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Genetic Effects of the Recessive Sex-Linked Lethal Gene, Tortoise, on the Prenatal Mortality of Mice

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THE GENETIC EFFECTS OF THE RECESSIVE SEX-LINKED LETHAL GENE, TORTOISE, ON THE PRENATAL MORTALITY OF MICE

by

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A Thesis
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TITLE OF THESIS

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ABSTRACT

Data on the prenatal mortality rate among embryos from the heterozygous Tortoise (To/+) show a statistically significant increase above the prenatal mortality rate determined for embryos from the normal (+/+) females. This suggests that the Tortoise gene is translated prior to day 6½ post vaginal plug. The prolonged post implantation viability observed for embryos with the Tortoise gene indicates that the lethal effect of this gene is dependent on the interaction between the embryo genome and intra-uterine factors.

ACKNOWLEDGMENT

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The investigation of embryonic mortality in mice due to a mutant gene facilitates an understanding of the causes of abnormal development in similarly inherited human diseases. By identifying how mutant genes alter normal development, an insight into how genes control the developing organism may be attained (Green, 1966).

The changes induced by a mutant gene on the normal developmental pattern of an organism are genetic, physiologic, biochemical, and morphological in nature. The mutant gene can accomplish these changes by altering the natural process of DNA replication, transcription (the process by which deoxyribonucleic acid (DNA) codes for messenger ribonucleic acid (mRNA)), translation (the process by which messenger RNA codes for proteins), and the post translational utilization of the protein synthesized. If transcription or translation are altered, proteins necessary to normally developing embryos may not be synthesized in appropriate amounts. The physiologic changes that occur result in the accumulation of products which may ultimately result in the death of the organism. Morphologic changes result from the accumulation of molecular errors leading to embryos with anatomical modifications incompatible with life. All the above contribute to the phenotypic expression of the lethal

mutant gene.

The phenotypic expression of a recessive sex-linked lethal gene may assume three forms. (1) It can make its expression in the homozygous state (normally observable only in females). (2) It can make its expression in the heterozygous state (normally observable in heterozygous females showing an appropriate number of cells with the mutant gene active). (3) It can make its expression in the hemizygote individual (normally observable only in males). This project is concerned with determining the time at which the recessive sex-linked lethal gene Tortoise exerts its lethal effect on mouse embryos. In general, the recessive sex-linked Tortoise gene exerts its effect prenatally. Nearly all the hemizygous Tortoise males and a limited number of Tortoise heterozygous females are lost. Such expression of the Tortoise lethal recessive gene will result in a deviation from the expected Mendelian segregation ratio. By using the Punnet square to demonstrate the mating between a heterozygous (To/+) female and a hemizygous (+/Y) normal male, this deviation of the progeny ratio becomes evident:

		Female Gametes: To	Female Gametes: +
Male Gametes	+	To/+♀	+/+♀
	Y	To/Y♂ (dies)	+/Y♂

Nearly all hemizygous males which inherit the Tortoise gene and some of the heterozygous (To/+) females will die. This results in a ratio of 3♀'s to 1♂ at birth.

Among affected animals, there are various degrees of expression of the Tortoise gene. These can be explained by the Lyon's Hypothesis and/or the Grüneberg's Hypothesis.

The Grüneberg hypothesis postulates that in the mammalian X chromosome there exists a center from which an inhibition of gene action spreads along the X chromosome with diminishing intensity (Grüneberg, 1967). For this to occur, two X chromosomes must be present. Grüneberg (1967) further states that "both centers are activated together and that the inhibiting action spreads along both X chromosomes." However, this does not necessarily mean that both chromosomes are inactivated equally. Cells with chromosomes on which the normal gene is expressed will exhibit the normal genotype, and those cells in which the chromosome with the mutant gene is expressed will exhibit the abnormal characteristics.

The Lyon's hypothesis postulates the random inactivation of one or the other of the two X chromosomes at an early stage of development, the cells with the mutant phenotype having the X chromosome with the normal gene inactive and vice versa (Lyon, 1968). This inactivation can be of either the maternal or paternal X chromosome and is irreversible so that all the subsequent generations of that cell will have the same chromosome inactive. This

hypothesis predicts that for sex-linked genes the heterozygous females will be mosaics, that is, the tissues in which a sex-linked gene is active should exhibit both normal and abnormal features characteristic of the random inactivation (Green, 1966). If the Lyon's hypothesis is true, those tissues in which more mutant genes are inactivated than normal ones will appear normal, and those that have more normal genes inactive than mutant ones will exhibit the abnormal characteristics. This is one way in which the different degrees of gene expression in the heterozygous (To/+) females can be explained.

The embryo is also subject to non-genetic factors which control or influence its development. Evidence in support of the fact that the age of the dam has an influence in prenatal mortality has been reported by Hollander and Strong (1950). They observed that there was an increased embryonic mortality in older female mice.

Parity, the state of having borne offspring, also plays a factor in the prenatal development of the mouse. It has been found that the number of corpora lutea tends to be considerable lower in females pregnant for the first time than in later pregnancies (Hollander and Strong, 1950; and Rugh, 1968). This may be due to the "insufficiency of the corpora lutea to supply the progesterone requirements of the excessive number of implantations" (Bowman and Roberts, 1958).

The ovulation rate has also been found to be an important factor in embryonic mortality as demonstrated by Bowman and Roberts (1958). They found that before implantation, the loss of eggs increased as the number of eggs shed per horn increased. They further state "as the number of eggs shed into a uterine horn increases the probability of each individual egg implanting decreases." The ovulation rate has been found to be genetically related by Land (1970). He reported that Follicle Stimulating Hormone (FSH) activity and ovarian sensitivity which control the rate of ovulation were correlated as measured by the response to Pregnant Mare Serum (PMS). Bradford (1969), working on the assumption that following superovulation the ability of female mice to gestate a larger litter might be improved because of a large number of embryos, found that selection for litter size resulted in an increase in the ovulation rate but he did not find the opposite to be true. In other words, an increase in the ovulation rate did not result in an increase in the litter size. One factor which might limit the size of the litter or the total number of implants per mouse surviving to birth is "the level of some substance circulating in the maternal blood supply" (Bowman and Roberts, 1958). This substance could be a prostaglandin. A prostaglandin is an acid composed of twenty carbon atoms. It is a lipid soluble compound biosynthesized from the essential fatty acids which

are present in the seminal fluid and extracts from some of the accessory genital glands (Eliasson, 1963). Nutting and Cammarata (1969), while working with rats into which PGE_2 or $PGF_{2\alpha}$ was injected subcutaneously during the first seven days of pregnancy, demonstrated that: (1) the prostaglandins had an antifertility action after implantation resulting in the reabsorption of the implantation sites; (2) that the prostaglandin inhibited implantation of the zygote; and (3) that there was a delay in the transport of the fertilized ova to the uterus as a result of the effects of the prostaglandin on smooth muscle activity.

MATERIALS AND METHODS

Animals

The female mice used in these experiments were litter mate homozygous C57BL/6J normal (+/+) and heterozygous C57BL/6J mutant (To/+). These animals were purchased from Jackson Laboratory, Maine or were produced in our laboratory at Northern Michigan University. All animals appeared to be in good physical condition at the time of their use.

Matings

Sets of litter mates, normal and tortoise-shell, were mated pairwise to males of the C57BL/6J strain. Mating was determined by the presence of the vaginal plug. The morning when the vaginal plug was observed is designated as day 1 in this study.

Collection of ovaries and uterus

Females were sacrificed by cervical dislocation on day 6½ to 15½ post-vaginal plug. The intact uterus, oviduct and ovaries were immediately removed and placed in a petri dish containing cold physiological saline (0.9% NaCl in distilled water). The ovary and corresponding horn of the uterus were given code numbers and transferred to separate petri dishes.

Corpus Luteum count

The ovaries were cleaned of all adhering tissue, transferred to a depression slide and the number of corpora lutea were counted under the dissecting microscope. This was done by inserting a pin through the ovary and rotating it noting the starting point. The number of corpus luteum counted was used as an estimate of the number of eggs ovulated by the female. There was some difficulty in obtaining an accurate count of corpora lutea in all stages of pregnancy observed in this study. If the corpora lutea count was less than the number of embryos implanted, the data was excluded from the analyses. The data was recorded for both normal (+/+) and mutant (To/+) female mice as outlined in table 1.

Implantation (count) sites

The number of implantations were scored by counting the number of embryos present in the uterus. Both the number of normal and abnormal implantations were recorded. There is a localized swelling at each implantation site and this denotes the fact that the blastocyst is beginning to grow rapidly. Identification of implantation sites and abnormal embryos that died early presented difficulties. The early 6½, 7½, 8½ day uteri were observed after clearing and bleaching to take advantage of pronounced vascular changes which occur soon after the

blastocyst contacts the endometrium (Orsini, 1962).

Table 1. Experimental layout for recording the number of corpora lutea and implantations counted

GENOTYPE	DAYS POST VAGINAL PLUG	DETERMINATIONS
To/+	6½ 15½	1 10
+/+	6½ 15½	

In order to detect abnormal embryos, it was necessary at times to stretch the uterus. These abnormal embryos are smaller than the living or normally developing ones, and they are characterized by a lack of color or bleaching because circulation to the fetus has failed (Hollander and Strong, 1950). The implantations were recorded according to the outline in Table 1.

