to exceed normal males, but he was unable to significantly modify the secondary sexual characteristics or libido in adult males. Hildemann (1954) also says that female sexual characteristics may be developed in sexually undifferentiated fish, but not in mature males by feeding or injecting estrogens. However, Berkowitz (1941) stated that estrogen feeding checked the color development and caused the fading of colors present after three months of treatment.

The writer was unable to find any studies concerning the comparable effects of different concentrations of diethylstilbestrol upon the guppy.

CHAPTER III

MATERIALS AND METHODS

The fish used for the experiments were guppies, Lebistes reticulatus. The fish were from brood stock kept in the Aquatic Biology Laboratory at Oklahoma State University, and were thoroughly adjusted to laboratory conditions.

The adult brood fish were confined within rectangular nets suspended in fifty-gallon tanks (Fig. 1). The nets were constructed of nylon "fish net". The hexagonal spaces of the net are approximately two millimeters in diameter. The spaces permitted the new-born fish to escape from the adults into the water surrounding the net soon after being born.

The young fish were dipped out each day, and placed in fifty-gallon tanks containing tap water that had first been allowed to stand for several days for temperature adjustment and the elimination of chlorine. In this way it was possible to produce, and keep, considerable numbers of fish of different known ages available for testing purposes. The water containing the fish was maintained at 80 $\pm$ 5° F. Over 3000 fish were used in the course of the different tests.

During the experiments, the animals were kept in aquaria measuring 36 centimeters long, 21 centimeters wide, and 24 centimeters deep. The aquaria were filled to a depth of about 20 centimeters, giving a volume of approximately 15,000 cubic centimeters of water per tank. A small amount of air from a large, centrally located compressor was supplied to

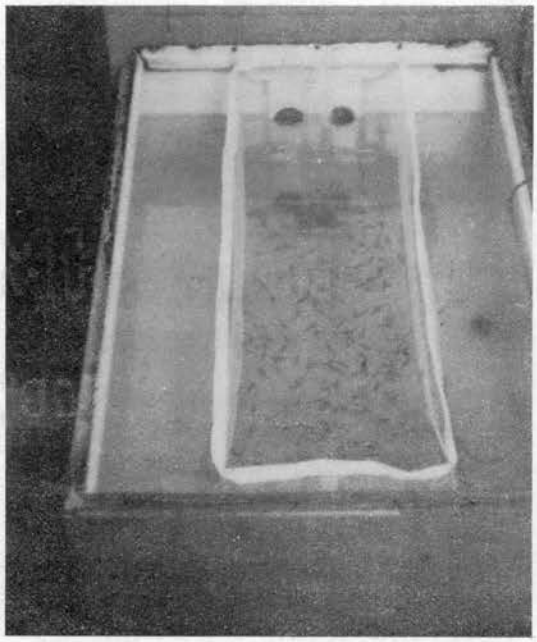


Figure 1. Brood tanks in which fish were raised for test purposes. Adult fish were confined within the nets. New-born fish escaped through the net-openings into the water outside, from which they were removed to separate holding tanks.

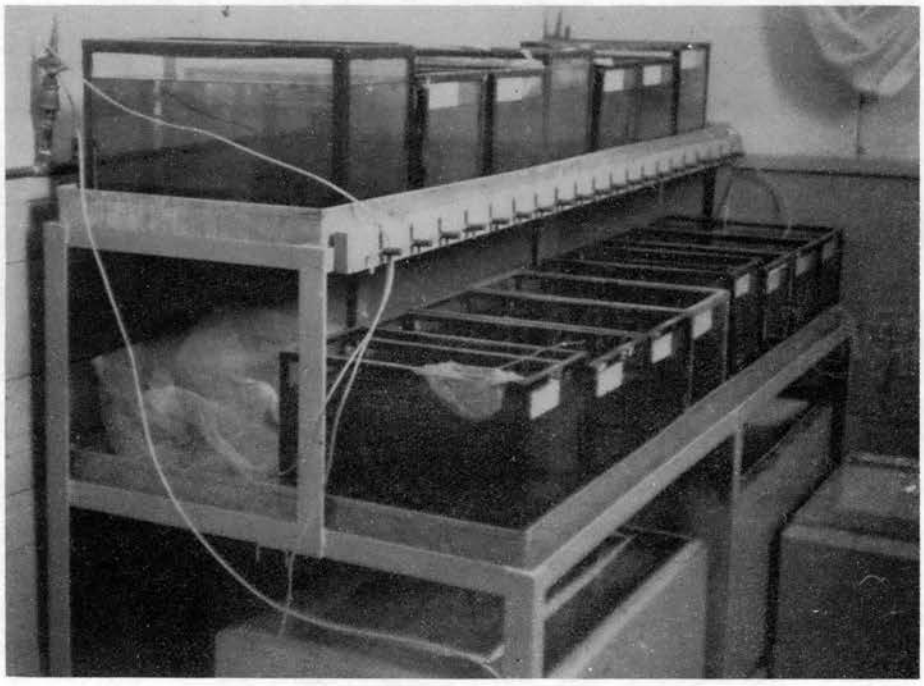


Figure 2. Aquaria in which fish were kept while observing their reactions to the concentrations of diethylstilbestrol used in the various tests.

each aquarium through breaker stones. For most of the tests each experimental group and its control was started with fifty fish per aquarium.

Aquaria used in the weighing experiments were washed and cleaned each week. Tap water, which had been allowed to stand in open fifty-gallon containers for several days to become "conditioned" was used for filling the aquaria after cleaning. The period of time between cleaning the aquaria used for testing behavioral and reproductive responses was sometimes longer than a week.

Five snails each of three different species /Heliosoma trivolvis (Say), Pomacea paludosa (Say), Melanopsis sp./ were kept in each of the aquaria to help consume any uneaten food that remained in the water after each feeding. The snails furnished some idea of the effect, if any, the hormone had on mollusks. The only plant used in the aquaria was Elodea. Some of this plant was placed in each of the tanks to furnish new-born young a place of escape from the adult fish.

The fish were fed daily with a food prepared from commercial chicken pellets and dried, powdered eggs. These two ingredients were ground and thoroughly mixed by running them several times through an electric coffee grinder, using the finest ("drip-grind") adjustment (Fig. 3). Extensive feeding experiments carried on previously by Dr. W. H. Irwin of Oklahoma State University (personal communication) had shown this to be a highly satisfactory food for producing and maintaining rapid and vigorous growth in the guppy.

Various methods of administering hormones to fish have been tried. Injection was used by Berkowitz (1937) and Eversole (1941). The administration of the powdered hormone to the water or to the food was shown to be quite effective (Berkowitz, 1938; Tavolga, 1949; Hildemann, 1954).

The injection method was rejected in these tests, because the time and expense involved would nullify any values it might have in fishery management. The powdered diethylstilbestrol was given to the experimental animals by adding it to the finely ground food and shaking the mixture vigorously. A stock mixture of one gram of diethylstilbestrol to 1000 grams of fish food was prepared, from which the concentrations desired for use in the various experiments could be prepared.

Because of the number of aquaria used and the time element involved, it was necessary to devise some method of measuring the quantity of food to be given at a feeding, other than weighing the food for the fish in each individual aquarium. Dippers were made by brazing heavy pieces of brass wire to .22 calibre brass cartridge cases (Fig. 5). Such dippers assured a quite uniform rate of feeding for each aquarium. The drawing of the cartridge feed cup through the food at each feeding also helped to provide a thorough and continuous mixing of the food and hormone. Separate dippers were made and used for each food jar containing a different concentration of the hormone.

There was no way to be sure that each fish consumed the same amount of food and hormone. Tavolga (1948) has shown that, when the water from an aquarium in which an androgenic hormone of relatively low solubility had been fed is circulated through a filter into another aquarium, the same reaction was produced on the fish in the second aquarium as in the aquarium to which the hormone had been originally added.

As a check to see if diethylstilbestrol also went into sufficient solution to produce reproductive and behavioral changes, the following experiment was set up. Fifty young, sexually mature, adult guppies were placed in a net 28 centimeters long and 15 centimeters wide (Fig. 4).

Figure 3. Coffee grinder used to grind chicken lay pellets into fine particles. Powdered egg was then run through the grinder with the granules to assure a uniform mixing of the ingredients used in making the fish food.

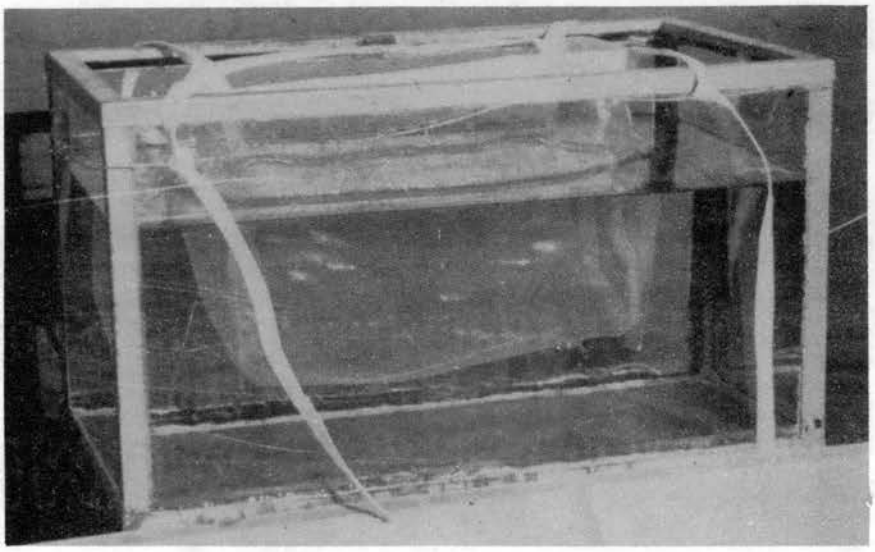


Figure 4. Arrangement of net for confining fish when testing to see the results of adding small amounts of diethylstilbestrol daily to the water. The chemical, mixed with fine sand, was placed in the water outside of the nets.

The net was suspended in the water of one of the experimental aquaria to a depth of 15 centimeters. Diethylstilbestrol was then mixed with fine sand, and added to the water outside the net each day in small amounts. The fish inside the net were fed regular, untreated food. These fish displayed the same effects as those fed treated food, showing that dissolution was in an amount great enough to produce an effect upon the animals.

Control tanks containing fish of the same age group as the experimental animals were used simultaneously with each experiment. These fish were fed at the same rate as the experimental fish, using food from the same "batch", except that the hormone was omitted.

The fish were fed the same amount every day for the duration of each experiment. Any fish that died were promptly removed upon discovery.

The fish were observed several times a day in order to note and record visible changes in sexual behaviors and secondary sexual characteristics.

Young fish, new-born to one week old, were used to study the effects of different concentrations upon the rate of growth. Replicates were used for each concentration tested. Control tanks were also duplicated.

The fish were weighed at the start of the testing, and each week thereafter for a period of seven weeks. The fish of each aquarium were weighed together, and the average weight determined at each weighing. Weekly weighings of the fish were discontinued after seven weeks, as the controls and the fish treated at the lower concentrations started to show the size differentiation characteristic of the two sexes.

