DEVELOPMENT OF A SYSTEM FOR ASCERTAINMENT OF DOMINANT LETHAL EVENTS IN *LEBISTES RETICULATUS*

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DEVELOPMENT OF A SYSTEM FOR ASCERTAINMENT OF DOMINANT LETHAL EVENTS IN LEBISTES RETICULATUS

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SUMMARY

The need to screen artificial chemical pollutants has become apparent from the volume of such materials which are introduced into the water cycle and are, therefore, subject to unlimited dispersal. The present study demonstrates the activity of one such agent, triethylenemelamine, in a test of dominant lethality in the guppy, Lebistes reticulatus. By one method, doses of 0.1, 0.2, and 0.4 mg/kg, in addition to a control sham treatment, are injected into mature male guppies subsequently mated to virgin females. Secondly, mature male fish are allowed to swim in triethylenemelamine solution of known concentration for a period of twenty-four hours. These are similarly mated to virgin female guppies. Ten days following matings, the females are dissected, and evidence of dominant lethal events are ascertained from percentage of dead embryos and total number of embryos. By analysis of variance dose effects are demonstrated to be significant on both measures, resulting in higher percentages of dead embryos and lower total number of embryos with increasing dose.

CHAPTER I

INTRODUCTION

Concern over the need to regulate potentially harmful man-made products is among the foremost controversies between environmentalists and those who find use for such agents both commercially and otherwise. Since the discovery during World War II that nitrogen mustard [methyl-Di(2-chloroethyl)amine] had cellular inhibitory properties similar to x-rays (Auerbach, Robson, and Carr, 1947), a great variety of chemicals has come under scrutiny for their mutagenic activity. Compounding the problem is the fact that such chemicals often have a variety of effects, only the most recently discovered of which may be harmful. Thus, in the majority of mutagenic screening, suspect agents have already become useful for a variety of purposes.

Perhaps the most widely known group of chemical mutagens are the alkylating agents. In the present study, triethylenemelamine [2,4,6-tris-(aziridinyl)-s-triazine; TEM] has been selected for the induction of dominant lethal events in the common guppy, Lebistes reticulatus. TEM is a trifunctional alkylating agent derived from ethyleneimine. Its polyfunctional nature is the basis for the extreme mutagenic activity it possesses. Both intra and interstrand crosslinks may

result from the formation of diguaninyl derivatives with adjacent GpG or opposite GpC sequences. In the latter case, complete DNA strand separation is prevented.

TEM has been shown by thin-layer chromatography to become unstable in acidic environments, with a total breakdown almost immediately at pH 3.0 and a 95% reduction in twenty-four hours at pH 5.0 (Beroza and Borkovec, 1964). Smith et al. (1958), working with humans, used ^{14}C labeling of the triazine ring and found that up to 88% of the injected radiocarbon was excreted from the system in the first twenty-four hours. In addition, the ^{14}C level in the blood level dropped by 99% in the same period. They also determined that all of the TEM was metabolized, the primary products being ethanolamine and cyanuric acid.

Comparative mutagenic effects have shown a 4×10^{-4} molar dose of TEM to correspond with between 6,000 and 18,000 r of x irradition (Fahmy and Fahmy, 1956). Auerbach (1958) has reported that aberrations resulting in lethality are usually small deletions in the case of TEM, while the effect of x-rays results mainly from translocations. This apparently results from a greater inability of TEM induced breaks to rejoin.

Somewhat prior to its discovery as a mutagen, TEM found use as an orally administered chemotherapeutic agent, primarily in the treatment of chronic lymphocytic leukemia (Rundles et al., 1958). Its use as a chemosterilant in the house fly,

Musca domestica, has also been described (Lachance et al., 1969). Commercially, it has been used as a crosslinking agent in textiles, in the manufacture of resins, and in the water-proofing of cellophane. Walpole (1958) determined that TEM injections increased the incidence of lung tumors in strain A mice, but induction of sarcoma by subcutaneous injection in mice and rats required the addition of croton oil, indicating TEM's role in carcinogenesis to be that of an initiator.

Toxocity levels for this and other polyfunctional alkylating agents have been determined by Murphy et al. (1958) on adult and fetal rats, where the respective LD_{50}'s were determined to be 1.25 mg/kg and 0.55 mg/kg. The former is comparable to an LD_{50} of 1.0 mg/kg and for the same strain of rat (Wistar) as determined by Phillips and Thiersch (1950). They also determined that adult mice were somewhat more resistant, exhibiting an LD_{50} at 2.67 mg/kg. In this regard the toxicity of TEM was comparable to that of nitrogen mustard. Cattanach (1966) found a greater toxicity in older male mice (age one year) than in three of four month old mice of the same strain. Similarly, doses of a given magnitude had more severe effects on fertility in surviving older mice.