A total of 200 female mice were used, 100 normal female (+/+) and 100 heterozygous females carrying the Tortoise gene (To/+).

The number of post implantation deaths is related to the number implanted, therefore, to obtain unbiased statistical comparisons between tortoise and normal females for post implantation mortality, the observed number of dead embryos was adjusted to correct for the difference between the two genotypes in the number of eggs implanted. The adjusted data were subjected to the Regression Analysis test (Woolf, 1968).

RESULTS

The mean number of corpora lutea observed in mutant and normal female mice at specific stages of gestation is shown in Figure I. The two lines are in close agreement which suggests that the genotype of the dam has no effect on the mean number of eggs shed, i.e. the presence of the Tortoise gene does not significantly alter the number of eggs ovulated by the female.

The Analysis of Variance test comparing the observed number of corpora lutea (eggs shed) indicates no statistically significant difference between the two genotypes (Table 2).

The comparison to determine the effect of fetal age or age sacrificed on the number of corpora lutea is statistically nonsignificant. This is expected because the number of eggs ovulated is independent of post conception time, stage of gestation and fetal age. The preimplantation loss is sumarized in Figure II. This was determined by the difference between the number of eggs ovulated and the number of eggs implanted. The value for the mutant genotype is consistently larger than the value for the normal genotype.

The mean number of implants for each genotype is plotted against days post vaginal plug in Figure III. Both

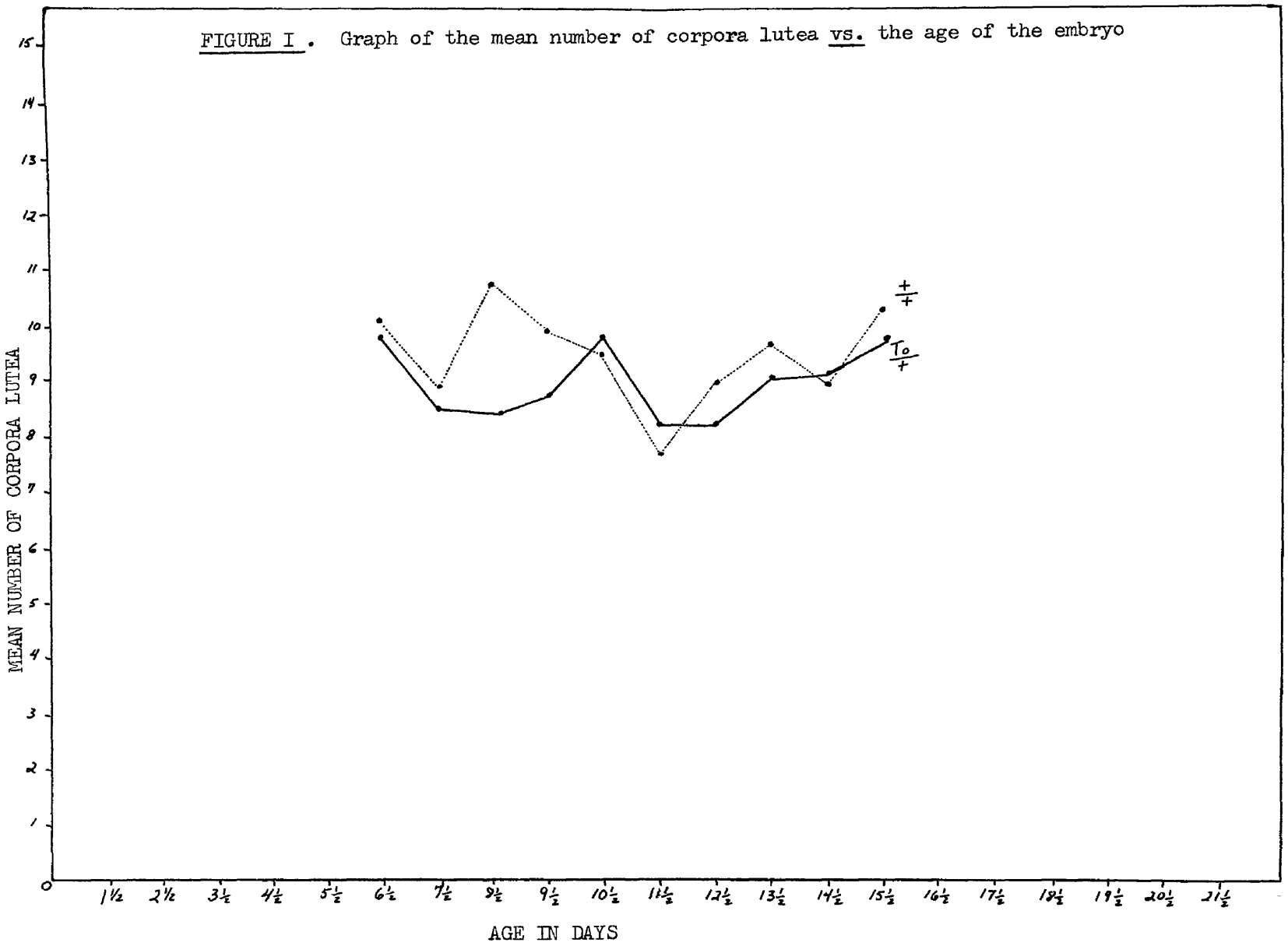


FIGURE I . Graph of the mean number of corpora lutea vs. the age of the embryo

Table 2. Analysis of Variance for Corpora Lutea Count.

Source of Variation	d.f.	S.S.	M.S.	F	Probability
Total	199	1609.52	8.0880		
Genotype(Dam)	1	13.52	13.52	1.6282	>0.05
Age(Fetus)	9	102.82	11.4244	1.3759	>0.05
Age x Gen.	9	0.0	0.0	0.0	
Error	180	1494.40	8.3032		