The method of weighing described below was based upon a procedure developed by Dr. W. H. Irwin of the Oklahoma State University Zoology

Staff during his feeding experiments. This method permitted quite accurate weighing of even new-born to one-week-old fish.

A small, basket-like container was made from fine-mesh copper screen (Fig. 6). A bail was attached so that the weighing basket could be suspended from one arm of a Christian Becker "Chainomatic" balance. This wire basket was "tared" each time by weighing it three separate times and computing an average, following the same procedure of wetting and drying that would be used in the actual weighing.

The fish to be weighed in each case were carefully poured into the wire container. A damp cellulose sponge was pressed quickly but firmly over the outside of the basket to absorb any excess water clinging to the wire or fish. The basket was then suspended from the scale balance by the bail, and the scale balanced. No attempt was made to read the weight registered on the scale until the fish had been returned to the water. The weight was then recorded. No fish were observed to be lost as a result of the weighing procedure. The fish were counted each time they were weighed so that the average weight for that test group could be calculated (Appendix D).

Weight records were also made of some of the fish that had been treated, and later returned to regular food for a period of time. Lastly, the fish were compared with the controls to see what effect the returning of treated fish to a regular diet might have, if any, upon the growth.

In order to check the effect upon reproduction a careful examination of each aquarium, both test and control, was made from one to several times a day for the presence of new-born young. Care was taken during the changing of water to make sure no small fish were overlooked.

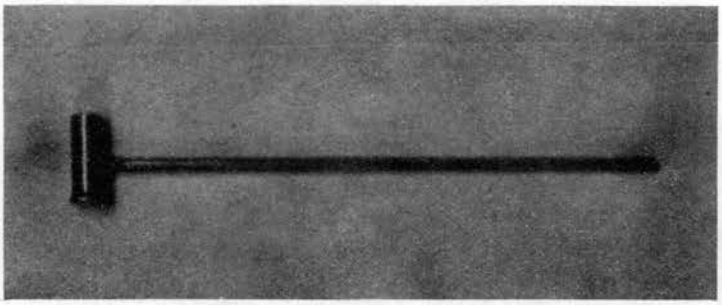


Figure 5. Dipper made from .22 calibre rifle shell, used to facilitate the feeding of relatively uniform amounts of the diethylstilbestrol-containing mixtures to the fish in the test aquaria.

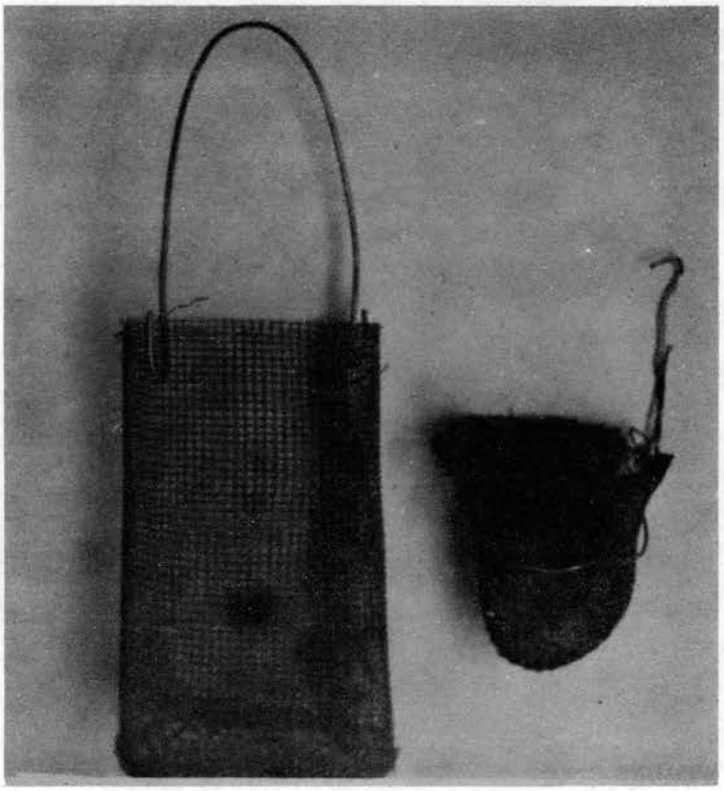


Figure 6. Baskets of fine-mesh screen used in weighing the fish being tested. The very small individuals were weighed in the small basket to the right, adult fish in the larger basket to the left.

CHAPTER IV

TOXIC EFFECTS OF DIETHYLSTILBESTROL WHEN MIXED WITH WATER

The writer was unable to find a discussion of the comparative toxic effects of different concentrations of diethylstilbestrol upon fish. Berkowitz (1941) used varying dosages and durations of estrogens, including stilbestrol, on the guppy in an attempt to obtain a more complete and more consistent sex reversal in the gonad than had previously been obtained. Berkowitz administered the stilbestrol by injection. Fraps, et al. (1956) found that multiple implants of diethylstilbestrol tablets in nine-weeks old chickens produced results which were not in proportion to the dosage levels.

In the current study, tests were run to determine what toxic reactions would be experienced by the guppy when it was exposed for a short time to high concentrations of the hormone added to the water. Lower concentrations were used in later, more prolonged, tests for determining the effects upon behavior, reproduction, and growth.

One gram of finely powdered diethylstilbestrol was added to 1000 cubic centimeters of distilled water to make a stock solution. The mixture was then shaken vigorously, and enough added to the different aquaria to produce concentrations of approximately 1, 5, and 10 parts per million in the water. Two aquaria were prepared with each of the concentrations. Because diethylstilbestrol is relatively insoluble in water, the actual concentration in the water of each aquarium was undoubtedly considerably

less than the 1, 5, and 10 ppm mentioned above. These concentrations produced severe toxic reactions within a short time, and they were used as the upper limits to which the fish were exposed during the following tests.

One hundred fifty fish were placed in each aquarium. The fish consisted of two age groups. Guppies, new-born to two weeks old, were placed in each of the concentrations to check the effects upon young fish. The adult fish used consisted of young, sexually mature individuals.

Neither young nor adult fish showed adverse reactions when first introduced into the three concentrations. They fed readily, and in the aquaria containing the adult fish, sexual behavior appeared to be no different than for the fish in the control aquaria.

Adult guppies react sooner and more violently to the presence of the hormone than do young fish. No deaths were noted among the young fish the first three days in the 1-ppm concentration, and only a few among the adults. However, 50 of the adults in the 5-ppm, and 51 in the 10-ppm concentrations were dead by the third day. Only one young fish died in the 5-ppm concentration in the first three days, and only seven in the 10-ppm concentration.

Snails (Heliosoma trivolvis) died over-night in the 5- and 10-ppm concentrations. Snails of the same species replaced in this water 10 days later were not killed, suggesting that there had been considerable decomposition of the chemical during that length of time.

Fifty fish were removed from each of the aquaria after three days exposure to the different concentrations and placed in aquaria containing fresh, conditioned water. The fish were observed for after-effects resulting from exposure to the different concentrations of the hormone.

Of the young fish left in the hormone-containing water, only three were alive in the 10-ppm concentration, and two in the 5-ppm concentration, after a period of 10 days. There was very little difference between the lethal effect upon young and adults, as only a few more adults than young remained alive in either of the two higher concentrations at the close of the 10-day period.

Several of the adult fish from the 5-ppm concentration had developed large hemorrhage spots on their backs and sides by the tenth day (seven days after removal from the hormone-containing water). Hemorrhaging appeared in some individuals as long as 20 days after exposure to the chemical. Death soon followed the appearance of the hemorrhagic condition. No hemorrhages developed in the remaining fish after 20 days.

A few of the adult fish developed a wild, "cork-screw" swimming motion, during which condition they would progress spirally through the water (much like the motion of a swimming Paramecium). These fish would then sink to the bottom of the aquaria for a time, lie quietly for a few seconds, and then again spiral wildly through the water. Death usually followed within a period of 24 hours.

Of the fish left in the 1-ppm concentration, many of those surviving had become thin and emaciated after 20 days exposure in spite of generous daily feedings of hormone-free food. The fish which were most emaciated rested on the bottom, apparently unable to maintain themselves above the bottom of the aquarium. Movements consisted of spasmodic "swimming-in-place", during which the fish neither rose nor progressed in the water. Even among the less severely affected fish, swimming was erratic.

The fish varied widely in their response to the hormone. While some reacted violently and exhibited one or more of the toxic effects

mentioned above, others in the same concentration showed little to no adverse effects. Also, fish exposed for extended periods to a lighter dosage usually developed the same type of severe reactions shown by those fish exposed to a heavier dosage for a shorter time. The variation in response of the guppies described above agrees with the findings of Adams (1946) concerning the effect of diethylstilbestrol on the newt, Triturus viridescens.

Witschi and Crown (1937), using the androgenic hormone testosterone propionate in water with the swordtail, Xiphophorus helleri, found it caused abortion or resorption of the young within one to two days, and that all large eggs were resorbed in adult females. New-born fish were found in the aquaria containing both the 1- and 5-ppm concentrations 24 hours after the adult fish were placed in the water in tests conducted with diethylstilbestrol. The young appeared to be fully-developed, healthy individuals. No more young were found in any of the aquaria containing the different concentrations used in testing, even though many of the females were apparently pregnant when introduced into the hormone-containing water. The writer was unable to find evidence that abortion had taken place. However, the visible signs of pregnancy (distended abdomen, a dark posterio-lateral area or gravid spot on the side of the abdomen) disappeared with continued exposure to the different concentrations used.

When fish were removed to fresh, conditioned water for six weeks after being exposed for three days to concentrations of 1 and 5 ppm the survival rate varied but little from that of the control (Table I). The mortality rate approached fifty percent by the end of six weeks for both the young and adult fish exposed to a concentration of 10 ppm.

TABLE I

MORTALITY RATE FOR GUPPIES EXPOSED FOR THREE DAYS TO CONCENTRATIONS OF 1, 5, and 10 PARTS PER MILLION DIETHYLSTILBESTROL IN THE WATER

Concentration	Adults		Young	
	Number removed to fresh water after 3 days exposure	Number alive at the end of 6 weeks	Number removed to fresh water after 3 days exposure	Number alive at the end of 6 weeks
Control	50	48	50	48
1 ppm	50	46	50	40
5 ppm	50	48	50	43
10 ppm	50	26	50	29

There seems to be some disagreement as to whether the effect of diethylstilbestrol upon fish is a stimulating or a sedative one. Hoar, et al. (1955) stated that goldfish immersed in the hormone were more active than untreated fish. Selye and Heard (1943) claimed that stilbestrol proved to be a very weak anesthetic for rats, but was comparatively much more potent for fish.

The writer was unable to detect a stimulating effect upon the guppy at any of the concentrations used. Fish placed in the different concentrations behaved no differently than in the controls. They appeared unable to detect the presence of the hormone in the water, even in the relatively lethal concentration of 10 ppm.