Mutation research with TEM has involved dominant lethal tests in Drosophila (Fahmy and Fahmy, 1954; Fano and Demerec, 1941), in mice (Epstein and Shafner, 1968; Bateman, 1960; Matter and Generoso, 1974), and in rats (Bateman, 1960). Cattanach (1967) has induced paternal sex chromosome loss in

male mouse post meiotic germ cells as well as paternal X chromosome abberations and autosomal mutations at specific loci. He has also used TEM for determination of induced specific loci mutation frequencies in mice as compared to spontaneous rates. The dose kinetics of TEM have been tested in prokaryotic systems by Iyer and Szybalski (1958).

Though the use of Lebistes in mutagenesis lacks the variety of investigations and the extensive literature of more familiar systems, considerable background information is available. Winge's (1927) description of a number of color genes on the heterogametic male's Y chromosome led to easy mutation detection in Lebistes by color polymorphisms. He also determined that the diploid chromosome number was 46. Goodrich et al. (1944) described two autosomal loci for chromatophore variations, providing a convenient system for tests of specific locus mutagenicity.

Schröder (1969a) applied from 500 to 2000 R x-radiation to primoridial germ cells in Lebistes and found differential viability effects on various germ cell stages, between successive generations, and between inbred and hybrid strains. Quantitative effects on neonatal guppies by acute (1000 R) x-radiation has also been demonstrated (Schröder, 1969b). He found a trend in treated guppies toward a broader fish of decreasing length and fewer number of vertebrae. Schröder and Holzberg (1972) showed an increase in viability of certain color phenotypes (wild type and blue) following 1000 R x-rays,

and estimated the mutation rate of guppies by such treatment to fall between Mus musculus and Drosophila. All but one of the original eighteen genes described by Winge (1927) were on sex chromosomes, and several were demonstrated to be capable of crossover between the X and Y chromosomes. Schröder (1969c) showed that this X-Y crossover was increased by spermatogonial exposure to x-rays. Teleosts have previously been used in dominant lethal studies by Purdom and Woodhead (1973) and Egami and Hyodo-Taguchi (1973). The former involved chronic irradiation of male and female Lebistes, and the latter was a similar study in the medaka, Oryzias latipes.

The present study is an attempt to blend knowledge of chemical mutagenesis from Drosophila and mammalian studies with a teleost system. The significance of such research lies in the dispersive potential of soluble chemical mutagens via the water cycle and the potential usefulness of a preliminary mutagenic screen for such water soluble mutagenic pollutants employing Lebistes. Lebistes has been chosen since its genetics have been worked out to some degree, though mutagenic studies involving it have concerned themselves primarily with radiation effects. This work follows two lines of mutagenic treatment. The first involves the application of several known doses of TEM to male Lebistes subsequently mated to virgin females of the species. Secondly, males are exposed for specified periods of time to TEM via known concentrations in the swimming media, with similar matings established following

treatment. Evidence of dominant lethal events will be based on total number of embryos and by percentages of live and dead embryos.

CHAPTER II

MATERIALS AND METHODS

Stocks and Maintenance Supplies

An initial outbred Lebistes stock was obtained from Petz, Inc. of Atlanta, and was occasionally supplemented by other purchases from local pet and fish dealers. These were used for development of procedures for all pilot experiments, and for the initial matings set up for the production of fry. For the experimental procedure described herein, the major stock was obtained from Raley Brothers Fish Farm, Gibsonton, Florida. This stock supplied both males for treatment and young which were selectively reared to isolate virgin females.

Stocks were maintained on a commercially available flake food produced by Hartz Mountain, Inc., containing recommended proportions of protein (48%), fat (2%), and fiber (5%). Feeding was done once daily--generally in the mornings--in amounts which would be devoured before settling. Food which settled and remained untouched for several minutes was removed by siphoning to defer bacterial buildup.