FIGURE II. Graph of pre-implantation loss

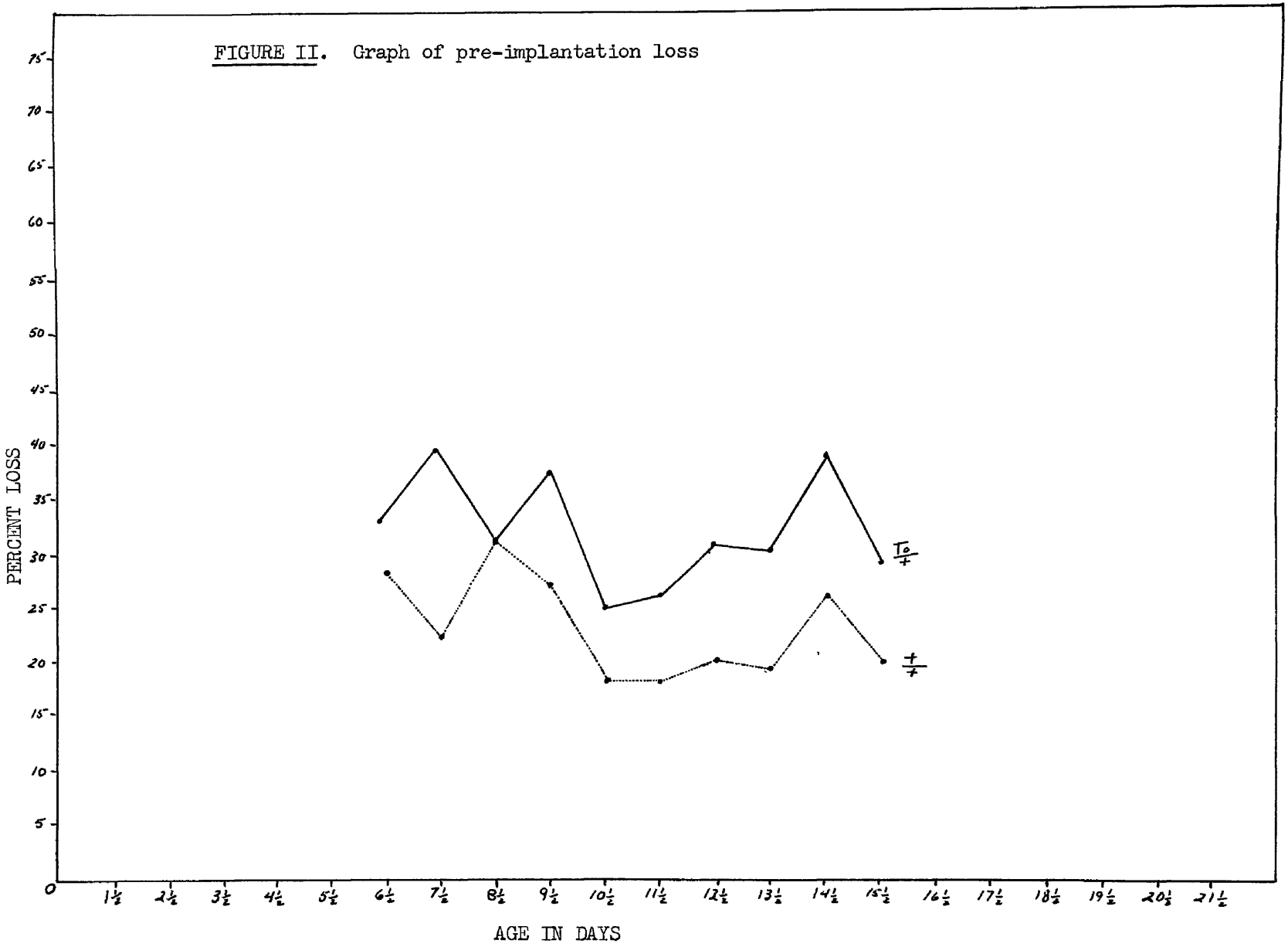
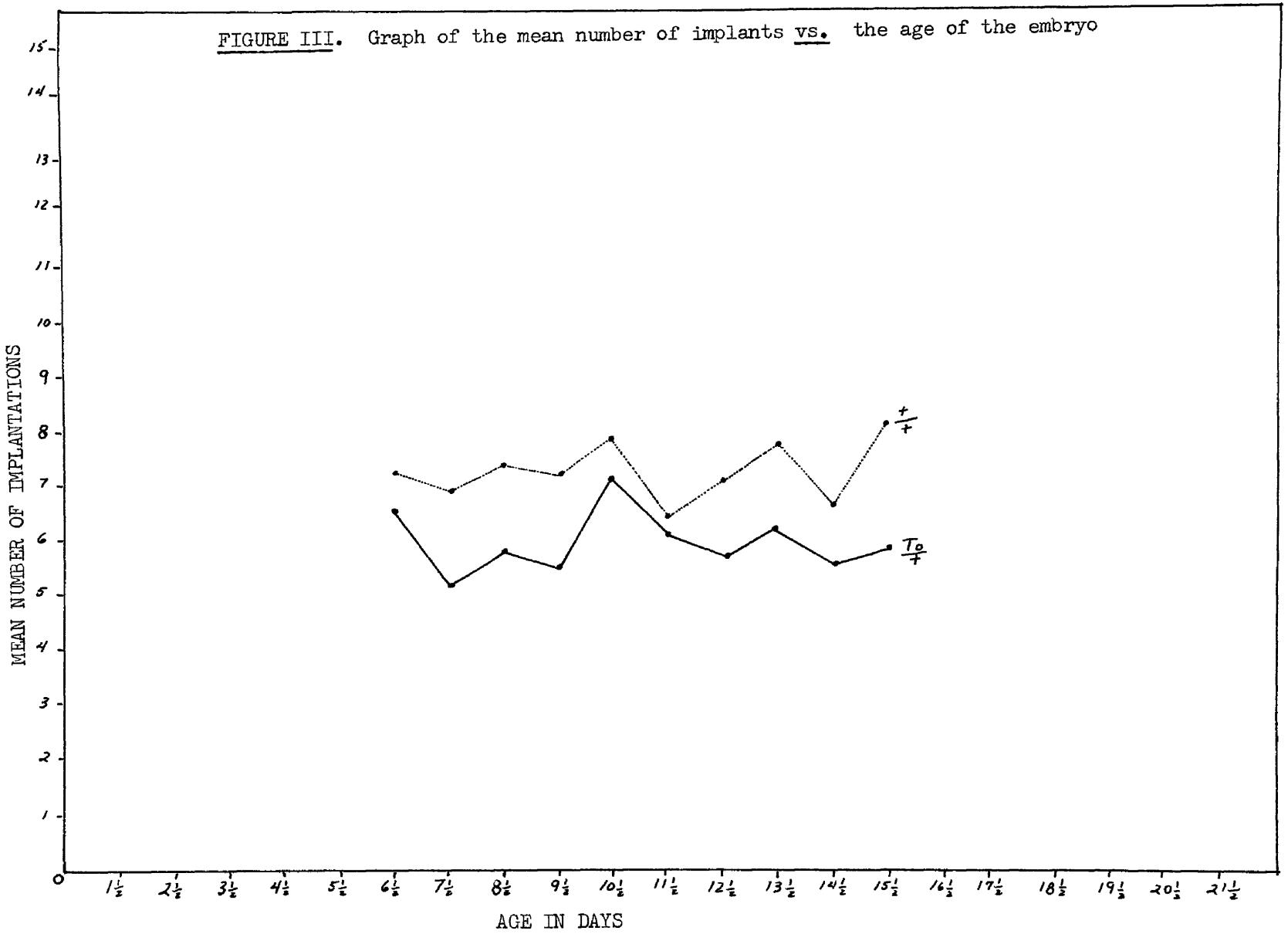


FIGURE III. Graph of the mean number of implants vs. the age of the embryo



plots show similar trends, with the exception that the line for mutant genotype is consistently lower than that of the normal genotype. This suggests that a larger number of embryos are lost and that the lethality of the Tortoise gene is translated by day 6½ post vaginal plug.

The Analysis of Variance test comparing the number of implants observed in the mutant and normal mice indicates statistically significant differences in the mean number of eggs implanted by each genotype (Table 3). The number of implants estimated decreases significantly as the pregnancy advances. This is because, as gestation progresses, it becomes more difficult to identify neoimplantation losses.

Within age group (day), regression coefficients for the number of embryos implanted on the number of eggs shed were calculated for both normal and mutant genotypes to determine if a linear relationship exists between the number of eggs shed and the number of eggs implanted. Each regression coefficient was tested for statistical significance using the "t" test (Woolf, 1968). If the calculated regression coefficient was significantly different from zero, the observed number of implantations was adjusted to make them independent of the number of eggs shed. Use of the adjusted number in future tests of significance permits unbiased estimates of the effect of genotype on post implantation loss. The regression coefficients and tests of significance are sumarized in Table 4. Significant linear relationships

Table 3. Analysis of Variance for Implantation Count.

Source of Variation	d.f.	S.S.	M.S.	F	Probability
Total	199	249.52	1.2538		
Genotype	1	72	72	93.1677**	< 0.01
Age	9	46.02	5.113	6.6162**	< 0.01
Age x Gen.	9	0	0	0	
Error	180	139.12	0.7728		

* significant at 0.05
** significant at 0.01

Table 4. Within Day Regression for the Number of Eggs Implanted on the Number of Eggs Shed.

Genotype	Age in Days	Number of Litters	Number of C.L.	Number of Implants	Regression Coefficient
To/+	6½	10	98	66	0.0952
	7½	10	85	52	0.5679*
	8½	10	84	58	0.4530**
	9½	10	87	55	0.7237**
	10½	10	96	72	0.5219**
	11½	10	82	61	0.5911**
	12½	10	82	57	0.5693**
	13½	10	90	62	0.5324**
	14½	10	91	56	0.4129**
	15½	10	97	69	0.2579
Summary		100	892	608	0.5205
+/+	6½	10	101	73	0.4876**
	7½	10	89	69	0.4737**
	8½	10	107	74	0.6050**
	9½	10	99	72	0.6992**
	10½	10	95	79	0.7837**
	11½	10	77	64	0.7885**
	12½	10	89	71	0.6895**
	13½	10	96	78	0.6777**
	14½	10	89	66	0.6411**
	15½	10	102	82	0.6010**
Summary		100	944	728	0.6243

* significant at 0.05 level of probability
** significant at 0.01 level of probability

exist in days 6½ through 15½ in normal mice. Regression coefficients for the mutant mice are significant on days 7½, 8½, 9½, 10½, 11½, 12½, 13½, and 14½.

The Analysis of Variance on the adjusted number of implants between the mutant and normal mice indicates a significant difference in the number of eggs implanted by each genotype (Table 5). The genotype, therefore, has been found to be a highly significant factor indicating that the Tortoise gene is exerting its lethal effect, and that these losses increase with the age of the embryo. The postimplantation loss is summarized in Figure IV.

Within day, regression of the number alive on the number implanted was also obtained. This was done to determine if there exists a linear relationship between the number of implants and the number of live embryos observed (Table 6). Regression coefficients are significant in days 8½, 9½, 10½, and 11½ for mutant mice.

An Analysis of Variance of the adjusted value for the number of live embryos (adjusted for the within day regression coefficient) was obtained for both the mutant and normal mice. There is a highly significant difference in the mean number of live embryos for each genotype as shown in Table 7. This is because the Tortoise gene has already been translated and there is greater mortality in the embryos from the heterozygous (To/+) females. The total prenatal loss associated with each genotype is presented in Figure V.

Table 5. Analysis of Variance for Adjusted Implantation Count.