Fish exposed to rather heavy concentrations of the hormone for a time may appear to be somewhat anesthetized. For example, it was noted that treated fish were less prone to attempt to jump from their containers during the weighing or while the aquaria were being washed than were untreated

fish. The writer is inclined to believe the lethargy was due to illness or a weakened condition caused by the toxicity of the diethylstilbestrol, rather than to an anesthetized condition.

CHAPTER V

TOXIC CONDITIONS RESULTING FROM MIXING DIETHYLSTILBESTROL WITH THE FOOD

Since the 1-, 5-, and 10-ppm concentrations of diethylstilbestrol in water soon produced severe, observable toxic results, the concentrations were obviously too great to be used for tests involving prolonged exposure to the chemical. Mixing the powdered hormone with the finely-ground fish food was adopted as the most practical method of administering the chemical to the fish in minute and measured amounts. Other workers, such as Hildemann (1954) and Hamon (1946) found that hormones could be given effectively to fish in this way.

Tests were made with the following concentrations, given in parts per million diethylstilbestrol in fish food: 1, 5, 8, 10, 12, 14, 20, 40, 60, 80, and 100. All tests were started with 50 young, sexually mature guppies in each aquarium. One-fourth gram of the hormone-food mixture, measured by means of the dippers described earlier, was fed to the fish in each of the aquaria once a day. From appearances, the fish readily consumed most of the mixture within a short time after it was placed in the water. Excess food was eaten by the snails kept in the aquaria as scavengers.

It was impossible to tell if the tiny particles of powdered hormone adhered to the finely-ground food and were consumed directly by the fish, or whether they dispersed into the water and went, at least partially,

into solution. If all of the hormone contained in a day's feeding of the 5-ppm hormone-food concentration were to have gone into solution, the water would have contained 8.3×10^{-11} grams per cubic centimeter of water in the aquarium.

Concentrations of less than 20 ppm showed no significant differences in the number of guppies surviving over the control. A rather sharp increase in mortality occurred between the 20- and 40-ppm concentrations. There was but little difference between the mortality produced by the 40- and 60-ppm concentrations of the hormone. Another sharp increase occurred between the 60- and 80-ppm concentrations. One hundred parts per million, though killing the greatest number of fish of any of the concentrations tried, was only slightly more lethal than the 80-ppm concentration (Fig. 7 and 8).

In later tests, involving very young fish which were fed a 5-ppm concentration, the fish showed little difference in survival rate from their control until about the sixth week of treatment (Fig. 9). The difference in the survival rate of the very young fish fed a concentration of 20 ppm became apparent around the third week of feeding. After the seventh week there was only a gradual increase in the numbers dying among the fish fed the 20-ppm concentration over those fed the 5-ppm concentration. At the end of the seventh week, sixty-nine percent of the fish fed the 5-ppm concentration were still surviving, while fifty-seven percent remained alive at the end of the thirteenth week. Sixty-two percent of the fish fed the 20-ppm concentration were alive at the end of the seventh week, and forty-eight percent survived at the end of the thirteenth week of feeding.

A 100-ppm concentration of diethylstilbestrol in the food caused a

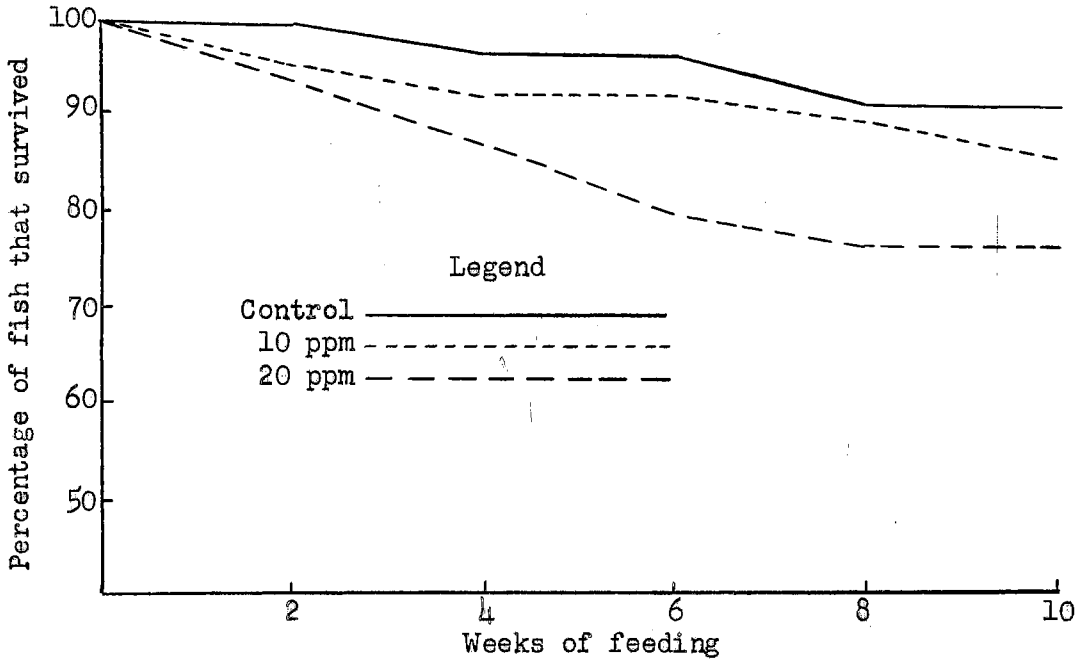


Figure 7. Percentage of adult fish fed concentrations of 10 and 20 ppm diethylstilbestrol mixed with their food surviving at two week intervals over a period of 10 weeks.

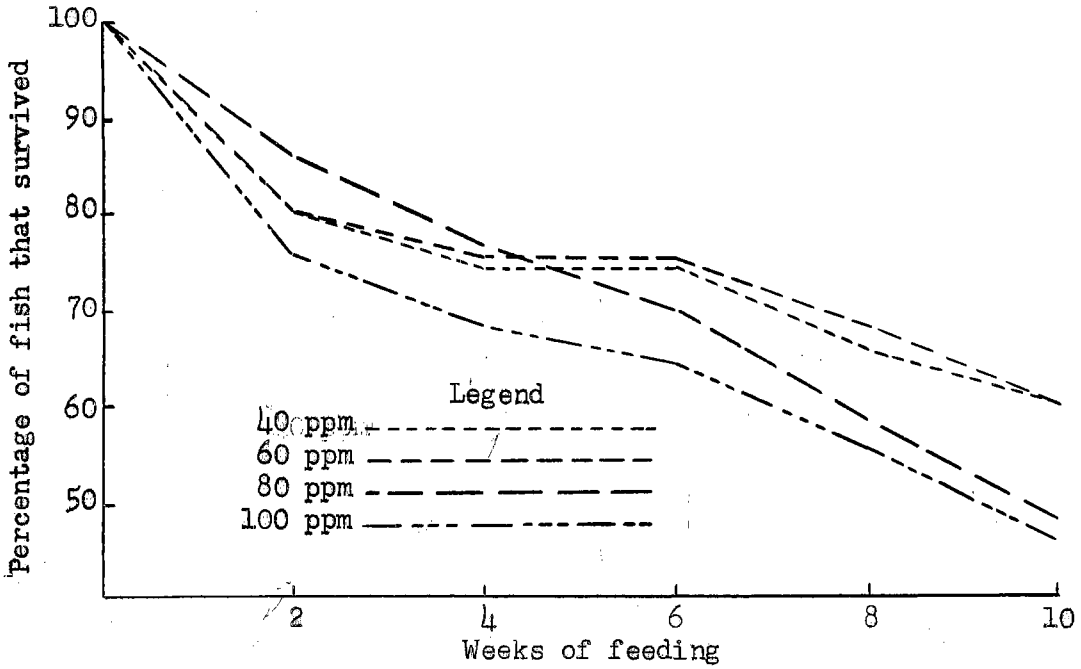


Figure 8. Percentage of adult fish fed concentrations of 40, 60, 80, and 100 ppm diethylstilbestrol mixed with their food surviving at two week intervals over a period of 10 weeks.

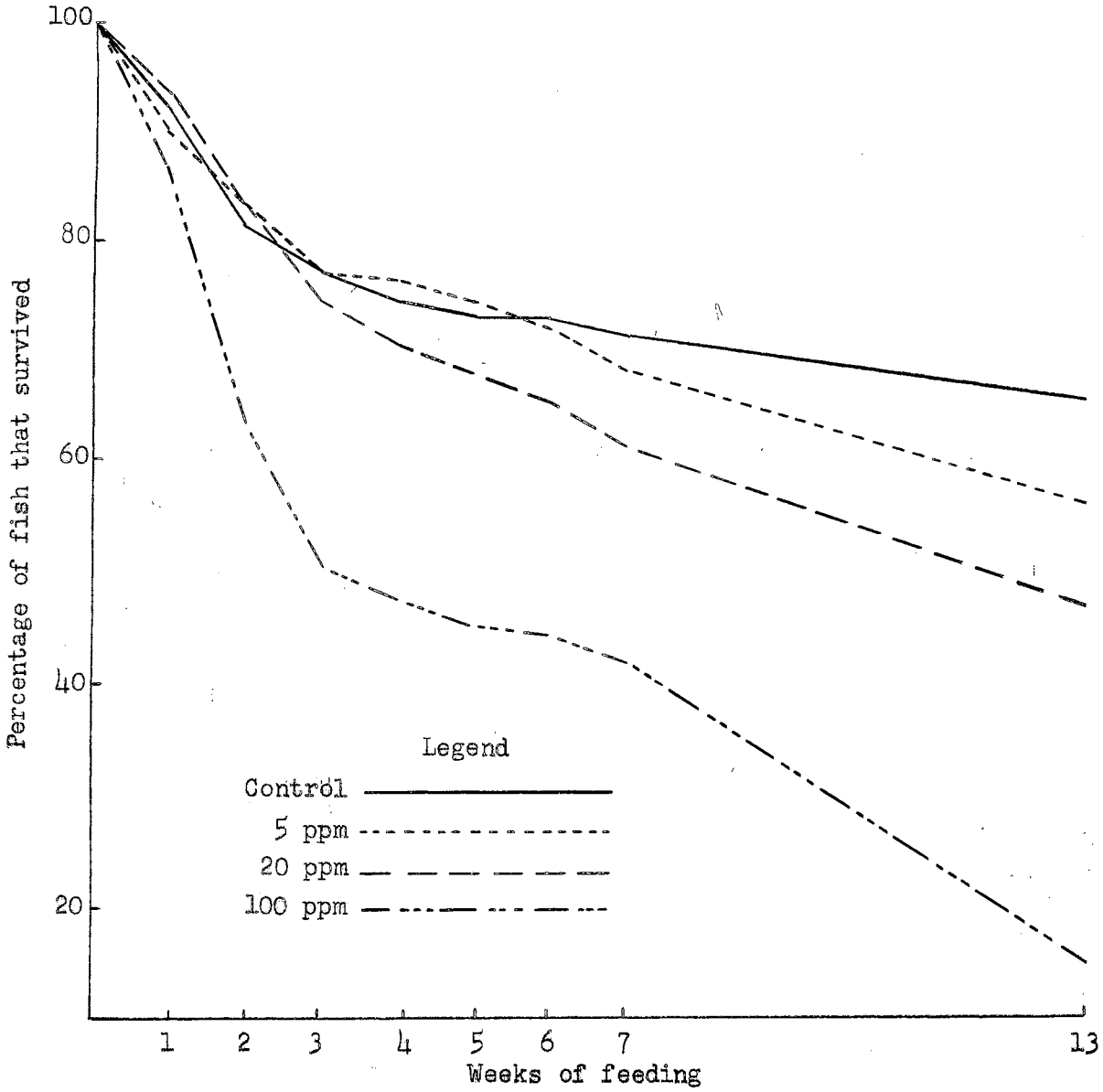


Figure 9. Percentage of young (1-7 day old) fish surviving at weekly intervals. Diethylstilbestrol was given with the food in the concentrations listed in the legend. Regular weekly counting was discontinued after seven weeks, but feeding of the diethylstilbestrol-containing food was continued. A final count was made six weeks following the last weekly count.

rapid drop in the number of young fish that survived the first three weeks of feeding, with nearly fifty percent mortality during this time. The death rate then ran nearly parallel to that resulting from feeding the 20-ppm hormone-food mixture until the seventh week. Only sixteen percent of the small fish fed the 100-ppm concentration remained alive by the thirteenth week.

