78-B5A

Title: Project 78-B5A. Insect Pathology and Storage Control

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I. Objectives: To investigate naturally occurring pathogens in NOW field populations and to test other microbial agents for NOW control.

II. Interpretive Summary: Chronic stunt virus (CSV) is a specific and highly virulent pathogen of the navel orangeworm. Attempts have been made in our laboratory to transmit this virus to several other species of moths, but so far only larvae of NOW have proven to be susceptible.

The virus is extremely small, about 1-millionth of an inch (25 nm) in diameter. As far as we know, virus particles must be eaten along with contaminated materials, e.g. almond hulls, in order for infection to take place. We have no evidence for congenital transmission of CSV, however, this is an area requiring more study.

Since the site of virus invasion is highly restricted to certain types of larval blood cells (hemocytes), we believe that once the virus passes through the larval gut wall, infection very rapidly spreads to all the susceptible cells. The infected blood cells are destroyed in the process of viral replication, and as a result the normal hormonal balance of the larva is destroyed. When a young larva becomes infected, the disease is usually fatal in a matter of a few days. However, when older larvae (half-grown) are infected the disease causes an immediate interference with growth and development. Such infected larvae may continue to survive in a stunted condition for many days (depending upon the intensity of the infection) before they die of the disease. Feeding essentially stops during this chronic period of infection.

During the past year we have been attempting to quantitate the relationship between larval mortality and the intensity of infection, as measured by the virus dose. Since it is not feasible to quantitate virus concentrations by counting the number of virus particles in a given suspension, we have tried to standardize the potency of our preparations by relating the virulence of a given dose to the quantity of infected larval tissue in the suspension. This method of equating larval mortality to dose has generally been satisfactory in our tests with very young larvae.

We have also conducted a series of tests to obtain a better understanding of the phenomenon of chronic stunting that occurs when older larvae become infected. These studies have provided useful data on the comparative larval size and weight of healthy and diseased populations. However, the "chronic-state" of infection needs to be tested further, especially in regards to the reduction in larval feeding activity and the ultimate mortality that occurs before adult emergence.

The effect of naturally occurring CSV on wild populations of NOW would be extremely difficult to assess, because CSV apparently occurs only infrequently in nature. It seems likely, however, that the incidence of CSV and its beneficial influence could be promoted by disseminating the virus by combining it with artificial baits and lures, such feeding stimulants or a pheromone.

III. Experimental Procedure:

Chronic Stunt Virus.--Dosage-mortality tests.--The effect of CSV concentration on larval mortality was studied exclusively with neonate (newly hatched) larvae less than 24 hr old. Serial dilutions of known concentrations of CSV stock suspensions were used. Larvae were reared individually in small plastic cups (2 ml size) containing 1 ml of agar-base diet (codling moth) with an overlay of 10µl of virus suspension. Twenty larvae were assayed with each dilution of virus in each test. Tests were repeated 4 times. Mortality was noted at intervals of 5, 7, 10 and 14 days from the test start. Tests were ended after 14 days, at which time the control larvae reached maturity.

A test to determine the mortality of larvae reared in bran diet plus CSV was also conducted, and repeated 3 times. In this test 4 replicates of 30 larvae each were reared on 20 g of diet + 2 ml of 4 serial dilutions of CSV. Because NOW develops more slowly on bran diet, tests were terminated after 21 days (27^{o}C).

Larval Stunting.--Preliminary studies were conducted with 10 day old larvae (late 3rd instar) reared on bran diet containing 5 serial dilutions of a CSV suspension. The degree of stunting was determined by directly comparing the frequency distributions of larval lengths of the diseased and control populations. When the control larval populations

(n = ca. 80-100) reached maturity (21 days at 27°C) all larvae were removed from the diets and fixed in hot (75°C) 80% alcohol. Fixed larvae were stored in 80% alcohol until measured. Larval length was measured at magnifications of 7X or 3.5X using an eyepiece micrometer mounted in a stereomicroscope. Although conversion factors were determined, all data reported here are presented in micrometer scale units for convenience. Measurements of larval head capsule width were obtained in the same manner.