Source of Variation	d.f.	S.S.	M.S.	F	Probability
Total	199	278.421	72.989		
Genotype	1	67.745	67.745	70.861**	< 0.01
Age	9	31.862	3.540	3.703*	< 0.05
Age x Gen.	9	6.729	0.748	0.782	
Error	180	172.085	0.956		

* significant at 0.05
** significant at 0.01

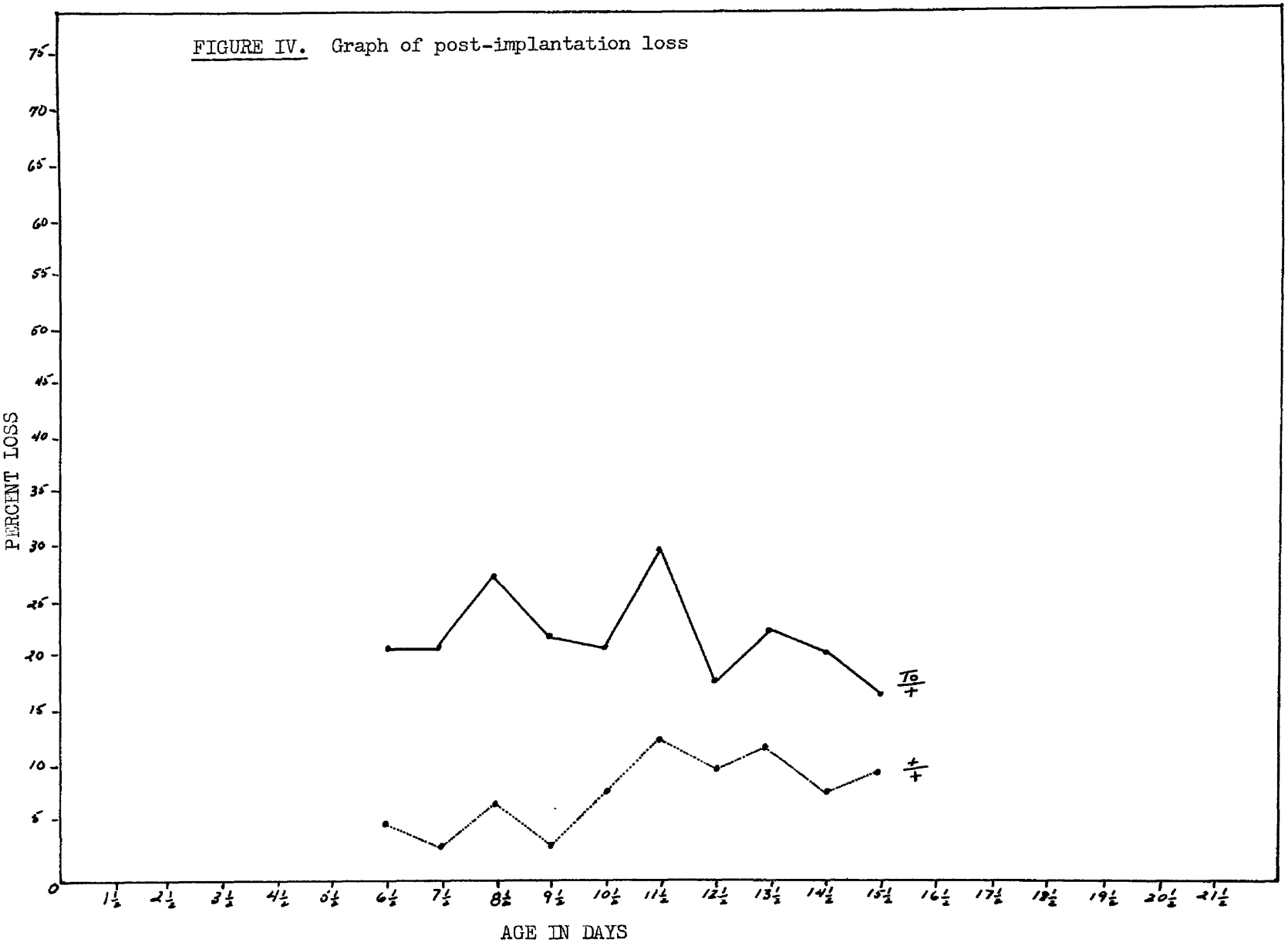


FIGURE IV. Graph of post-implantation loss
PERCENT LOSS
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
1½ 2½ 3½ 4½ 5½ 6½ 7½ 8½ 9½ 10½ 11½ 12½ 13½ 14½ 15½ 16½ 17½ 18½ 19½ 20½ 21½
AGE IN DAYS
To/+
+/+

Table 6. Within Day Regression on the Number of Live Embryos on the Number of Eggs Implanted.

Genotype	Age of Litters	Number of Litters	Number of Implants	Number of Live Emb.	Regression Coefficient
To/+	6½	10	66	52	0.9062
	7½	10	52	41	0.5000
	8½	10	58	42	0.4204*
	9½	10	55	43	0.6164*
	10½	10	72	57	0.5432**
	11½	10	61	43	0.6424*
	12½	10	57	47	0.7674
	13½	10	62	48	0.6935
	14½	10	56	44	0.9791
	15½	10	69	57	0.3469
Summary		100	608	474	0.6868
+/+	6½	10	73	69	0.7114
	7½	10	69	67	0.8546
	8½	10	74	69	0.7674
	9½	10	72	70	0.8333
	10½	10	79	73	0.8277
	11½	10	64	56	0.8589
	12½	10	71	64	0.9896
	13½	10	78	69	0.7403*
	14½	10	66	61	0.8252
	15½	10	82	74	0.6538
Summary		100	728	672	0.8219

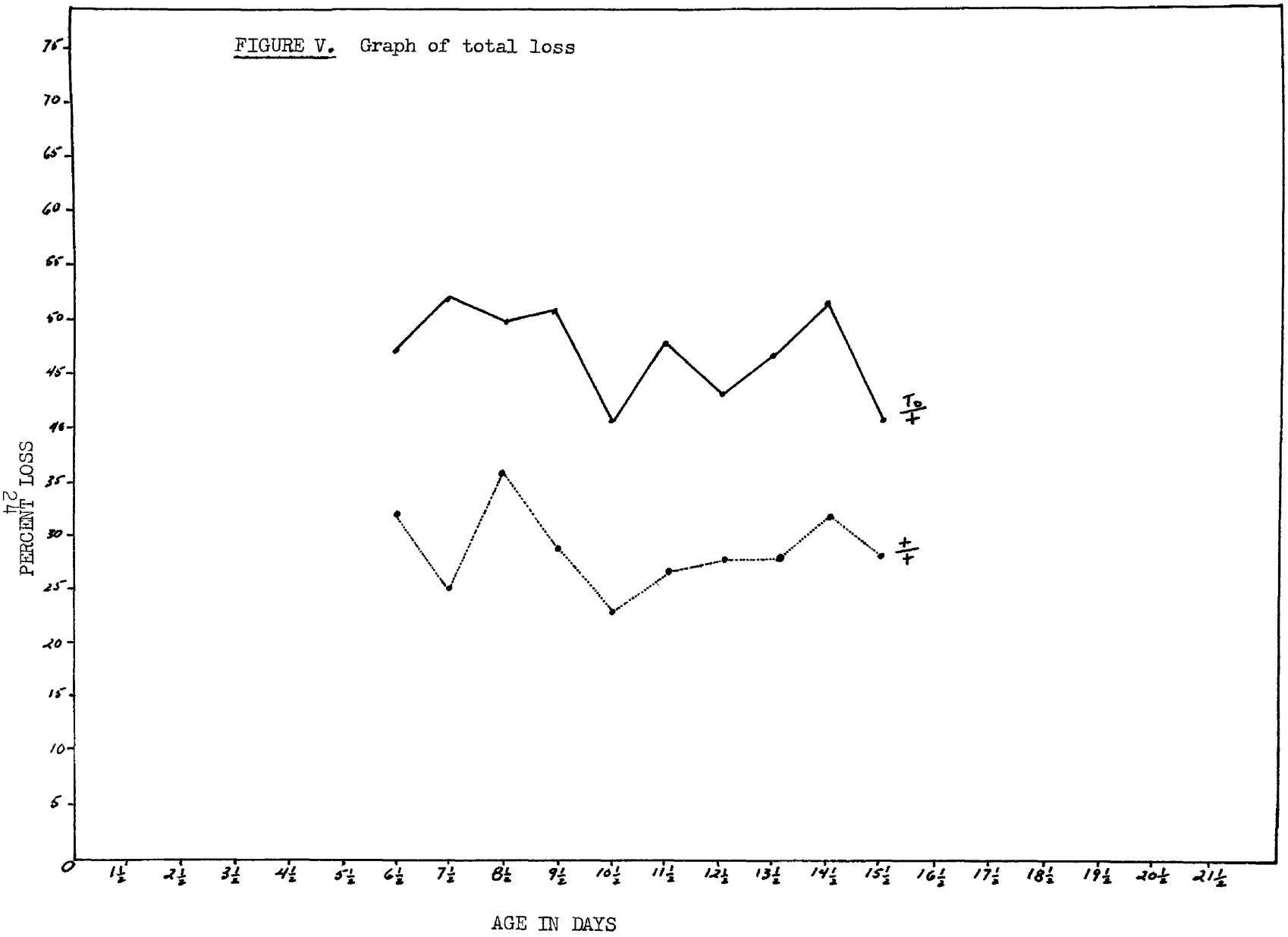
* significant at 0.05 level of probability
** significant at 0.01 level of probability

Table 7. Analysis of Variance for Adjusted Live Count.