No cases of hemorrhage were found in fish fed hormone-food concentrations of 10 ppm or less. One male guppy with hemorrhagic spots was observed among the fish fed 12 ppm 89 days after the start of the test. Hemorrhagic areas developed on two of the fish fed the 20-ppm concentration 30 days from the first feeding. One of these was hemorrhaging only slightly, while the other was severely affected, with enlarged, hematic areas on its back and head. In other tests using the 20-ppm hormone-food mixture, hemorrhages developed as early as 15 and as late as 88 days from the start of feeding. Two fish fed the 60-ppm concentration and one fed the 80-ppm concentration developed hemorrhages within 21 days from the first feeding. Hemorrhages appeared on the back of one fish 57 days from the start of feeding the 80-ppm hormone concentration. The greater number of cases of hemorrhage found as resulting from feeding the 20-ppm concentration was apparently due to the larger number of fish which were fed this concentration in the various tests, not to a greater percentage of occurrence at this level.

No cases of hemorrhage were found among the very young fish which were fed the 5-, 20-, and 100-ppm hormone-food concentrations in the later tests of the effect of the hormone upon the growth of young fish. Hemorrhages were found only in fish that were mature or nearly mature at the start of the diethylstilbestrol feeding period.

Exposures for prolonged periods sometimes caused the abdomen to become quite swollen and distended. This condition was especially noticeable in the males, as it caused them to assume the general appearance of a gravid female. The swollen area appeared to be more toward the anterior end than the enlarged abdomen of a pregnant female. Dissection of such fish disclosed a large accumulation of fat in the coelomic cavity.

Extremely thin fish developed in all concentrations from 5 ppm upward. In the 5-ppm concentrations a few fish became noticeably thin 16 days from the start of feeding. The wasted condition of the body appeared in one of the fish fed the 10-ppm hormone-food concentration within 10 days from the first feeding. The higher concentrations resulted in many of the fish becoming quite thin and emaciated. This condition was most readily observed in the case of the larger females. Sixty days after starting the test, many of the guppies receiving the 100-ppm mixture had obviously lost much weight, in spite of being fed regularly each day. By 75 days from the start of the feeding, most of the large fish fed the 100-ppm concentration were dead. The remaining fish were small, weak, and thin.

Analysis of Percentage of Survival of Fish Subjected to Four Levels of Treatment

A statistical analysis was made of the percentage of survival of the young guppies given the 5-, 20-, and 100-ppm concentration of diethylstilbestrol.

It was felt the percentage of survival of the fish subjected to the four levels of treatment could be described by an exponential curve of the form:

$$Y = ab^X,$$

where

Y = percentage of survival at week x

a and b = constants determined by the level of treatment

x = week of treatment

In order to estimate a and b, the logarithms of the percentages of survival were taken, making linear regression techniques possible. The estimates are given in Table II.

TABLE II

CONSTANTS DESCRIBING PERCENT SURVIVAL OF FISH SUBJECTED TO DIFFERENT LEVELS OF DIETHYLSTILBESTROL

Level	a	b
Control	.8590	.9775
5 ppm	.9026	.9642
20 ppm	.9120	.9484
100 ppm	.8742	.8816

Note that the size of the b constant decreases as the level of treatment increases. This indicates that the curves for the higher levels of treatment decrease more rapidly through time. In order to determine whether the decrease in survival was truly different from one level of treatment to another, a test of significance was made on the b values while still in the logarithm form, and each curve was found to be significantly different from the others at the .05 level of probability. This means that the percent survival follows a different curve for each level of treatment, and the curve becomes progressively more steep as the level of treatment is increased.

CHAPTER VI

LOWERED RESISTANCE OF TREATED FISH TO COLD

During the experiments discussed in this paper, the fish were kept at a temperature of 80 $\pm$ 5° Fahrenheit. Following the conclusion of the weighing experiment, the remaining fish used in these tests were moved to a room in which the temperature could not be controlled. However, the writer continued to feed the guppies the treated food in the concentrations used during the weighing experiments. A sudden fall cold wave caused a sharp over-night drop in the temperature of the water in the aquaria to about 55° F. The following morning all but one of the fish that had been fed the 100-ppm concentration of the hormone were dead. Five were dead in each of the groups fed the 5- and 20-ppm concentrations. There were no dead fish in the controls. The treated fish had been fed the hormone for some five months at the time of the temperature drop.

The above information would suggest that the treated fish had a lower resistance to cold than did the untreated fish. There is a possibility that the fish might also have a lowered resistance to other stress in the environment as a result of exposure to diethylstilbestrol.

CHAPTER VII

THE DEVELOPMENT OF TOLERANCE TO DIETHYLSTILBESTROL FOLLOWING PROLONGED TREATMENT

Fifty adult males were placed together in an aquarium, and fed fish food containing 20 ppm diethylstilbestrol. At least one of the various debilitating conditions described earlier in this paper (locomotor ataxia, hemorrhage, emaciation, etc.) occurred among the fish at some time during the first three months of the treatment. Twenty-one of the 50 remained alive at the end of the third month. There were no bright sexual colors among the fish. Though considerable "homosexual" behavior had been observed between the males during the first few weeks of the test, no such behavior was in evidence at the end of the third month.

Three months later (six months following the beginning of hormone administration) 10 fish remained alive. The fish had a feminine shape and were considerably larger than normal males, averaging 426.5 milligrams, compared to an average of 150 milligrams for control males. All appeared to be in excellent health. The characteristic bright male colors had returned, along with renewed sexual interest. Some of the males again pursued other males and thrust at them with their gonopodia. No deaths occurred during the last six weeks.

The reappearance of sexual coloration and mating behavior, and the lack of visible toxic reactions among the persisting fish, even though they had been fed from the same hormone-containing mixture through the

six-month period, suggests the development of a tolerance toward diethylstilbestrol (at least the 20-ppm concentration) following prolonged, continuous exposure of fish which were mature adults when the treatment was begun.

Feeding of the 20-ppm concentration of the chemical to sexually undifferentiated guppies produced somewhat different results. One hundred young, immature guppies were treated for over six months. As in the case of the adult fish mentioned above, none of the 20 fish that survived displayed any visible ill effects from the prolonged exposure to the hormone. All appeared to be in excellent condition. However, no male characteristics were visible among any of the fish. All 20 appeared to be females. Evidently the estrogenic influence of diethylstilbestrol was great enough to prevent sexual dimorphism.

The writer believes the inhibiting effect of the chemical upon the undeveloped testes of the young males prevented the production of the hormones influencing color, male body shape, and gonopodium in sufficient amounts to bring about the appearance of these characters.

CHAPTER VIII

EFFECTS OF DIFFERENT CONCENTRATIONS OF DIETHYLSTILBESTROL UPON SECONDARY SEXUAL CHARACTERISTICS AND REPRODUCTION OF ADULT GUPPIES

The males of the guppy are very conspicuously colored. The anal fin is modified into a gonopodium. Females lack the bright colors, have no gonopodia, and are normally larger than the adult males. Usually the pregnant females can be readily recognized by the presence of a dark area on the side of the abdomen, which some authors (Berkowitz, 1938; Hildemann, 1954) have referred to as the "gravid spot".

Aronson and Clark (1951) stated that

> One of the most striking features of the sexual behavior in this species is the manner in which the males consistently pursue the females, and the great frequency with which the males jab at the genital regions of the females with a momentary thrust of the highly modified anal fin, or gonopodium.

The female breeds when one inch long and the male when three-fourths inch long. The sexually mature adult female has broods every four weeks or so (Axelrod and Schultz, 1935). A single mating may furnish four or five broods. Division of size appears at about 35 days. The gonopodium becomes noticeable in approximately 40 days and coloration 50 to 60 days after birth (Berkowitz, 1938).

The above characteristics of the guppy were used in comparing the effects upon secondary sexual characteristics of the concentrations of diethylstilbestrol used in the various tests. Concentrations of 5, 8,

10, 12, 14, and 20 ppm were tried, in an attempt to determine (1) the approximate level at which changes could be produced, and (2) the effect upon the production of young, first, during the administration of the hormone and, second, upon discontinuing the feeding of the chemical.

All fish used in these experiments were removed from the same brood tank and divided at random into aquaria. Fifty fish were used in each test group. Replicates were not made due to the lack of sufficient aquaria and aquaria space. Feeding of the concentrations mentioned was continued for 100 days. The fish were then returned to regular food for 100 days.

Five Parts Per Million

A hormone-food concentration of 5 ppm had no visible effect upon either the secondary sexual characters or the behavior of sexually mature fish. There was no fading of the distinctive male coloration. The males actively pursued the females throughout the treatment, and the production of young was not inhibited.

Eight and Ten Parts Per Million

The adult males fed the 8- and 10-ppm concentrations were only slightly colored after 75 days of treatment. During this time the characteristic colors had gradually faded until they were almost absent. This agrees with the findings of Berkowitz (1941), who reported that estrogen feeding inhibited further development of color and that, after three months, fading occurred.

The gonopodium, once established, was not affected. In the above test group, sexual activity, in the form of the males pursuing and

thrusting at the females with their gonopodia, was almost absent after 75 days of treatment. What little sexual activity was observed lacked the aggressiveness seen among the fish in the control aquaria. Some of the males had assumed the typical shape of a female.

Thirty days after returning the fish to the regular food the colors of the males were obviously more intense than when the regular feeding was resumed. Sexual behavior appeared to have returned to near normal within fifty days after discontinuing the hormone feeding.

Twelve and Fourteen Parts Per Million

Sexually mature male fish fed the 12- and 14-ppm diethylstilbestrol-food mixture had very little bright coloration after 75 days of feeding. Sexual activity had almost entirely ceased. Some of the males fed this concentration still retained a faint coloration at the end of the 100-day hormone feeding period. The colors had increased slightly by 42 days after resuming regular feeding. Males had by this time started to display renewed interest in the females, although the pursuit behavior lacked the vigor found among the control animals.