Equipment and Laboratory Conditions

Fish were housed in ten, fifteen, and twenty gallon aquaria, the total volume of all aquaria used varying from twenty to seventy-five gallons depending upon present stock

size. Tap water was used to fill the tanks, and was either allowed to sit for several days for purification purposes or was treated with a commercial thiosulfate solution prepared for removal of chlorine and flourine. Aquaria were not covered and the resulting evaporation and refilling allowed approximately a ten percent turnover in water weekly. A carbon filtration system, utilizing Dynaflo filter #425 (Metaframe, Inc.) was used with each tank, and carbon and fiber filter materials were changed approximately monthly. In addition to the open aquaria tops, the filtration inflow was arranged so as to provide aeration to the water. Aeration stones were used only on occasions when stocks were unusually large and aquaria became crowded. No flora was placed in the tanks.

Temperatures were held constant at 78° F (25.5° C) by vertical heaters suspended from the top edges of aquaria sides. In instances when fish were brought into the lab, several hours of temperature equilibration were allowed to prevent death from temperature shock. When dead fish were found in any of the aquaria, they were immediately removed. If losses began to increase rapidly in a particular tank, the water was completely changed and both the filtration system and the tank itself were thoroughly cleaned.

Rearing of Experimental Animals

Male and female guppies used in the experiment were reared from fry obtained in mid-July of 1975. From a total stock of several thousand fish, approximately one thousand

sexually immature young were separated from larger fish and reared in a group. Since the characteristic male colors become evident several weeks before the fish are sexually mature (Goodrich et al., 1944), daily removal of those beginning to display colors assured that selected females would retain their virginity. This is necessary because female Lebistes retain sperm and are capable of bearing eight to nine successive broods from a single mating (Goodrich et al., 1944). At the time of initiation of experimental matings in late December, virtually all surving fish had reached sexual maturity. Since the collection of virgin females was the limiting factor in beginning these matings, they were effected as soon as the required number were on hand.

Mutagenic Agent and Dose Determination

The mutagen chosen for experimental treatment was the trifuctional alkylating agent, triethylenemelamine (TEM). This was originally supplied by Dr. E. W. Cantrall of Lederle Laboratories. Since the definitive experiments actually were initiated one year later, a fresh sample supplied by Dr. Eugene Soares of the National Institute of Environmental Health was used. TEM solutions were made in fish ringer solutions, containing 6.5 g NaCl, 0.42 g KCl, and 0.25 g $CaCl_2$ per liter of distilled water. Five male fish, approximately the age and size desired for matings, were weighed and a mean weight was determined. Based on this average and an injection volume of

2×10^{-6} liter (which was determined in pilot work to be the most accurately measurable volume providing minimal leakage), the respective solutions were prepared. Hamilton microliter syringes equipped with 31 gauge needes were used for injections. These same solutions were used for the swimming experiment.

Experimental Procedure

Two methods were employed for the induction of dominant lethal events, both involving treatments applied to male fish subsequently mated to virgin females. Method One involved the intraperitoneal injection of male Lebistes with doses of 0.0 (sham treatment), 0.1, 0.2, and 0.4 mg/kg TEM. In method two, male fish were placed in the solutions used for method one injections and allowed to swim for a period of twenty-four hours. As a control, fish swimming in isotonic saline (used as a basis for injection solutions) for the same twenty-four hours were used. These experimental groupings thus provided for four doses by each of two application procedures, or eight total groups. Ten males were used in each group, and a twenty-four hour period of recovery following treatment was permitted before matings ensued. Pilot experiments had previously indicated that death by injection injury normally occurred within that amount of time, if at all.

On day one following treatments, ten virgin females were placed with each experimental and control group of ten males in fresh medium for mass matings. These were removed

after twenty-four hours, and subsequent groups of females were introduced into the same male tanks. A series of five such twenty-four hour mass matings were effected, yielding a total of fifty females in each of the experimental and control groups, or a total of 400 matings. Each mating group of ten females was maintained in a one liter beaker following removal from the male tanks.

Scoring of Results and Statistical Analysis

Ten days following each mating period, female fish were decapitated and dissected, and numbers of live and dead embryos (based on differences in size, coloration, and vascularization determined in pilot experiments), were recorded for each female successfully mated. Percent dead embryos were converted by the arcsine transformation and total embryos by the square root transformation of Mousteller and Youtz (1961) for normalization of data distribution. A three way analysis of variance in percent dead embryos and total embryos was calculated, considering methods of treatment, mutagenic dose, and day of mating. One way analysis of variance for arcsine transformed percent dead embryos and square root transformation of total embryos each versus method, dose, and day, respectively, were also calculated.