Source of Variation	d.f.	S.S.	M.S.	F	Probability
Total	199	323.765	195.034		
Genotype	1	193.218	193.218	289.309**	< 0.01
Age	9	6.174	0.686	1.027	> 0.05
Age x Gen.	9	4.158	0.462	0.692	> 0.05
Error	180	120.215	0.668		

** significant at 0.01

FIGURE V. Graph of total loss



DISCUSSION AND CONCLUSION

The method used in this study to determine the time of death for embryos with the recessive sex-linked lethal gene was to examine progressively younger litters. Working backwards in time, from 14½ days to 6½ days, the younger litters were scored for dead and abnormal embryos. It was expected that at a specific time the proportion of dead and abnormal embryos would be equal among the offspring of homozygous (+/+) and heterozygous Tortoise (To/+) females. This time would antedate the first expression or translation of the Tortoise gene. At an interval close to this time, one would expect to find an increase in the proportion of dead and abnormal embryos among the offspring of the heterozygous Tortoise (To/+) females. The occurrence of these embryos, clustered in time, would indicate approximately the initial age at which the lethality of the mutant gene is expressed or it would mark the end of the time when the embryos carrying the lethal gene are viable.

The data on prenatal mortality rate among embryos from the heterozygous Tortoise (To/+) females show a statistically significant increase above the prenatal mortality rate associated with embryos from the normal (+/+) females. The interpretation is that the lethality of the recessive

sex-linked gene is expressed prior to the earliest, post-vaginal plug, age at sacrifice. The observed differences in preimplantation loss between the eggs of homozygous normal (+/+, To/+, +/y and To/y) females further suggest that the Tortoise gene is translated by day four postvaginal plug. The observed preimplantation loss attributable to the lethality of the Tortoise gene was less than the expected proportion (25 percent loss expected). If, however, the values observed for the preimplantation and postimplantation are combined, the expected proportion is achieved. This agrees with the work of Constance (1970). It is therefore concluded that the termination of the viability of embryos carrying the Tortoise gene may be delayed. This appears to be dependent on intra-uterine factors such as the genotype of the neighboring embryos and the number of embryos in the uterine horn.

The data analysed support the idea that the recessive sex-linked lethal gene Tortoise is translated by day four post vaginal plug. The embryos with the Tortoise gene have a variable postimplantation survival time. The loss of viability for these embryos seems to be dependent on the interaction between the embryo genome and intra-uterine factors.

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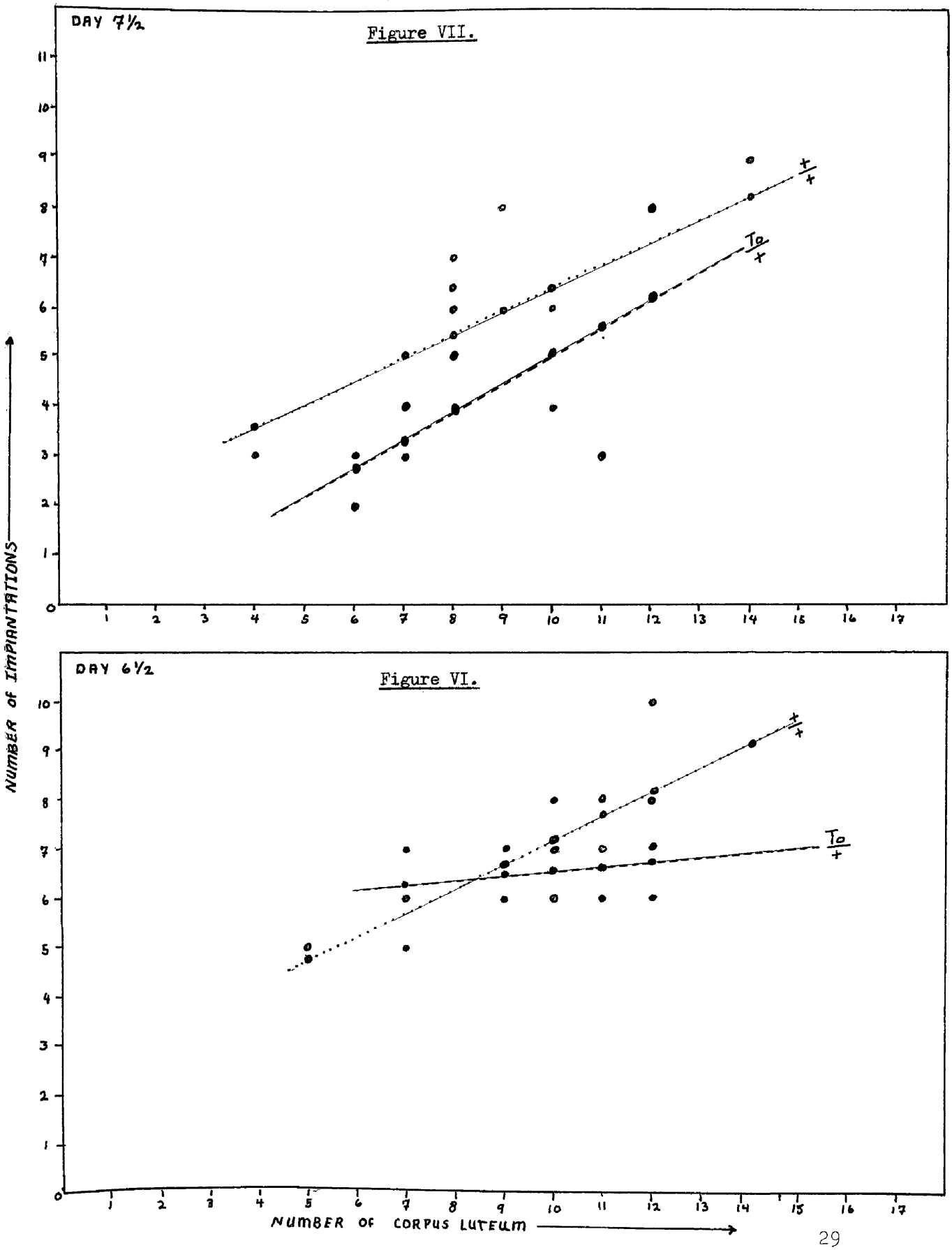
APPENDIX

Plots of the linear regression of number implanted on corpus luteum count.

Figures VI through XV - Plots of the regression line of the number of corpus luteum on the number of implanted eggs



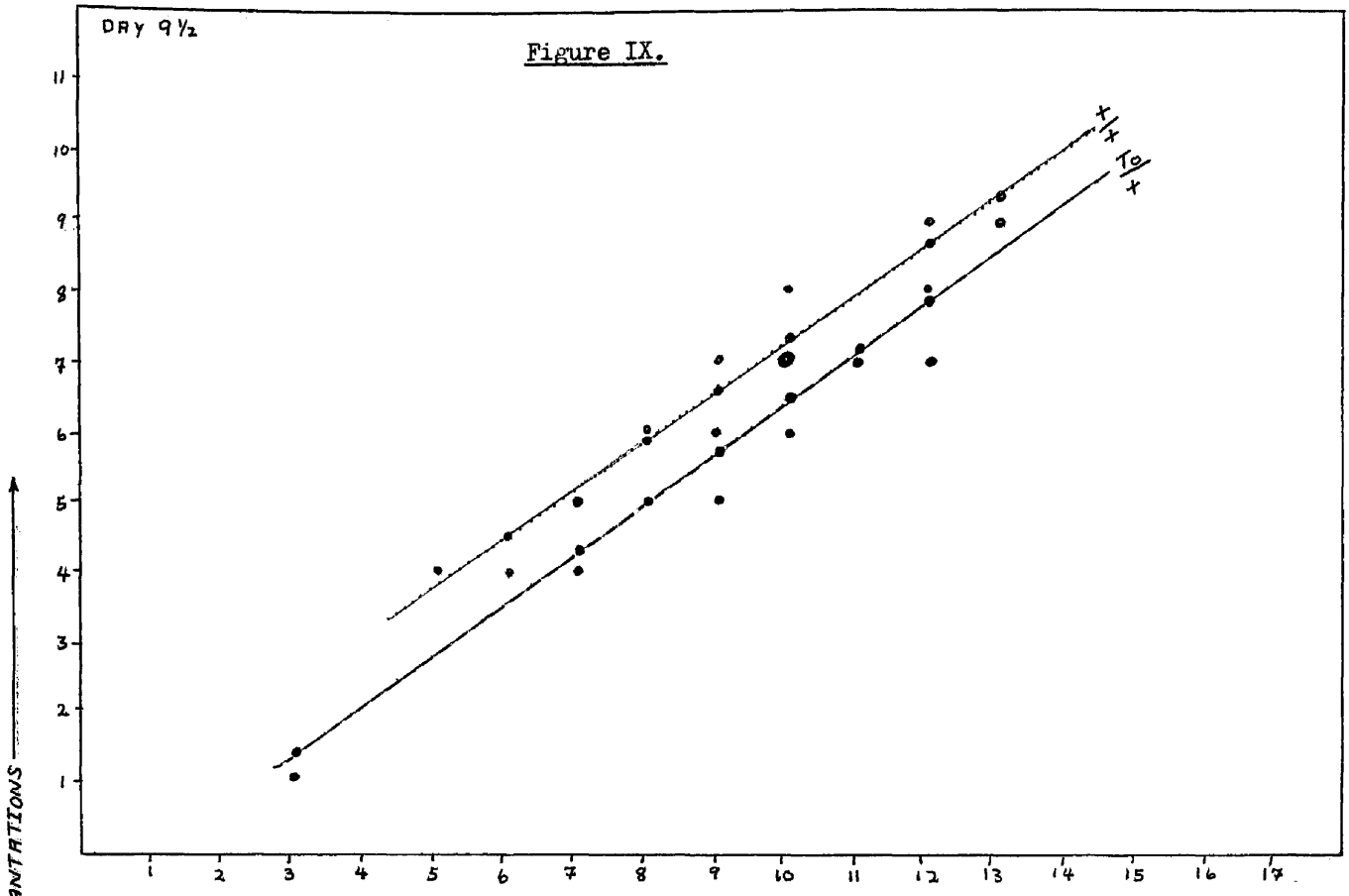


Figure IX.