Twenty Parts Per Million

The bright colors, of the adult males fed the 20-ppm concentrations, had completely disappeared within 75 days. Sexual behavior had ceased to be evident.

Only faint coloration was noticeable among the males 40 days after resuming regular food. Males displayed practically no interest in the females at this time. By 56 days coloration was only slightly more intense. Some of the large, faintly-colored, female-shaped males

exhibited an active mating behavior, pursuing the females aggressively.

Higher Concentrations

Fish were fed the higher concentrations tested (40, 60, 80, 100 ppm) for only 50 days, at which time regular feeding was resumed.

Among the fish fed the 40-ppm concentration and higher of the hormone, sexual activity had ceased by the forty-third day, and the bright colors of the males had faded by the forty-seventh day. Seventy-five days after returning the guppies to regular food, the male coloration had started to return, but was still faint. By 100 days after the discontinuing of the hormone feeding, most of the males had assumed what appeared to be the normal bright colors. Some males, even during this length of time, had developed very little coloration. A few large, colorless, female-shaped fish could be recognized as males by the presence of the well-developed gonopodia.

Inhibition of Reproduction in Adult Guppies When Fed Diethylstilbestrol

All concentrations of 8 ppm and above had an inhibiting effect upon the production of young. The production of young following the return of the guppies to regular food was delayed. The inhibiting effect of the concentrations of diethylstilbestrol upon the production of young is shown in Tables III and IV.

At least one new-born young was found among each of the test groups during the first 30 days following the start of the tests, except those fed the 40-ppm concentration. Young were born 46 days from the start of the test in the aquarium containing the adults fed the 8-ppm concentration. No young were found after the first 30 days among fish fed the

10- to 100-ppm concentrations of diethylstilbestrol, nor among those fed 8 ppm after 46 days.

Young fish were first found among the guppies which received the 14-ppm concentration 64 days after discontinuing the hormone in the diet, one day later among those receiving the 12 ppm, and three days later among those given a diet containing 8 ppm diethylstilbestrol. Ninety-one days after discontinuing the hormone young guppies were born in the aquaria containing the fish that had received a concentration of 60 ppm and appeared four days later among the fish that had received a concentration of 20 ppm (Tables III and IV).

The experiment was terminated 100 days after discontinuing diethylstilbestrol in the diet.

TABLE III

THE INHIBITING EFFECT OF DIFFERENT CONCENTRATIONS OF DIETHYLSTILBESTROL DURING ADMINISTRATION UPON THE PRODUCTION OF YOUNG BY SEXUALLY MATURE ADULT GUPPIES

Days from start of test[2]	Number of young produced and days from first feeding young appeared in aquaria[1]								
	Control	8	10	12	14	20	40	60	80	100
30	35	4	3	1	1	1	0	7	10	9
46	9	3	0	0	0	0	0	0	0	0
51	10	0	0	0	0	0	0	0	0	0
73	6	0	0	0	0	0	0	0	0	0
75	7	0	0	0	0	0	0	0	0	0

[1]The concentration of diethylstilbestrol powder mixed with the food is expressed in parts per million. The fish were fed 0.25 gram of the mixture each day.

[2]Fish listed on the first date were born at some time during the 30-day period. The remaining numbers represent the day on which young were found in one or more of the aquaria.

TABLE IV

THE INHIBITING EFFECT OF DIFFERENT CONCENTRATIONS OF DIETHYLSTILBESTROL FOLLOWING DISCONTINUANCE OF TREATMENT UPON THE PRODUCTION OF YOUNG BY SEXUALLY MATURE ADULT GUPPIES

Days after returning to regular food**	Length of time before treated fish produced young following discontinuing of hormone administration*								
	Control	8	10	12	14	20	40	60	80	100
30	2	0	0	0	0	0	0	0	0	0
50	1	0	0	0	0	0	0	0	0	0
58	0	0	0	0	2	0	0	0	0	0
64	0	0	9	0	0	0	0	0	0	0
65	1	0	0	4	0	0	0	0	0	0
67	0	1	0	0	0	0	0	0	0	0
91	0	0	0	0	0	0	0	3	0	0
95	0	0	0	0	0	2	0	0	0	0

*The concentration of diethylstilbestrol powder given the fish is expressed in parts per million of regular fish food with which it was mixed.

**The experiment was discontinued after young fish were found among the adults that had received the higher diethylstilbestrol concentrations.

CHAPTER IX

THE EFFECTS OF DIETHYLSTILBESTROL UPON THE GROWTH OF VERY YOUNG GUPPIES AND THEIR REPRODUCTION WHEN GROWN

The following experiments were conducted to determine the effects of diethylstilbestrol upon the growth of young, immature guppies and upon their later reproduction. The fish used were one to seven days old at the start of the tests. Duplicate aquaria with 50 fish each were set up for the control and each concentration used.

Three concentrations of the hormone were chosen for the tests; 5, 20, and 100 ppm, mixed with the finely-ground food. The fish in each aquarium were fed approximately 0.25 gram of the hormone-food mixture each day. Measurements of the amount of food given was made by means of the dippers described under Materials and Methods.

A concentration of 5 ppm was chosen as the weakest mixture to be used, because earlier tests upon adult fish had shown this concentration to have very little effect upon survival and behavior.

A mixture containing 20 ppm was used because the results of previous tests suggested that this level of concentration, while being more toxic than lower concentrations, was necessary to insure suppression of sexual behavior and reproduction.

The concentration of 100 ppm was arbitrarily chosen as the upper limit.

Five Parts Per Million

The fish fed the 5-ppm concentration were quite varied in size by the end of the second week of feeding. Some appeared to have grown only slightly. Most of the fish appeared to be in excellent condition. One very thin fish was observed in each replicate. These two fish died within a few days.

Ten fish in Replicate Number 1 and seven in Replicate Number 2 had grown only slightly by the end of the fifth week of feeding. Only one fish appeared to be ill. All others were vigorous and active, and gave no indication of adverse toxic reaction to the presence of the hormone.

Several fish still showed very little increase in size, over that at the start, after 7 weeks of feeding. Many of the other fish had grown very rapidly, exceeding the size attained by the fish in either the control or other test aquaria.

The average increase in weight per fish throughout the 7-week feeding period was continuous and relatively uniform (Fig. 10). The average weights of the fish consistently exceeded that of all other fish tested, including the control. No period of weight loss, or period of no gain, occurred as was found at the 20- and 100-ppm levels.

Only one fish displayed conspicuous symptoms of toxicity at the last weekly weighing period. This fish exhibited the locomotor trouble described earlier under toxic effects.

Some males could be distinguished from females at this time. The larger males were uncolored, but had rather well-developed gonopodia. The largest male, though lacking coloration, was seen actively pursuing one of the larger females.

Regular weekly weighing was discontinued after the seventh week,

because sexual dimorphism was becoming apparent. Feeding of the 5-ppm concentration was continued, and a final weighing was made six weeks later. The males and females could then be distinguished, and the sexes receiving this concentration were weighed separately to find the average weight for each sex.

The average weight of the females was considerably higher at the end of 13 weeks from the beginning than that of either the control or the other treated animals of either sex. The average weight of the males exceeded the average weight of all other fish tested, including the females of the control group (Appendix D).

The largest males had not developed normal coloration, but could be separated from the females by their gonopodia and sexual behavior. The smallest males had the most brilliant colors. Only one very thin fish was found. No other evidence of toxic reaction was seen, and the remainder of the fish appeared to be in excellent condition. New-born young were found in both replicates at this time.

Twenty Parts Per Million

Fish fed the 20-ppm concentration, while not gaining as rapidly as those fed the 5-ppm mixture, had a greater average percentage of growth during the first week than did those in the control (Fig. 10).

There was a very small percentage of gain in weight during the second week of feeding. This can be at least partly explained by the fact that each replicate at this time contained a number of fish that, though still alive, showed severe toxic effects to the hormone, and had become thin and emaciated. The remainder of the fish displayed no ill effects. The percentage of gain was based upon the average weight of all fish

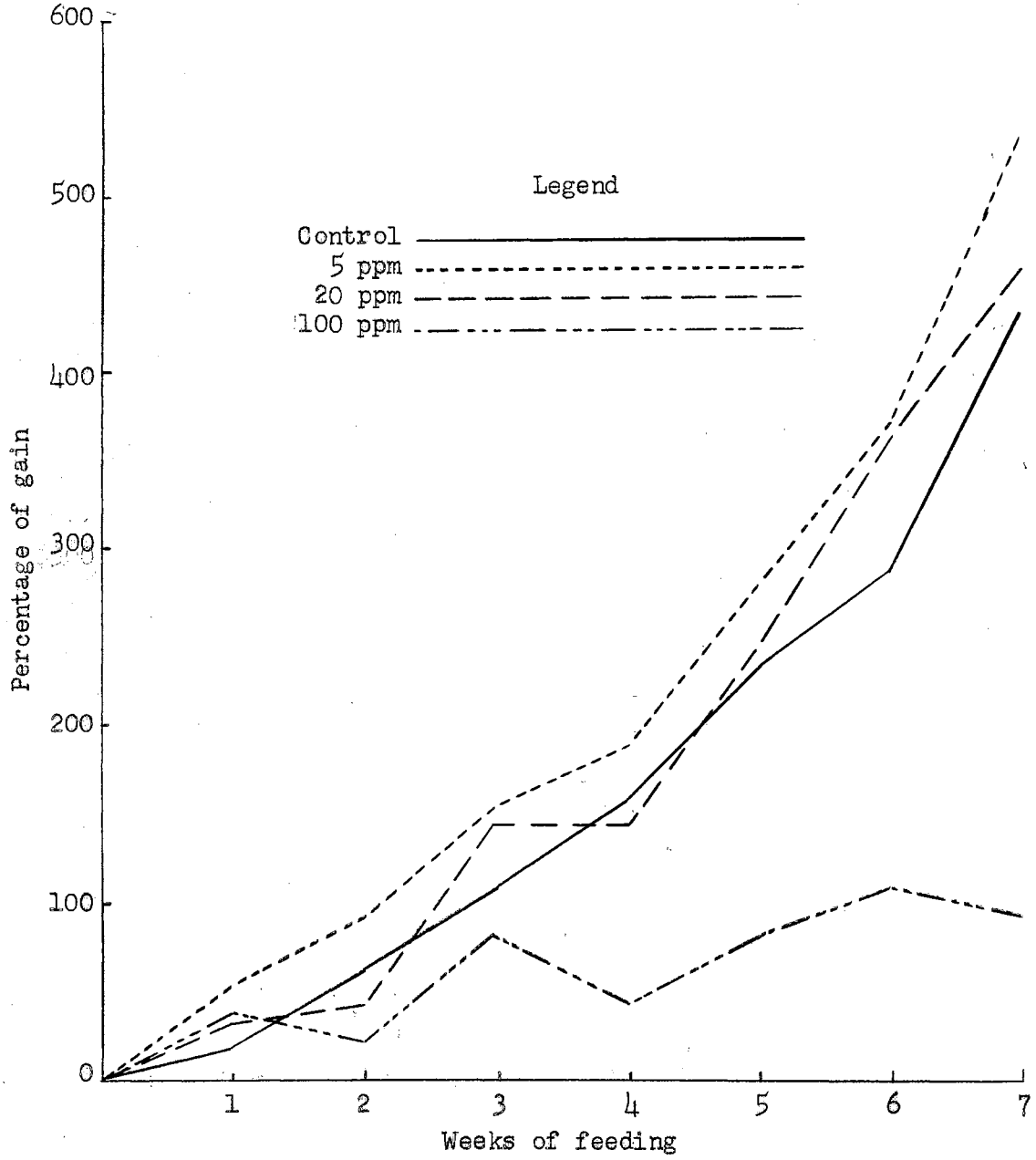


Figure 10. Percentage of gain over starting weight of young fish (1-7 days old) fed diethylstilbestrol-containing food in the concentrations listed in the legend. Regular weekly weighing was discontinued after seven weeks, as the control and fish fed the 5-ppm concentration were beginning to show sexual dimorphism.

alive at the time of weighing. The inclusion of the emaciated individuals mentioned above resulted in an average percentage of gain that was only slightly above that of the previous week.