CHAPTER III

RESULTS

From total numbers of live and dead embryos in each mating, the arcsine transformed percentage dead and square root transformation of total embryos were analyzed for effects on variance by method (TEM injections or swimming in TEM solutions); by dose (0.0, 0.1, 0.2, 0.4 mg/kg and injection solutions for swimming); and by day of mating following treatment (1-5). Data are based on a total (in all cells) of 253 successful matings out of 400 which were set up. A portion of these unsuccessful matings resulted from death of females prior to dissection, and the remainder from failure of fertilization or, possibly, early deaths not ascertained by these methods.

Table 1 is an analysis of variance table for arcsine transformed percent dead embryos versus dose, method, and day of mating. From the significance figures of F values (where significance is accepted for $p < .05$), it is apparent that the day of mating provided virtually no variation in group mean values. On the other hand, method of treatment produced highly significant effects on the percentage of dead embryos ($p \simeq .008$) and effects due to dose were highly significant ($p \simeq .001$). Similarly, Table 2, which is an analysis of variance for the square root transformation of total embryos,

Table 1. Three Way Analysis of Variance.

Arcsine Transformed Percent Dead Embryos
Versus Method, Dose, and Day

Source of Variation	Sum of Squares	Degrees Freedom	Mean Square	F
Main Effects	72741.839	8	9092.730	37.444*
Method	1746.096	1	1746.096	7.190*
Dose	68071.231	3	22690.410	93.439*
Day	90.136	4	22.534	.093
Two Way Interactions	2381.185	19	125.326	.516
Method x Dose	387.996	3	129.332	.533
Method x Day	438.008	4	109.502	.451
Dose x Day	1560.142	12	130.012	.535
Three Way Interactions	2028.362	12	169.030	.696
Method x Dose x Day	2028.362	12	169.030	.696

*$p < .01$

Table 2. Three Way Analysis of Variance

Square Root Transformation of Total Implants
Versus Method, Dose, and Day

Source of Variation	Sum of Squares	Degrees Freedom	Mean Square	F
Main Effects	56.801	8	7.100	6.028*
Method	16.014	1	16.014	13.595*
Dose	32.679	3	10.893	9.248*
Day	1.989	4	.497	.422
Two Way Interactions	16.146	19	.850	.721
Method x Dose	4.013	3	1.338	1.136
Method x Day	4.487	4	1.122	.952
Dose x Day	7.750	12	.646	.548
Three Way Interactions	8.853	12	.738	.626
Method x Dose x Day	8.853	12	.738	.626

*$p < .01$

shows no effect on variation in group means related to day of mating, but significant effects are indicated due to method of treatment ($p \simeq .001$) and dose ($p \simeq .001$). For total numbers of live and dead embryos with dose and method groups, see the Appendix. One way analyses of variance were also computed for the individual cases of arcsine transformed percent dead embryos and square root transformations of total embryos versus method and versus dose.

Figures 1 and 2 are dose response curves for percent dead embryos and total embryos with separate regressions for each treatment. Each consists of points plotted for group means within each dose as a variable dependent upon the dose. The computations for the regression coefficient and Y intercept were carried out as outlined in Biometry by Sokal and Rohlf (1969). The slope of both lines are significant at the .05 level for percent dead embryos versus dose, but slope of both lines for total embryos versus dose are not significant.