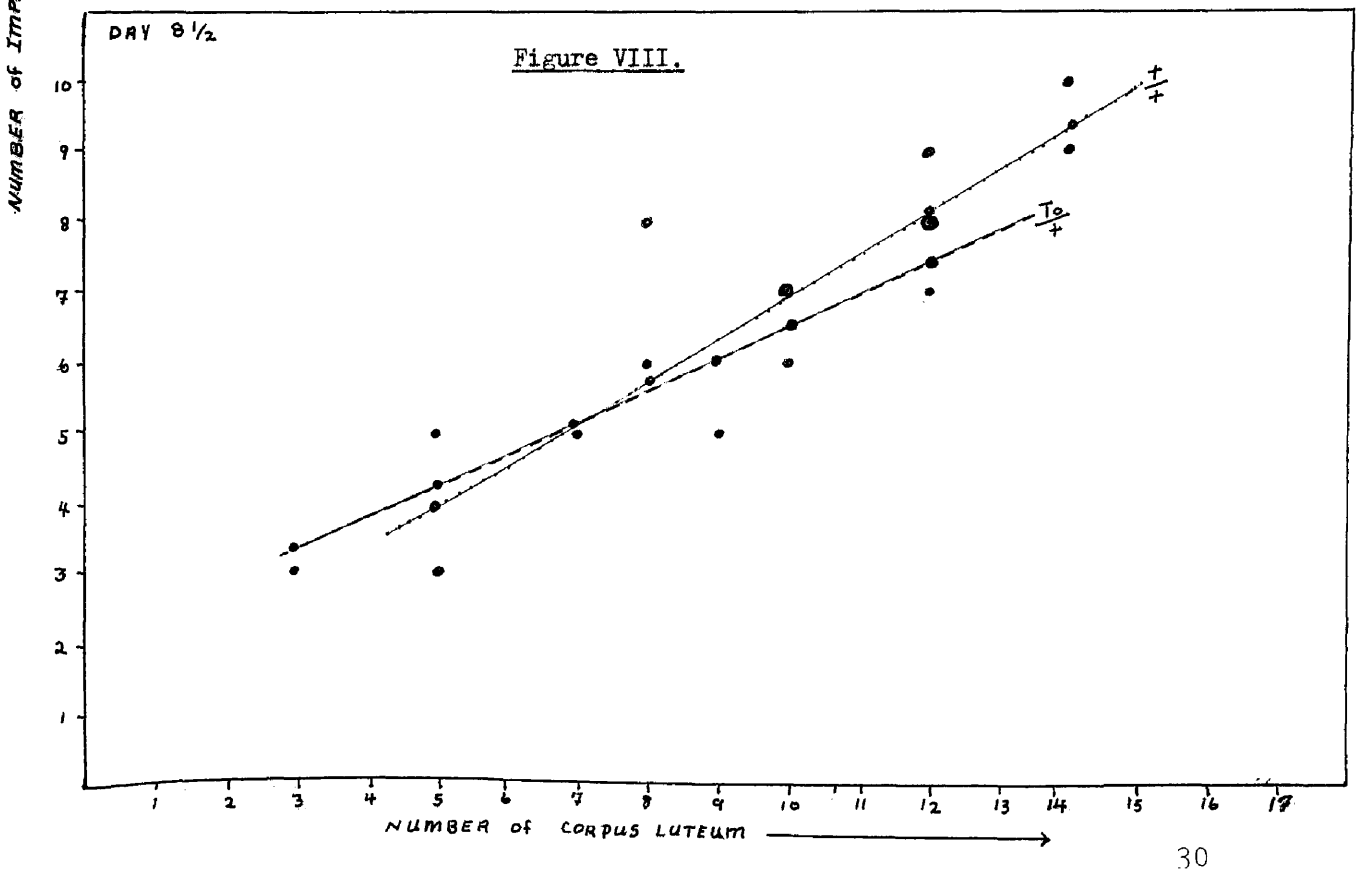


Figure VIII.

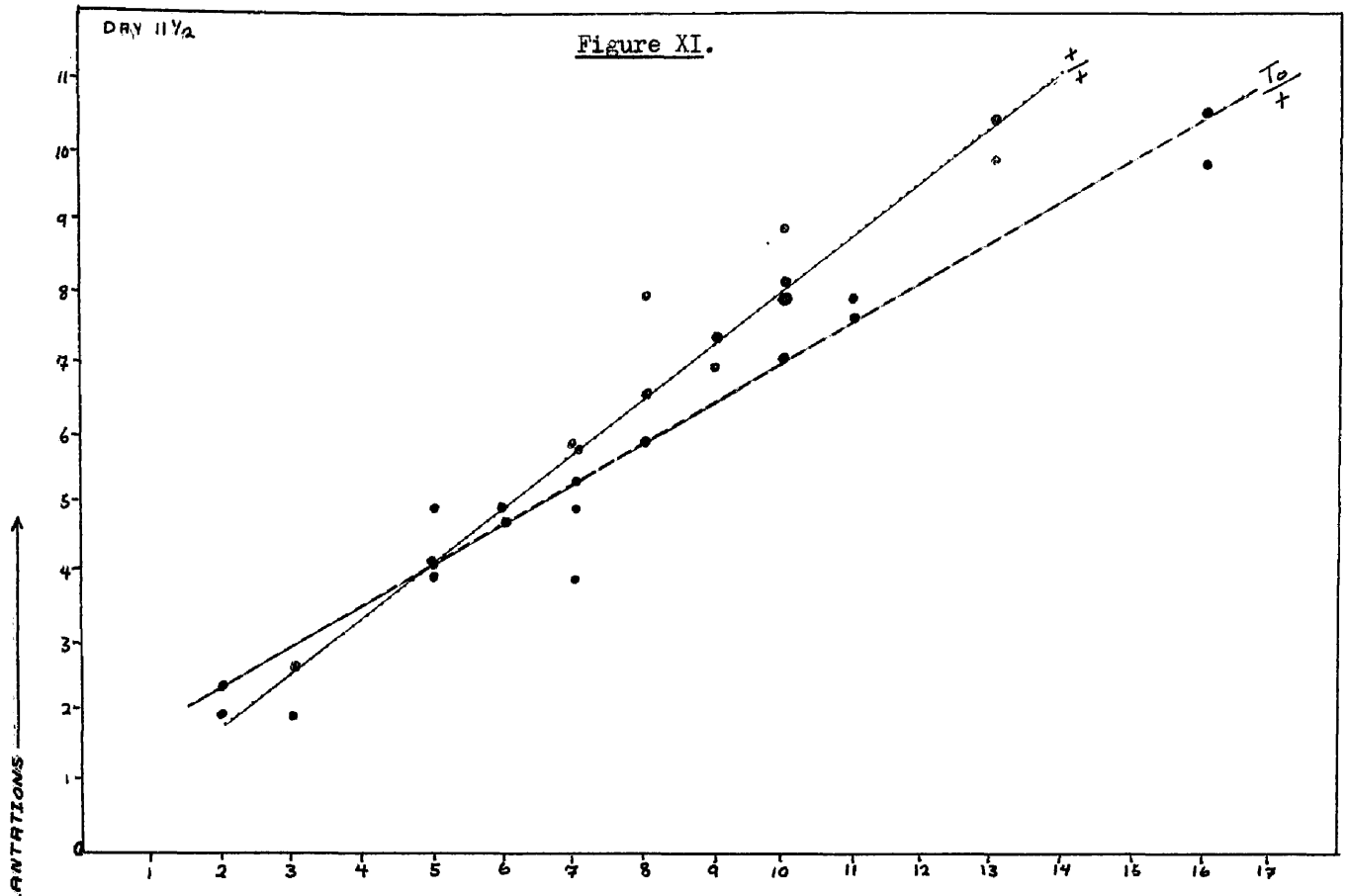


Figure XI.