There was an abrupt increase in the average weight of the fish fed the 20-ppm concentration during the third week. Most of the thin fish that were included in the average weight the week before were now dead, while the majority of the remaining fish appeared to be healthy.

The fish made practically no gain in weight during the fourth week of feeding. A number of the fish had reacted to the toxic effects of the chemical, and were in poor physical condition. In addition to the presence of some very thin fish, several of the smallest had turned extremely dark, a condition past experience had shown that, while not necessarily due to the hormone, was soon followed by the death of the fish.

The percentage of weight increase rose abruptly from the end of the fourth week and passed that made by the control during the fifth week. All remaining fish appeared to be in excellent condition at the last weekly weighing period.

Only one fish had developed any of the characteristic male color by the end of seven weeks of feeding the 20-ppm concentration of the hormone. This fish appeared to be a female. No fish that could be distinguished as a male by the presence of a gonopodium had developed the normal coloration expected. No sexual activity was noted.

As in the fish fed the 5-ppm hormone-food mixture, regular weekly weighing was discontinued at the end of seven weeks. Feeding of the 20-ppm concentration was continued for an additional six weeks. The fish were then weighed for the last time. All fish had developed a more or

less feminine shape, and it was impossible to distinguish the males from the females by shape, color, or behavior. For this reason, all fish were weighed together, and the total average percentage of gain calculated (Appendix D). This percentage was considerably less than that made by either the males or females of the fish fed the 5-ppm concentration or the females of the control.

One Hundred Parts Per Million

Young guppies given food containing 100 ppm diethylstilbestrol gained weight at a slightly higher rate than those given 20 ppm of the hormone during the first week. The average weight at the end of the second week showed a substantial loss of weight over that of the first weighing period. Several fish exhibited the locomotor difficulty described earlier. Each replicate also contained at least six very small, thin fish.

A marked increase in weight occurred during the third week of feeding, similar to that which took place during the same week of feeding the 20-ppm concentration. It was noted that most of the sickly fish present the previous week were gone, which no doubt was the reason for the rather large increase in average weight. The surviving fish appeared to be in excellent health. They were of a rather uniformly small size, showing much less variation between the largest and smallest fish than was found among the fish of the control or those fed the 5- and 20-ppm concentrations.

An even greater loss of average weight occurred during the fourth week of feeding than took place during the second week. Several extremely thin fish were present among those weighed at this time.

A slow but steady increase in average percentage of gain took place during the fifth and sixth weeks of feeding. However, a loss from the previous week took place during the seventh and last week of regular weighing.

No sexual dimorphism was evident among the fish, and males could not be distinguished from the females. All fish had a juvenile appearance. Feeding of the hormone was continued, but weekly weighing was not. Six weeks later the surviving fish were again weighed to find the percentage of gain. The fish were weighed together, as the sexes could not be distinguished. The retarding effect of the 100-ppm concentration of the diethylstilbestrol was so pronounced that the fish had less than doubled their starting weight in over 90 days.

Analysis of Gain

An analysis of variance was conducted on the gains of the fish on the four treatments over seven periods encompassing 49 days. The analysis and the accompanying values of F are given in Table VII.

TABLE VII

ANALYSIS OF VARIANCE ON GAINS OF FISH

Source	d.f.	M.S.	F
Treatments	3	775.10	7.56**
Error (a)	18	102.64	
Periods	6	1,449.18	155.16**
Reps x Trts	4	55.24	5.91**
Error (b)	24	9.34	

**Probability less than .01 of chance value this large

The fact that the periods are significantly different is merely a reflection of the growth curve. The significance of the replicate mean square is an indication that there was a detectable lack of uniformity between the two groups of fish receiving the same treatment. The main purpose of the analysis, however, was to determine whether the treatments affected gain differentially. The fact that the treatments mean square is significant is certainly a strong indication that this is the case.

Since the analysis of variance leads to the conclusion that there are differential treatment effects, it is desirable to determine which treatments are different with respect to gain. Table VI presents the mean differences between all pairs of treatments and indicates which differences are significant.

TABLE VI

MEAN DIFFERENCES AMONG ALL TREATMENT COMPARISONS

Treatment:	5 ppm	20 ppm	100 ppm
Control	-2.41	0.61	14.05**
5 ppm		3.03	16.46**
20 ppm			13.44**

**Probability less than .01 of chance difference this large ($LSD_{.01}$ = 7.79).

The analysis of variance indicated there were differences among the treatment means, therefore the Least Significant Difference at the .01 level of probability ($LSD_{.01}$) was used to detect the differences. It indicates that the gain was significantly lower for those fish receiving 100 ppm irrespective to the group with which they are compared. However,

the differences among the other groups are so small that they must be attributed to chance.

CHAPTER X

GROWTH RESPONSE OF ADULT GUPPIES WHEN RETURNED TO A REGULAR DIET FROM DIETHYLSTILBESTROL-CONTAINING FOOD

The following test was made to determine if size changes would occur among adult fish returned to a regular diet, after having been fed hormone-containing food for 75 days. Mature adult fish were used in the test. Weights were made 100 days after the diethylstilbestrol mixture was discontinued. Males and females were weighed separately.

Adult males of all treated groups exceeded the size attained by the males in the controls. The greatest weight average was attained by those fish that had received the 20-ppm level of concentration of diethylstilbestrol powder in their food. The average weights for each treated group are given in Table VII.

A limited number of tests were conducted using a prepared diethylstilbestrol-containing material supplied by the Eli Lilly Company, called STILBOSOL. The material was designed as an additive to livestock food. The STILBOSOL was diluted with fish food to obtain the concentration of diethylstilbestrol listed in Table VII. The greatest increase in average weight in males fed STILBOSOL occurred among those fed a concentration of 30 ppm diethylstilbestrol in their food, followed by those given food containing a 20-ppm concentration.

The average weight attained by the females was inversed to that attained by the males. The higher the concentration used, the lower the

average weight of the females (with one exception). Fraps, et al. (1956) found that, in general, when diethylstilbestrol was administered to poultry, the adverse effect upon semen volume increased with the increasing dosage. Though the semen production of the treated fish was not known, the greater number of pregnant females among both the controls and the fish receiving the lighter concentrations of the hormone would suggest that the findings of Fraps might also be true for guppies.

TABLE VII

GROWTH RESPONSE OF ADULT MALE AND FEMALE GUPPIES FED VARIOUS CONCENTRATIONS OF DIETHYLSTILBESTROL WHEN RETURNED TO REGULAR FOOD

The fish were mature adults at the start of the test. The concentrations listed for each group were fed for a period of 75 days. The weights given are the average weights for each test group 100 days after being returned to regular fish food. Weights are given in milligrams.

Concentration used (ppm)	Number of males	Average weight	Number of females	Average weight
	Diethylstilbestrol powder mixed with food			
8	18	165.9	21	590.3
10	22	201.6	20	490.4
12	22	242.2	11	458.7
14	26	233.9	17	434.4
20	18	257.5	22	366.9
	Control			
#1	24	146.9	23	572.0
#2	29	153.4	20	617.5
	STILBOSOL[1] mixed with food			
10	25	197.1	25	618.6
20	31	238.9	19	494.4
30	24	270.3	22	456.9
40	16	218.8	29	422.3
50	13	227.5	29	428.5

[1]STILBOSOL is a product of the Eli Lilly Company. It is a diethylstilbestrol-containing preparation for mixing with livestock feed.

CHAPTER XI

DISCUSSION

Evidence obtained from the study would indicate that diethylstilbestrol has only a limited value in fishery management.

Although fish given food containing the lower concentrations of the hormone outgained the controls, the increase in weight was primarily due to the greater size attained by the males, which tended to assume the larger configuration of the females. In those species of fishes having no difference in size between the sexes, or in species in which the male normally exceeds the female in size, the estrogenic effect of the hormone would be of doubtful value in producing greater growth. It might result in an actual descrease in the average size attained by the fish.

Diethylstilbestrol inhibited sexual behavior and the production of young in the guppy. The most effective level of concentration for this purpose was about 20 ppm in the food. Lower concentrations did not necessarily stop reproduction or sexual behavior completely. Field tests would be necessary to determine if reproduction of the larger species of fishes, such as are used in small impoundments, are affected at the same level of concentration as the guppy.

Heavy concentrations of the hormone, while exerting a inhibitive effect upon reproduction, also have a stunting effect upon members of both sexes. Heavy concentrations also have a detrimental effect upon the health and survival of the fish.

Powdered diethylstilbestrol did not appear to lose its strength when mixed with the finely ground fish food during the period of study. The bright colors of adult male guppies faded as readily when fed mixtures which had been stored for six to eight months as when they were fed freshly prepared concentrations of diethylstilbestrol and fish food. However, to avoid a possible loss in effectiveness due to storage, each test was started with new preparations. In tests that ran for an extended length of time, the old diethylstilbestrol-food mixtures were replaced by fresh preparations each three months.

It would be worthwhile to study the effect of the hormone in relation to its ability to inhibit reproduction in small impoundments. Fish such as the bluegill, Lepomis machrochirus Rafinesque, which commonly overpopulate ponds, would be especially vulnerable to the effects of the hormone during the spawning season. Dr. Roy W. Jones (personal communication) found that a concentration as low as one-tenth part per million of diethylstilbestrol in solution would stop mitosis in the developing embryo of the zebra fish, Brachydanio rerio. As bluegills nest in colonies, with the nests practically touching (Beckman, 1941), spreading of the hormone-containing food over the spawning beds would not only inhibit the production of eggs and sperm, but would further reduce numbers through the effect upon the mitotic processes of the developing embryos by the chemical dissolved in the water.