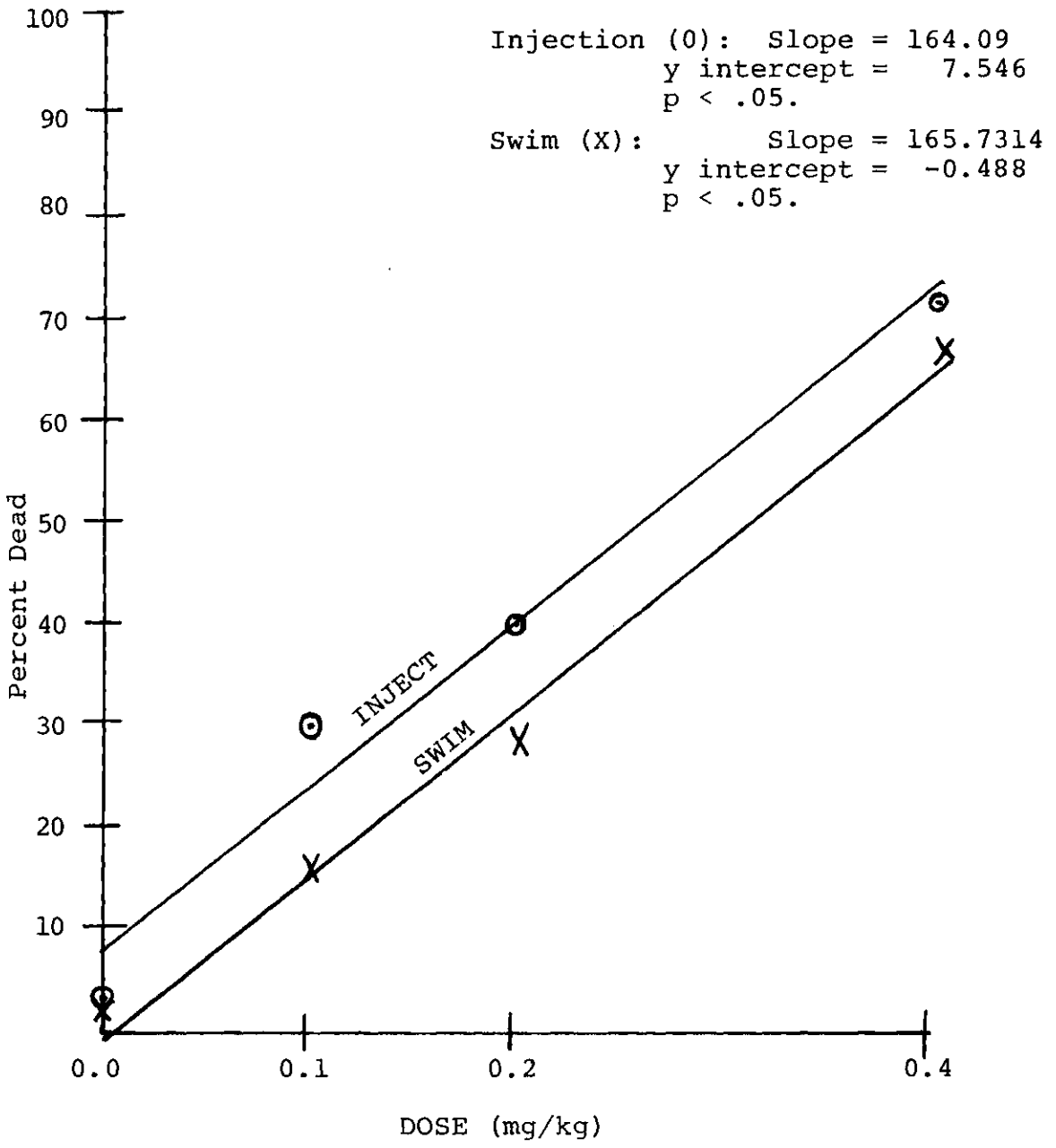

Figure 1. Percent Dead Embryos Versus Dose.