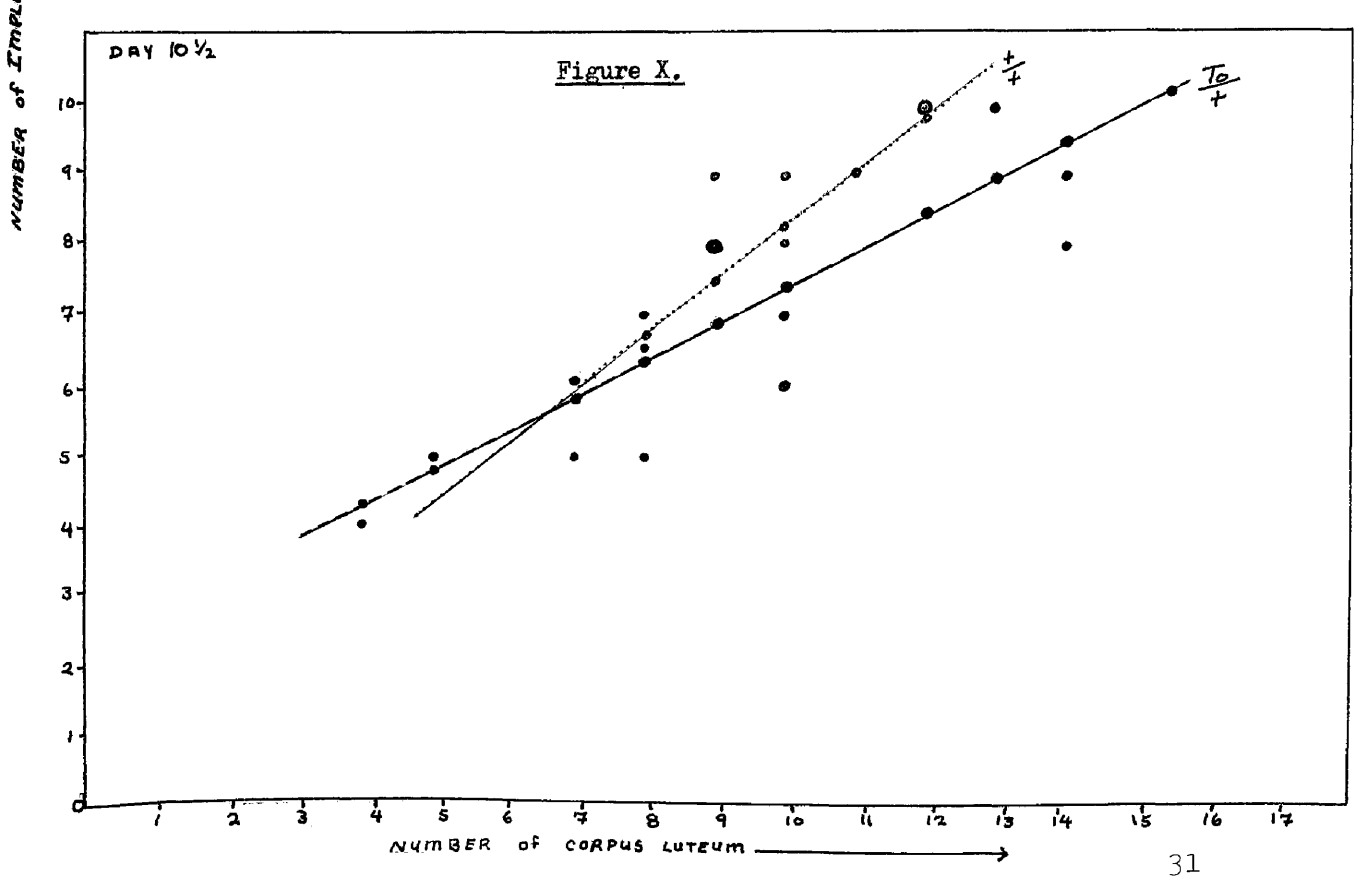
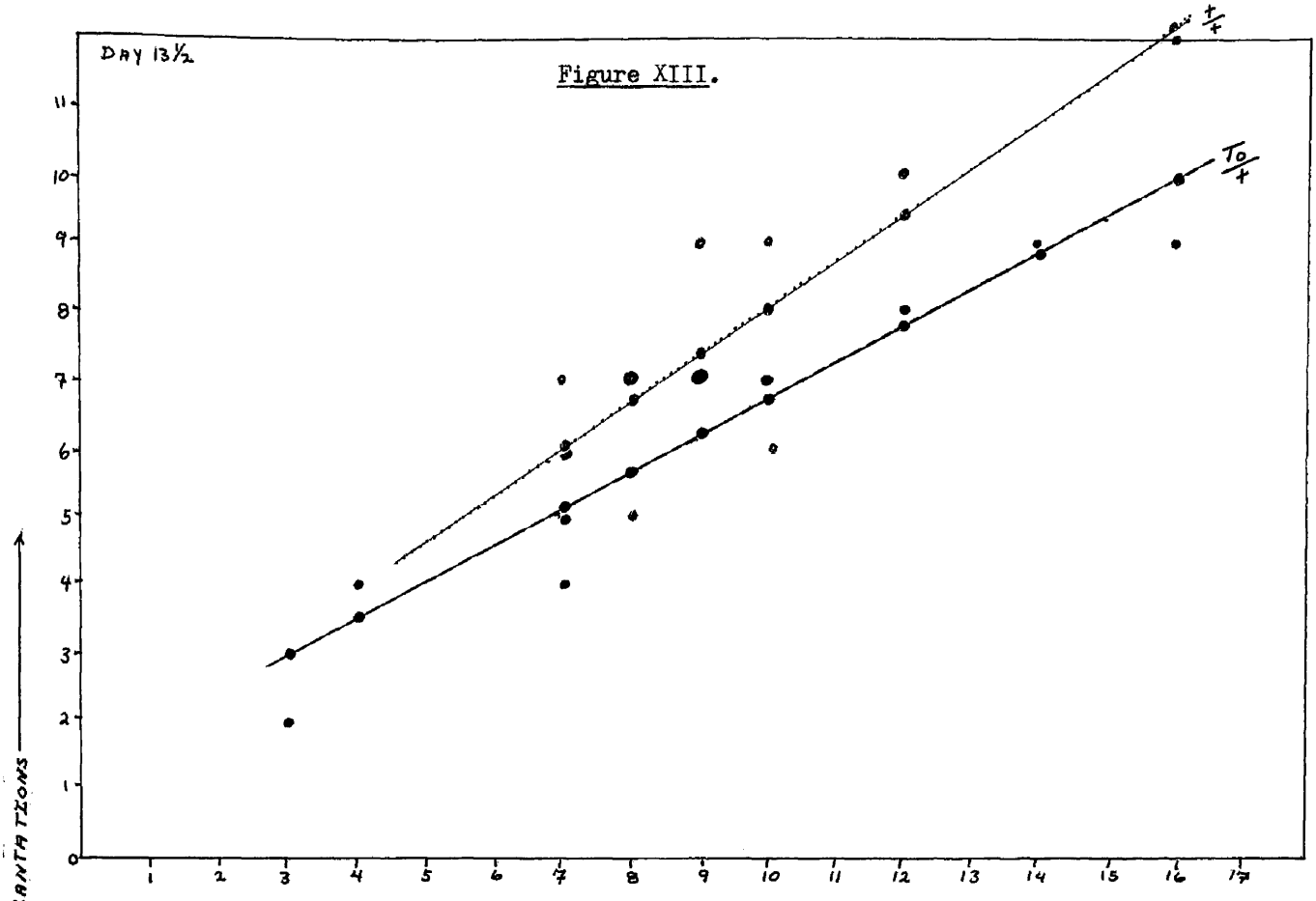


Figure X.



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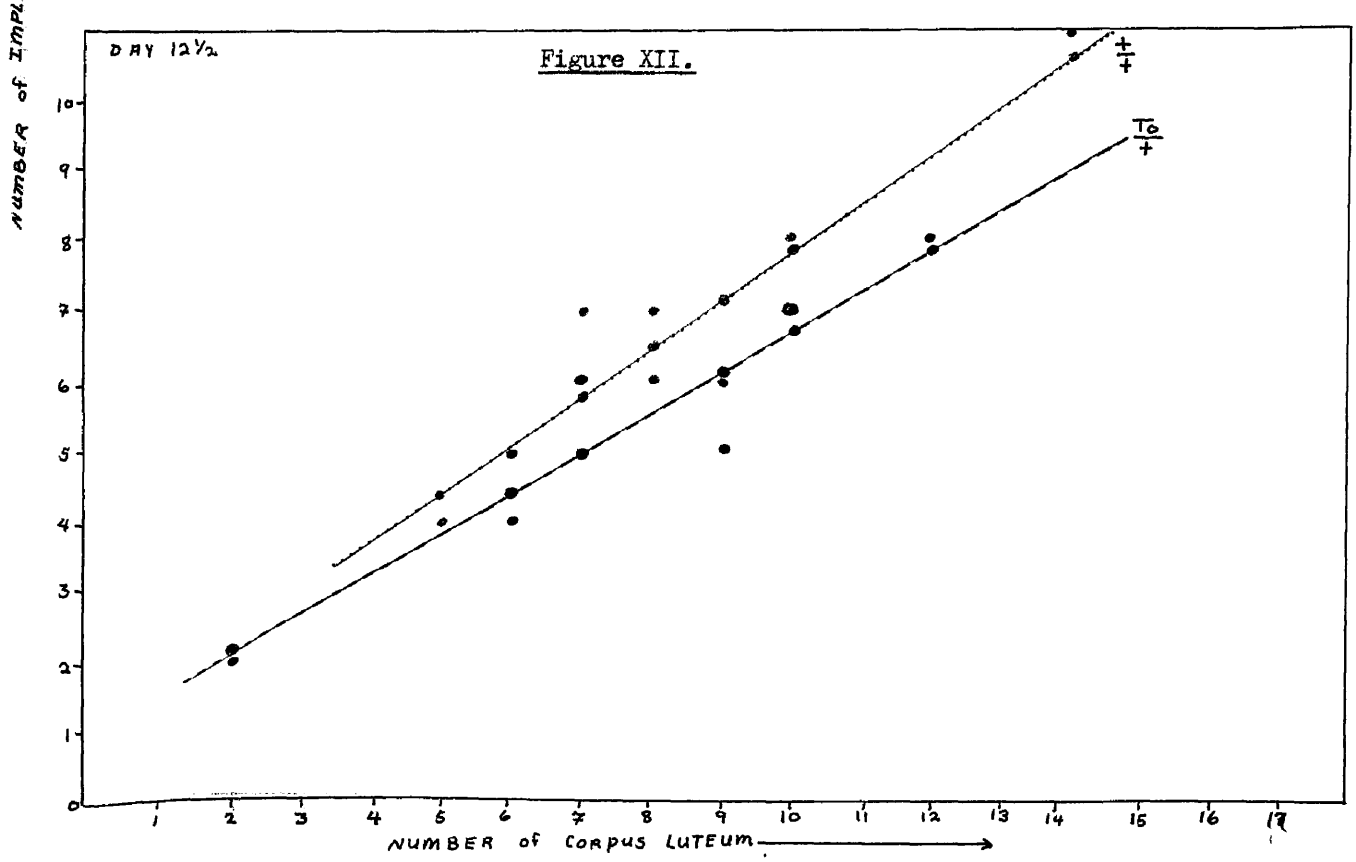