Altman (1959) showed that fish may be concentrated in certain selected areas of a pond by the use of commercial lay pellets. Stomach analysis proved the fish to be consuming the pellets introduced into the water. The writer had hoped that, as the chemical has an inhibiting effect upon the production of young, diethylstilbestrol could be added to

the food before compressing it into pellet form, and the hormone-containing pellets fed in selected feeding areas of a pond to help keep the numbers in check. However, since the study discussed in this paper was made, diethylstilbestrol has been shown to be a carcinogen. As a carcinogen, the chemical falls within the ban of the so-called Delaney clause which is set forth in Section 409 (c) (3) (A) of the Food Additives Amendment to the Federal Food, Drug, and Cosmetic Act. Under such circumstances, the Department of Health, Education, and Welfare of the Food and Drug Administration can no longer authorize the use of diethylstilbestrol as an additive to human or animal food.

The limitations placed upon the use of diethylstilbestrol would seriously restrict, if not eliminate, its use as a part of fish food to control populations, especially if the fish were being used for food as well as sport.

The data presented in this study suggest that excessive numbers of fish may be controlled in part by the use of estrogenic and androgenic substances.

CHAPTER XII

SUMMARY

1. The results of a study of the effects of the synthetic organic chemical, diethylstilbestrol, upon the guppy, Lebistes reticulatus, with special reference to the effects of different concentrations upon growth, sexual behavior, and reproduction are presented. The toxic effects of the different concentrations are also reported.

2. The hormone was found to produce several severe toxic conditions, especially at concentrations above 20 ppm in the food. Included among the pathological conditions were hemorrhage, locomotor trouble, emaciation, and fatty deposits in the coelomic cavity.

3. A 5-ppm concentration of the hormone in the food increased the rate of growth of young guppies. Relatively little toxicity was found and sterility was not produced among the fish treated at this level.

4. A concentration of about 20 or more parts per million stopped or inhibited reproduction and sexual behavior. Some toxic effects were noted at the 20-ppm level of concentration.

5. Adult male guppies which had lost their bright colors and libido following three months of receiving the 20-ppm concentration, regained their color and sexual interest three months later, in spite of continued administration of the chemical in their diet. Young, immature fish fed the same concentration for six months failed to display any male characteristics.

6. Inhibition of normal male coloration was not necessarily a concomitant of an inhibition of sexual behavior.

7. Young fish fed food containing 20 ppm diethylstilbestrol did not gain as fast as those fed the 5-ppm concentration, but did outgain the control animals over a 7-week period.

8. A concentration of 100 ppm of the hormone in the food had a definite retarding effect upon the rate of growth of young fish. Considerable numbers of the test fish were lost at this level of concentration, due to the toxic effects of the hormone.

9. Stopping of the reproductive behavior and the production of young was not permanent, even at the higher levels of concentration. The production of young was renewed several weeks following the discontinuing of diethylstilbestrol administration.

10. Government restrictions upon the use of diethylstilbestrol, due to its carcinogenic effects, and the limitation of the use of the chemical as a food additive is discussed.

11. The results of the study suggest that the populations of small impoundments can be partially controlled by the use of food additives affecting reproduction. Field studies using non-carcinogenic androgens and/or estrogens are recommended.

LITERATURE CITED

Adams, Elizabeth A. 1946. Sexual conditions in Triturus viridescens. IV. The effects of the administration of diethylstilbestrol on adult normal and castrated males. Jour. Exp. Zool., 101: 1-38.

Altman, Ralph W. 1959. Increased harvest of warm water fish through supplemental feeding. Thesis, Okla. State Univ. Libr. 116 p.

Aronson, Lester R., and Eugenia Clark. 1951. Sexual behavior in the guppy, Lebistes reticulatus (Peters). Zoologica, 26: 49-66.

Axelrod, H. R., and L. P. Schultz. 1955. Handbook of tropical aquarium fishes. McGraw-Hill Book Co., Inc., New York, 718 p.

Beckman, W. C. 1941. Meet Mr. Bluegill. Mich. Conserv., 10(7): 6-7, 11.

Berkowitz, Philip. 1938. The effects of estrogenic substances in Lebistes reticulatus (guppy). Anat. Rec., 71: 161-175.

_____. 1941 The effects of estrogenic substances in the fish (Lebistes reticulatus). Jour. Exp. Zool., 87: 233-240.

Black, J. C., and R. Gordon Booth. 1946. Capon production by the use of synthetic oestrogen (stilbestrol). Vet. Jour., 102: 41-44.

Bouvier, G., D. Sartori, and F. A. Torres. 1944. Ensaios de castração quimica de frangos. Biológico (São Paulo)., 15: 31-34.

Cohen, Herman. 1946. Effects of sex hormones on the development of the platyfish, Platypoecilus maculatus. Zoologica, 31: 121-128.

Dunn, Charles W. 1941. Stilbestrol-induced testicular degeneration in hypersexual males. Jour. Clin. Endocrinol., 1: 643-648.

Eversole, W. J. 1939. The effects of androgens upon the fish (Lebistes reticulatus). Endocrinol., 25: 328-330.

_____. 1941. The effects of pregneninolone and related steroids on sexual development in the fish (Lebistes reticulatus). Endocrinol., 28: 603-610.

Fraps, R. M., H. A. Sohn, and M. W. Olsen. 1956. Some effects of multiple pellet implants of diethylstilbestrol in 9-week old chickens. Poultry Sci., 35: 665-668.

Goodrich, H. B., J. E. Dee, C. M. Flynn, and Rowena N. Mercer. 1934. Germ cells and sex differentiations in Lebistes reticulatus. Biol. Bull., 67: 83-96.

Green, Morris. 1958. Gynecomastia and pseudoprecocious puberty following diethylstilbestrol exposure (case report). Jour. Diseases Children, 95: 637-639.

Hamon, Mary Vonne. 1946. Action des hormones sexuelles de synthese sur la morphologie extérne de Gambusia Holbrooki Gir. Bull. Soc. Hist. Nat. Afrique du Nord, 37: 122-141.

Hildemann, William H. 1954. Effect of sex hormones on the secondary sex characters of Lebistes reticulatus. Jour. Exp. Zool., 126: 1-16.

Hoar, W. S., M. H. A. Keeleyside, and R. S. Goodale. 1955. The effects of the thyroxine and gonadal steroids on the activity of salmon and goldfish. Can. Jour. Zool., 33: 428-439.

Kelly, Kathleen G. R., and R. S. Roberts. 1950. Stilboestrol implantation in cockerels. Vet. Rec., 62: 44-45.

Lorenz, F. W. 1945. The fattening action of orally administered synthetic estrogens as compared with diethylstilbestrol pellet implants. Poultry Sci., 24: 91-92.

Moreng, Robert E., Reece L. Bryant, and David G. Gosslee. 1956. Gonad and comb response of mature male chickens to diethylstilbestrol as influenced by breed and dosage. Poultry Sci., 35: 476-482.

Mulligan, R. M. 1947. Residual tissue changes in male dogs following cessation of orally administered stilbestrol. Amer. Jour. Path., 23: 299-311.

Neverla, G. J. 1944. The history of the discovery and isolation of the female sex hormones. New England Jour. Med., 230: 595-604.

Perry, T. W., F. N. Andrews, and W. M. Beeson. 1951. The effects of stilbestrol on suckling lambs. Jour. Anim. Sci., 10: 602-606.

Peterson, W. E., and W. L. Boyd. 1944. Stilbestrol helps solve sterility. Minn. Farm and Home Sci., 1: 3-4.

Roberts, W. L. 1950. What happens when pellet-inserted chicken heads are fed? Amer. Fur Breeder, 24: 17-18.

Scott, Joseph. 1944. The effects of the steroids on the skeleton of the poecilid fish Lebistes reticulatus. Zoologica, 29: 49-59.

Selye, Hans, and R. D. H. Heard. 1943. The fish assay for the anesthetic effect of the steroids. Anesthesiology, 4: 36-47.

Sharof, A., and E. S. E. Hafex. 1958. Effect of the synthetic oestrogen stilboestrol on pregnancy in the rabbit. Nature, 181: 1211.

Tavolga, Margaret C. 1949. Differential effects of estradiol, estradiol benzoate and pregneninolone on Platypoecilus maculatus. Zoologica, 34: 215-237.

Westermarch, H., and A. Aaltonen. 1950. Om kapunering av tuppar med stilbestrol (Caponization of roosters with stilbestrol). Nordisk Veterinarmed, 2: 405-414.

Witschi, E., and E. N. Crown. 1937. Hormones and sex determination in fishes and in frogs. Anat. Rec., 70: 121.

A P P E N D I C E S

APPENDIX A

EFFECT OF SELECTED CONCENTRATIONS OF DIETHYLSTILBESTROL UPON SURVIVAL OF ADULT GUPPIES*

Concentration of diethylstilbestrol in the food**	Start	2 weeks	4 weeks	6 weeks	8 weeks	10 weeks
Control	100	99	96	96	91	91
10	100	95	92	92	89	85
20	100	93	87	79	76	76
40	100	80	72	72	66	60
60	100	80	74	74	68	60
80	100	86	76	70	58	48
100	100	76	68	64	56	46

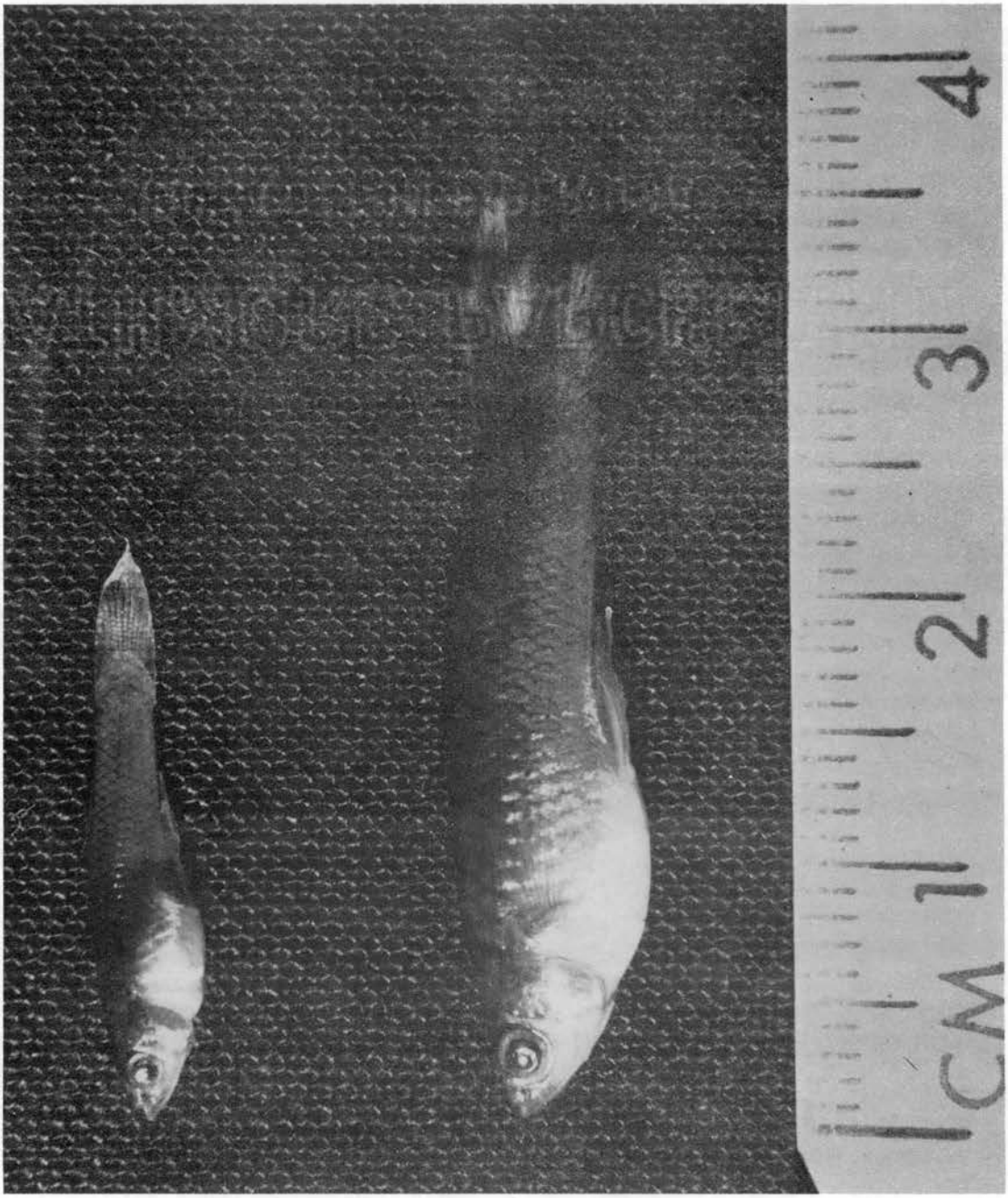
*Expressed in percentage of survival.
**Expressed in parts per million of fish food.