In one test (repeated twice with 4 replicates of 30 larvae each), the degree of stunting in diseased larval populations was measured at intervals of 7, 10, 13, 16, 20, and 40 days after the larvae were placed on the diet. Only one concentration of CSV was tested--50ng/g diet. Larval length was measured as described above. Although most of the stunted larvae in these tests had acquired fatal infections, the ultimate mortality that would normally result was not determined. The chronic state of disease that develops in older larvae and its relationship to long-term mortality will be explored in future studies, along with the influence of infection on feeding behavior and nutrition.

Tissue Culture Study.--Three attempts were made to culture CSV in a cell line (TN 368) from the cabbage looper, Trichoplusia ni. A suspension of CSV was cold sterilized by filtering through a 220 nm millipore filter. Filtrate was then added to cell cultures at the time of transfer to new media. The initial inoculum was assayed for CSV activity before and after the tests. Also, tissue cultures were assayed for possible CSV titer increase as an indication of virus replication. Serial dilutions of triturated cell cultures were prepared in TRIS-KCL buffer and assayed with neonate larvae.

Storage Tests.--Suspensions of CSV in PBS pH 7.2 have been held in storage at 4°C and assayed at various intervals for activity against neonate larvae.

IV. Results: Dosage-mortality tests--Figs. 1-3 show the mortality response of neonate larvae to CSV infection. These data relate the concentration of CSV in nanograms of infected larval tissue to the cm^2 of agar-base diet or grams of bran diet. Also, all data reflect response to only one stock concentration of CSV which was stored at $-20^{o}C$. When the virus suspension was applied as a surface application on agar-base diet, the LC_{50} was about 6 ng/cm^2. When the virus suspension was mixed into the bran diet, the LC_{50} was ca. 10^2 greater probably because of the dilution of the virus particles on the bran substrate. Figs. 1 and 2 show the same data analyzed in two different ways, but giving identical results in determination of the LC_{50} point.

Larval stunting.--Fig. 4 shows the size distribution of the survivors of one of the dosage-mortality tests presented in Fig. 3. One of these data points (at 0.5µg, n = 74) is further expanded in Fig. 5 as a histogram of the population size-frequency distribution. Obviously the survivors of this test were greatly influenced by CSV infection, although they survived until the test was terminated at 21 days after first exposure to the virus. Most of these larvae would have succumbed at a later date with chronic infections.

The degree of stunting that occurs when 10 day old larvae (3rd instar) become infected can be related to virus dose. Fig. 6 shows the mean larval length of populations exposed to 5 concentrations of CSV (5 to 50,000 nanograms/g). When compared to the size of healthy larvae, the mean percent reduction in length can be shown, as is presented in Fig. 7. Also, the same data can be expressed as the percent increase in size when compared to the mean length of the initial 10 day old larvae. The greatest difference, of course, occurs between the 2 populations exposed to the 2 extremes of virus dose; whereas relatively small degrees of difference exists between the intermediate doses. Figs. 9 and 10 are histograms showing the frequency distributions of larval size of the 2 populations reared on 50 ng and 50µg CSV/g diet. Note that there was a 1000-fold difference in these 2 virus concentrations, but there was only 17% difference in the degree of stunting (40% versus 57%, see Fig. 7). However, the ultimate mortality that would have occurred among the chronically diseased larvae was not

determined in this test.

The influence of the virus concentration on larval weight is shown in Fig. 11. Larval weight reduction (as a percent of healthy larval weight) is even more dramatic than reduction in length. This is especially evident in the difference between the 2 lowest concentrations tested (5.0 and 50 ng), --ca. 32% versus 69% reduction, respectively, when compared to the mean weight of the control larval population. The rate of larval weight reduction declined rapidly above 500 ng/g; this decline was also reflected in the larval length (Fig. 6).