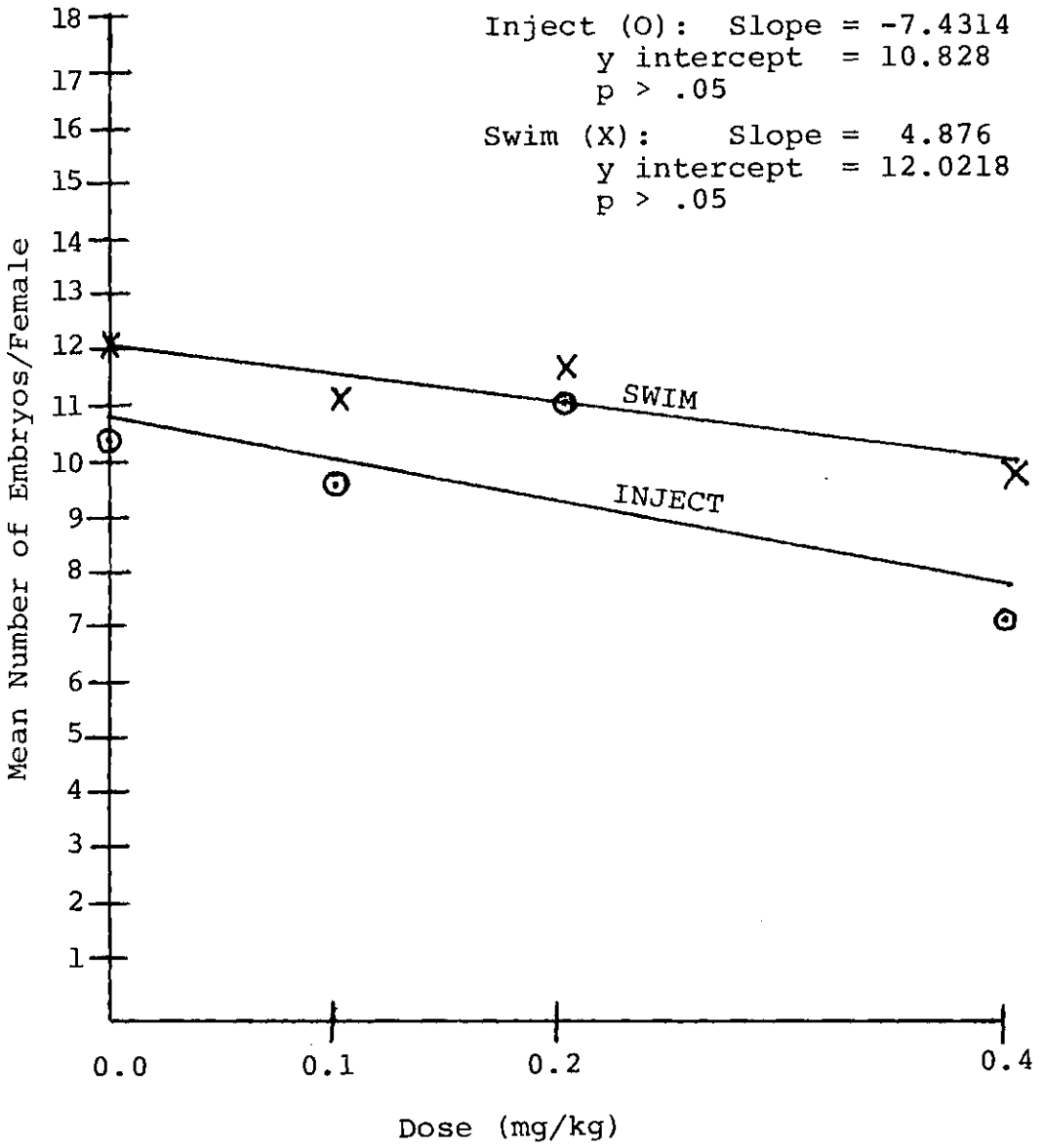

Figure 2. Mean Number of Embryos/Female Versus Dose.

CHAPTER IV

DISCUSSION OF RESULTS

Dose Effects

Although these results lead to more questions on various points, interpretation of some trends is rather straightforward. The main effects we see, upon both percentage of dead embryos and total number of embryos, result from dose and method of dose application. As far as variations in dose, increasing percentages of dead embryos with increased amount of mutagen concentration follow from our basic understanding of mutagenic effects. The highly significant nature of the differences demonstrated in our analyses of variance bears this out. There is some question as to the linearity of response, though our dose response points seem to support such a relationship. Generally, dominant lethal events might be expected to follow more closely a curvilinear response, characteristic of events due to two-hit phenomena (Dempster, 1941 and Fahmy and Fahmy, 1954). However, TEM is a polyfunctional agent, and may react with both strands of DNA to form covalent, interstrand crosslinks. Thus, one "hit" may result in a major aberration. This basic variation in dose response for dominant lethal mutations has been demonstrated by Matter and Generoso (1974) with EMS and TEM.

Results from those animals treated by emersion in the

TEM solutions lead to the same line of reasoning for the most part. Increasing concentration of the TEM solution resulted in a greater percentage of dead embryos. Data from these two methods are shown in the graph in Figure 1.

Effects of dose on total number of embryos, analyzed after square root transformation and with both methods of treatment pooled, were also significant. Figure 2 indicates the fit of linear regressions; however, the slopes of the lines are not significant at the .05 level. Further, data from the control and two lower doses do not suggest a general trend toward a reduction in total embryos with increasing dose, rather a reduction in number of embryos becomes apparent only at the highest (0.4 mg/kg) dose. This is consistent with the findings of Generoso et al. (1974) that preimplantation losses increase appreciably at higher doses only. These data strongly indicate that mechanisms responsible for late loss of embryos and operative at the lowest TEM dose employed, are different from those leading to reductions in numbers of embryos and presumably due to early loss of zygotes.