Appendix B

The upper fish was the largest individual among those fed food containing 100 ppm diethylstilbestrol from soon after birth for a period of six months. (Weight: 101.8 mg.).

The lower fish was an adult male fed food containing 20 ppm diethylstilbestrol for six months. Normal colors, which had disappeared during the first three months of feeding, were now quite conspicuous in the living specimen. (Weight: 482.2 mg.).

APPENDIX B

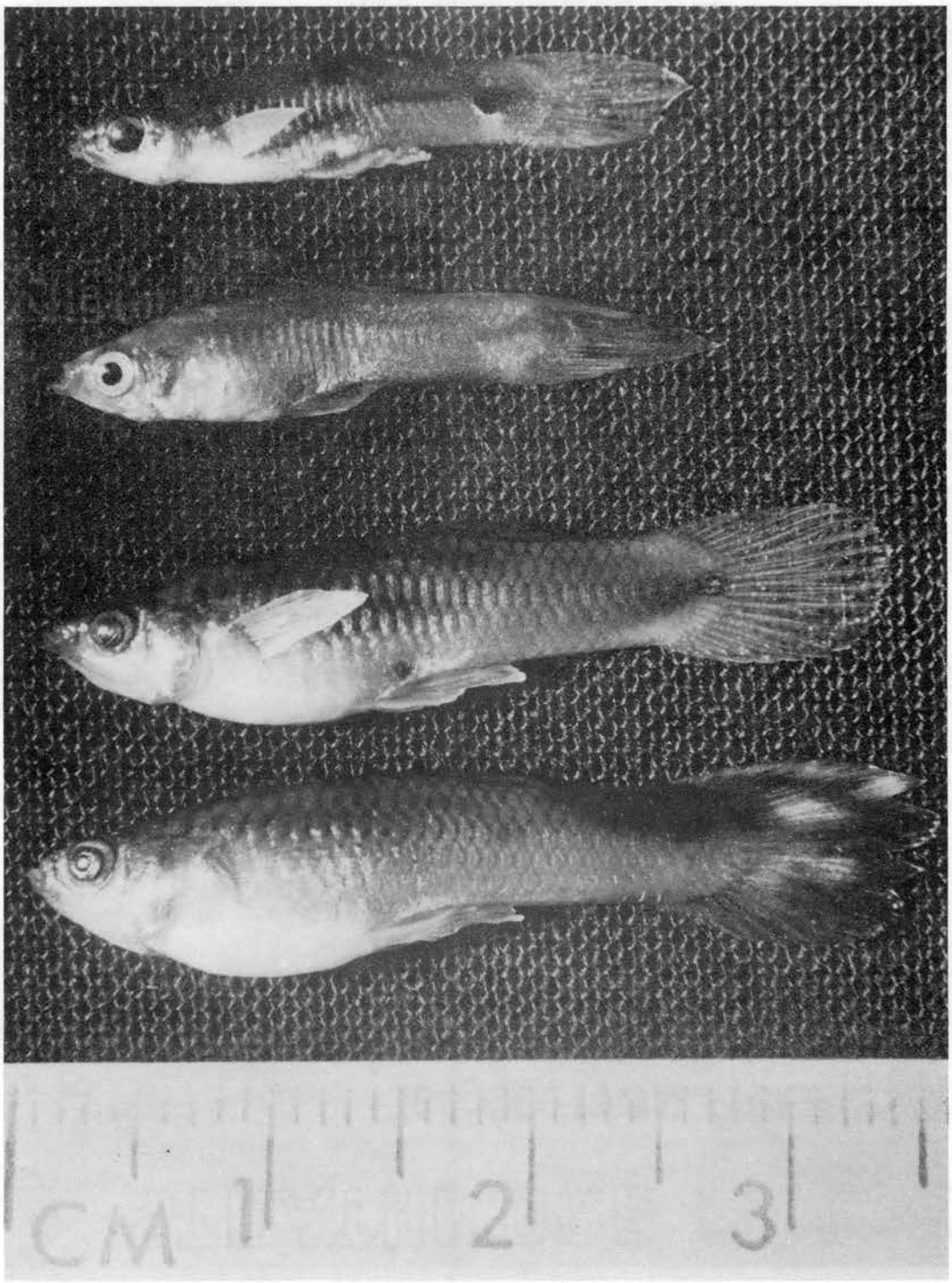


Appendix C

The two fish at the top of the picture were adult males from the control group. (Average weight: 150.1 mg.).

The two fish at the bottom of the picture were adult males of the same age group fed food containing 20 ppm diethylstilbestrol for six months. (Average weight: 426.5 mg.).

APPENDIX C



APPENDIX D

RATE OF GAIN OVER STARTING WEIGHT BY GUPPIES FED 5-, 20-, AND 100-PARTS PER MILLION CONCENTRATIONS OF DIETHYLSTILBESTROL MIXED WITH REGULAR FISH FOOD

Fish were 1 to 7 days old at the start of the test. The rate of gain or loss for each test group is expressed in percentage of average increase at each weighing period over the average weight at the start of the test

Concentration used		Weight at start in mg.	WEEKS 1		2		3		4		5		6		7		13*		
			Gain over starting weight		Gain over starting weight		Gain over starting weight		Gain over starting weight		Gain over starting weight		Gain over starting weight		Gain over starting weight		Gain over starting weight		
			mg	%	mg	%	mg	%	mg	%	mg	%	mg	%	mg	%	mg	%	
Control	1	11.6	2.8	24.1	8.3	71.6	13.7	118.1	19.9	171.6	28.0	241.4	34.9	300.9	48.8	420.7	85.8	739.7	♂
																	125.5	1081.9	♀
	2	11.8	1.3	11.0	7.0	59.3	11.3	95.8	15.8	133.9	27.0	228.8	32.0	271.2	53.1	450.0	94.5	800.9	♂
																	159.0	1347.5	♀
5 ppm	1	8.5	4.4	51.8	11.5	135.3	16.7	195.6	19.1	224.7	27.5	323.5	36.0	423.5	54.8	644.7	117.8	1385.9	♂
																	180.1	2118.8	♀
	2	12.5	6.7	53.6	5.8	46.4	13.5	108.0	18.9	151.2	29.5	236.0	40.5	324.0	52.8	422.4	136.5	1092.0	♂
																	198.3	1586.4	♀
20 ppm	1	9.2	5.7	62.0	6.7	72.8	17.3	188.0	16.6	180.0	31.6	343.5	43.2	469.6	53.8	584.8	126.0	1369.6	
	2	12.5	1.5	12.0	1.4	11.2	11.7	93.6	13.6	108.8	17.6	140.8	32.8	262.4	41.8	344.4	86.0	688.0	
100 ppm	1	11.2	4.1	36.6	3.0	26.8	14.5	129.5	4.6	41.1	6.0	53.6	10.7	95.5	7.6	67.9	24.7	220.5	
	2	12.3	4.9	39.8	1.7	13.8	3.4	27.6	5.7	46.3	12.8	104.1	14.1	117.1	13.8	112.2	17.9	145.5	

*Regular weekly weighing was discontinued at the end of seven weeks, because sexual dimorphism becomes apparent at about this time (Goodrich, et al., 1934). The surviving fish were weighed after an additional six weeks of feeding the diethylstilbestrol-food mixture. Males and females could be separated in the control and 5-ppm aquaria, and were weighed separately. Sexes could not be distinguished among the fish fed the food containing the 20- and 100-ppm concentrations. The figures for the last groups represent the average weight of all the fish in that group.

VITA

Robert Joe Boles

Candidate for the Degree of

Doctor of Philosophy

Thesis: EFFECTS OF SELECTED CONCENTRATIONS OF DIETHYLSTILBESTROL UPON THE GUPPY, LEBISTES RETICULATUS (PETERS)

Major Field: Zoology

Biographical:

Personal Data: Born at Brandsville, Missouri, November 7, 1916, the son of Walter E. and Ivy N. Boles.

Education: Attended grade school at Wilmore, Kansas; graduated from Wilmore Rural High School in 1933; received the Bachelor of Science degree from Southwestern College of Winfield, Kansas, with a major in Biology in May 1938; attended Biarritz American University, France, 1945; received Master of Science degree from Kansas State University, with a major in Zoology in May 1949; attended Michigan Biological Station, Cheboygan, Michigan, summer 1949; attended Rocky Mountain Biological Laboratory, summer 1957; completed requirements for the Doctor of Philosophy degree in May 1960.

Professional experience: Taught high school at Viola, Kansas, 1939-1940; taught Biology at Manhattan Senior High School, Manhattan, Kansas, 1940-1942; entered United States Army, 1942; received Battlefield Commission, Infantry, Germany, 1944; returned to Biology Department, Manhattan Senior High School, 1946-1958; assisted with research, Stored Grains, Department of Entomology, Kansas State University, summer 1958; Graduate Assistant, Department of Zoology, Oklahoma State University, 1958-1960.

Professional and honorary organizations: Order of Mound, Pi Gamma Mu (Southwestern College), Phi Delta Kappa, Sigma Xi, Phi Kappa Phi, Kansas Ornithological Society, Kansas State Teachers Association, National Education Association, Southwestern Association of Naturalists, Oklahoma Academy of Science.