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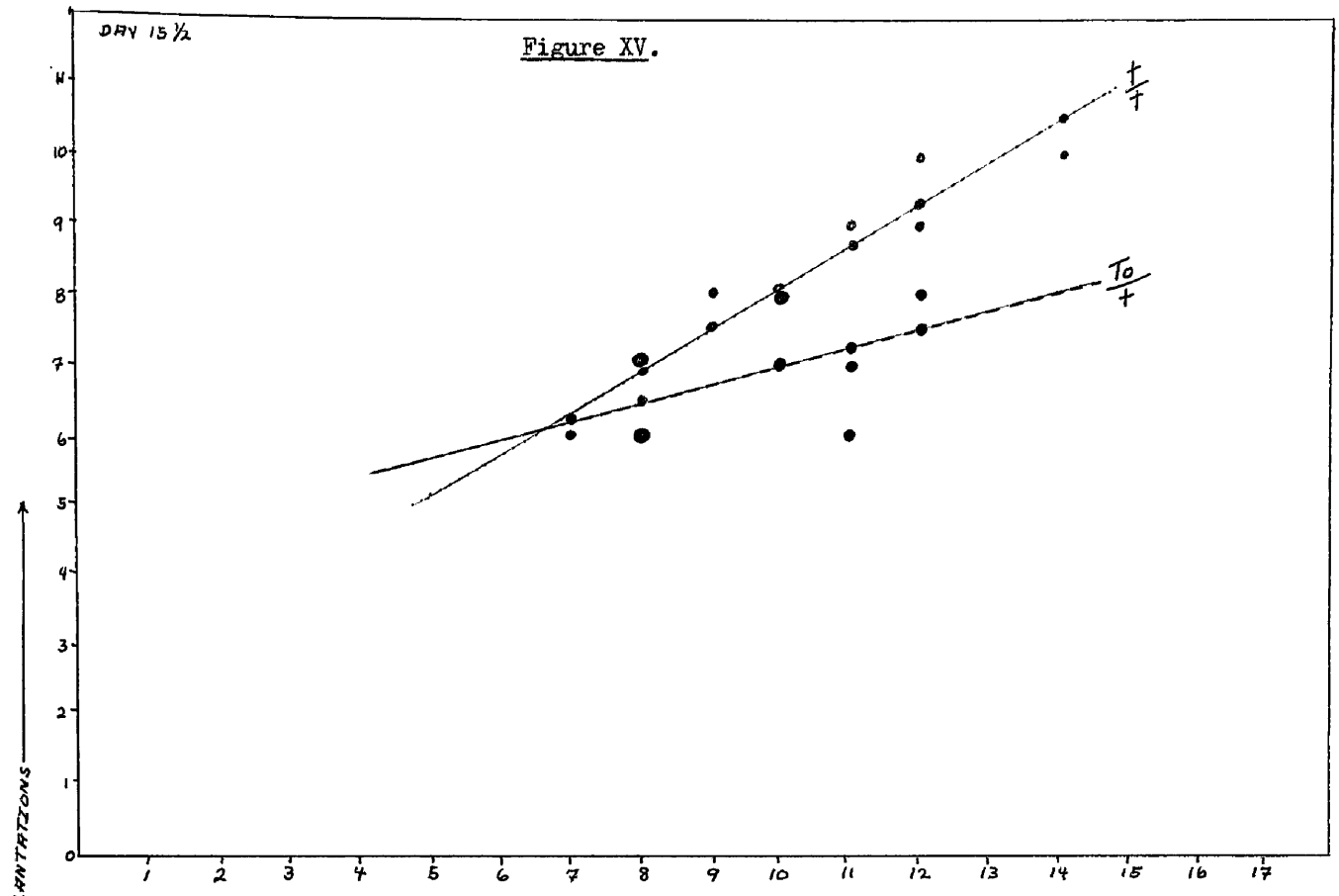

Figure XV.

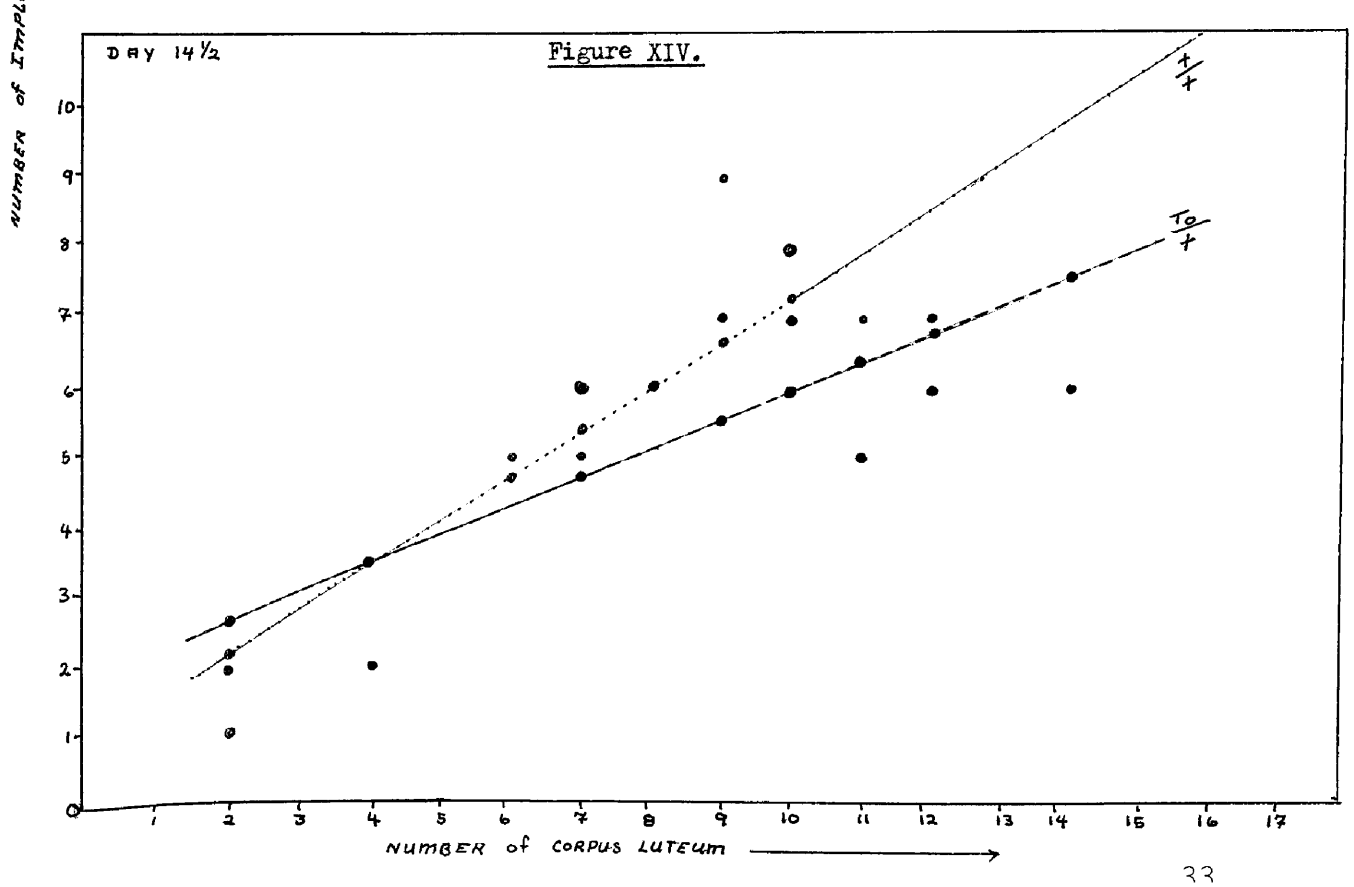

Figure XIV.