Effects of Methods

In addition to the group mean variations due to dosage, the two different methods of treatment--injection and swimming--also showed significantly different effects upon both percentage of dead embryos and total implants. In each case, the injection method had an apparently greater deleterious effect on viability. Unlike dosage effects, however, it is difficult

to compare precisely the implications of the differences in effects due to injection and swimming methods. The latter was an attempt to measure a mutagenic effect based only on emersion of an organism in solution of known mutagenic concentration for a given time. Certainly the measurement is useful since, in an environmental situation, the most readily measurable variant would be such a concentration. Until physiological data are available on the amount of a given chemical agent that may be taken in by an organism based on concentration in medium, duration of exposure, and other variables, this is a most useful method of obtaining comparative measurements. Indeed, what is certainly being dealt with is a dosage effect, but the methods of this study do not include a means of determining precise dose to gonad. For that matter, neither is it certain what the precise dose to gonad for a given I.P. injected dose is, but the relationship is likely more direct.

Problems of Dominant Lethality

The concept of dominant lethality is one which is not defined altogether clearly. Although there is little doubt that such events occur with a genetic basis, only cytological tests can prove whether chromosome aberrations result in a specific event. Muller, in his classic work demonstrating the mutagenic effects of x-rays on Drosophila (1927), found what he considered evidence of dominant lethal mutations in the overall reduction in egg counts and variations in the resulting

sex ratio in the F_1 generation.

Dominant lethal estimates may be based on reduced total numbers of embryos, or on reduced percentages of embryos which remain viable. The distinction here is between an early event versus a later effect on a more established embryo. This study has by design discounted one possible indication of early post fertilization lethality, that being females which were not found to be pregnant by the tenth day of gestation (the point of dissection). Many of these resulted from lack of copulation or fertilization, but certainly some may have been fertilized and the effects of dominant lethality may have been early enough to eliminate them from consideration. In fact, Fahmy and Fahmy (1954) found most unhatched Drosophila eggs in a dominant lethal study with TEM to have been fertilized. Thus, the only measure known of such early deaths are the total numbers of embryos for those fish which were found to be gravid. A. J. Bateman (1960) points out that TEM is known to reduce fertility in male rats--possibly by functional oligospermy--resulting in sperm incapable of fertilization. However, these are not considered dominant lethal mutations, which are usually defined for practical purposes as post-fertilization deaths.

Results obtained from the present study differ in methods of ascertainment of dead embryos from those of Purdom and Woodhead (1973). While they demonstrated dominant lethal effects induced by x-rays based on fecundity in the guppy, their measure was numbers of live births. They were unsuccessful in

attempts to distinguish early deaths by mullerian duct contents of gravid females. The present study uses counts of live and dead embryos from dissections conducted approximately 35% through gestation. Identifications were based on earlier pilot work which revealed readily identifiable differences between living and dead embryos. Dead embryos were distinguishable by size (approximately one-half that of live embryos at 10 days), and coloration (appearing very whitish, semiclear, and without the familiar black and silver coloring and red pigmented vascularization of viable embryos). Both were often found in the same litters.

Besides the question of timing of lethal events, there is the possibility of other effects. Probably the most important of these is toxicity. This has been measured in Wistar strain rats by Phillips and Thiersch (1950) and Murphy et al. (1958). Both found and LD_{50} in adult rats to be approximately 1.0-1.25 mg/kg TEM, and for rat fetuses an LD_{50} of 0.55 mg/kg was determined. Mice, however, were somewhat more resistant. Phillips and Thiersch (1950) measured the LD_{50} of CFW mice to be 2.66 mg/kg TEM. Virtually all deaths resulting from doses of less than the LD_{50} to several times LD_{50} did not occur for several days. In the present study, doses were well below LD_{50} estimates from earlier studies. Additionally, nearly all post-treatment male deaths followed immediately after injection, and were randomly distributed over all groups, including controls.

Measured against one other often used chemical mutagen, EMS, TEM appears to be a good choice in consideration of its effects as a mutagen and its toxicity levels. Matter and Generoso (1974) measured genetically effective doses and lethal doses for both chemicals. The respective ratio for EMS was 1:3.5, whereas, for TEM, it was 1:100. This lends some support to the hypothesis that we are dealing primarily with genetic effects, at least in regard to proportion of dead embryos.

Reliability of Measurements

A final question which deserves comment is the magnitude of the effects themselves, and whether they are consistent with the work of other investigators. Schröder (1969a) concluded that Lebistes is a little less sensitive to mutation than Mus musculus. Epstein and Shafner (1968) have employed a mitotic index based on dominant lethal mutations in mice treated with TEM. This index is a measure of deciduomata and late deaths divided by total implantations, and multiplied by 100 for a percentage result. They employed one dose coincidental with the present study, that of 0.2 mg/kg. This was determined to yield a mitotic index of 26 in mice, whereas our results from the I.P. injection method are computed to give an index of 32 for the 0.2 mg/kg dose in Lebistes. This is not consistent with Schröder's conclusions. However, it should be noted that Schröder's measurements were by radiation mutagenesis, involved a different strain of

Lebistes, and employed a measure other than dominant lethal events. Fahmy and Fahmy (1954), working with Drosophila, have indicated that, for equivalent mutagenic doses, TEM is a more effective inducer of dominant lethals than is x-radiation. This supports an explanation of the discrepancy between the present results and those of Schröder, being based upon differences in effect of mutagenic agent.

CHAPTER V

CONCLUSIONS

Results from this study confirm the hypothesis that triethylenemelamine is a potent agent in the induction of dominant lethal events in Lebistes reticulatus. It is evident that the application of mutagenic doses is not limited by artificial treatment (i.e. injection), but that some agents may also be assimilated by the organism in the aquatic environment. Effects due to dose are apparent: With increasing amount of agent in injections or swimming solution, lethal events increase. This is apparent from data both on percentage of dead embryos for the greatest dose, and on total embryos. The evidence also suggests that injection may be more effective than a twenty four hour emersion in injection medium of similar concentration.

Though it is not clear or even likely that every lethal event ascertained is genetic in origin, the simplest hypothesis is that many of these must be the result of chromosome aberrations, and therefore dominant lethal mutations. In addition, some lethal events do not occur at the early post-fertilization stage, but result in embryonic deaths sometime later. Lethal effects with TEM are apparent at dose levels far below those which could be expected to result in death from toxicity. In

this regard Lebistes responds similarly to other animals which have undergone similar treatment.

CHAPTER VI

RECOMMENDATIONS

The results of this work indicate the need for several areas of new or further testing:

1. Further studies on the effects of exposure to pollutant mutagens in aquatic solution by emersion of the experimental animals, and determination of dose assimilated and dose to gonad by such exposure.
2. Cytological evaluation of actual chromosome damage leading to dominant lethal events induced by chemical mutagens in fish.
3. Determination of toxicity levels of chemical mutagens in fish, and comparison of toxic levels between injected dose and those assimilated from the swimming environment.
4. Overall sensitivity of fish to chemical mutagens in comparison with Drosophila and mammalian systems.

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APPENDIX

APPENDIX

GROUP DATA TOTALS

Method	Dose (mg/kg)	Day	Live Embryos	Dead Embryos	No. Females
Inject	0.0	1	66	0	6
Inject	0.0	2	53	3	5
Inject	0.0	3	42	0	4
Inject	0.0	4	60	2	7
Inject	0.0	5	30	4	3
Inject	0.1	1	37	16	7
Inject	0.1	2	53	28	9
Inject	0.1	3	49	17	6
Inject	0.1	4	29	14	4
Inject	0.1	5	26	8	4
Inject	0.2	1	23	15	4
Inject	0.2	2	32	18	4
Inject	0.2	3	23	18	4
Inject	0.2	4	52	27	7
Inject	0.2	5	23	22	4
Inject	0.4	1	11	37	7
Inject	0.4	2	10	30	5
Inject	0.4	3	15	37	7
Inject	0.4	4	17	32	7

APPENDIX (CONTINUED)

Method	Dose (mg/kg)	Day	Live Embryos	Dead Embryos	No. Females
Inject	0.4	5	11	29	6
Swim	0.0	1	79	6	7
Swim	0.0	2	102	0	9
Swim	0.0	3	109	1	9
Swim	0.0	4	84	3	7
Swim	0.0	5	86	1	7
Swim	0.1	1	65	10	7
Swim	0.1	2	82	13	9
Swim	0.1	3	72	17	8
Swim	0.1	4	99	14	10
Swim	0.1	5	66	16	7
Swim	0.2	1	58	32	8
Swim	0.2	2	55	19	7
Swim	0.2	3	67	25	8
Swim	0.2	4	53	14	6
Swim	0.2	5	60	24	6
Swim	0.4	1	20	42	6
Swim	0.4	2	22	43	6
Swim	0.4	3	24	47	8
Swim	0.4	4	10	40	6
Swim	0.4	5	15	24	3