The progressive change in the frequency distribution of larval length of infected populations exposed to 50µg/g CSV is shown in a series of histograms (Figs. 12 to 17). These diseased populations were infected as 10 day old larvae and sampled on days 7, 10, 13, 16, 20, and 40 postinfection. The control population of larvae reached maturity on day 13; however, this population is repeated on the histograms of days 16 through 40 for comparative purposes. The greatest shift in mean population larval length occurred during the first 13 days. The infected populations grew relatively little, whereas the control larval populations reached mature size. From days 13 to 40 postinfection, the mean size of stunted populations essentially remained unchanged. Judging the amounts of accumulated frass in the treatment jars, there apparently was relatively little feeding activity by the older diseased larvae.

The frequency distribution of measurements of head capsule widths of some stunted populations is presented in Figs. 18-20. On day 7 postinfection the head capsules are fairly normal (Fig. 18); but by day 13, the head capsules no longer are representative of the larval age or instar (Fig. 19), and by day 40 postinfection (Fig. 20) the widths are similar to those of day 13. Thus, measurements of larval length, weight, and head capsule width reflect the drastic influence that CSV exerts on normal larval growth and development.

There is a possibility that the intensity of infection by CSV can be moderated during the early stage of disease. We have one observation of 3 larval populations that were exposed to temperatures above 30^{o}

for several days after the first few days of infection. The 3 populations were combined in the histogram shown in Fig. 21 and the graph in Fig. 22. Note the bimodal distribution of larval length. Although all larvae were stunted, there were 2 distinct populations of stuntedness. We have not pursued this phenomenon further, but it appears likely that approximately half of the larvae became slightly less susceptible due to the influence of the increased temperature.

Tissue and Culture Study:--There was no evidence of virus replication in the TN-368 cell line. The CSV inoculum proved highly active when assayed against neonate larvae. We concluded that TN-368 is not susceptible to CSV.

Storage Tests:--The CSV suspensions held at $4^{o}C$ have been active for all periods tested. The oldest batch of virus was 384 days old. The possible shift in LC_{50} of these suspensions was not studied. Activity was based on "normal" response (stunting and general mortality) observed in 10 day old larvae reared on bran diet contaminated with the virus in a ratio of 1:10 (vol/wt).

V. Discussion: --Dosate-mortality data were obtained from tests that were terminated after 14 days, at which time control larvae had reached mature size and were ready to enter the prepupal stage. Had these tests been continued for an additional 10 days, the resulting LC_{50} would have been shifted to a lower concentration because moribund larvae (counted as "alive") would have died. For comparative purposes, however, the 14-day test is adequate. Longer term mortality should be studied, however, especially as it relates to adult emergence, and the survival of infected individuals to produce stunted pupae and adults. As expected, neonate larvae are most susceptible and may succumb to infection in only a day or two. The chronic state of disease is usually associated with larvae that become infected after they have reached the late 3rd or early 4th instar. In these cases, the disease may bring about gradual mortality that may not be evident for several weeks.

Since CSV is restricted to the granular hemocytes, increasing the dose beyond a certain level is not reflected in a proportional increase in response. This is the usual type of relationship in dosage-mortality data, but it is even more evident with CSV because of the limited number of sites for invasion. We know that there is a change in the total and differential hemocyte counts of infected larvae as disease progresses, however, the details of these changes and how they relate to the disease syndrome is unknown.

The effect of temperature and UV on virus stability was not studied. A susceptible cell line would be a valuable tool to measure the influence of such environmental factors on the virus; assays with tissue cultures are highly sensitive to shifts in virulence. Determination of plaque forming units and $TCID_{50}$ are routine techniques for virus assays of this type.

Stability of virus in the almond orchard is just as important as effective distribution of the virus to the target site.

.9, 141

14 days, Mortal

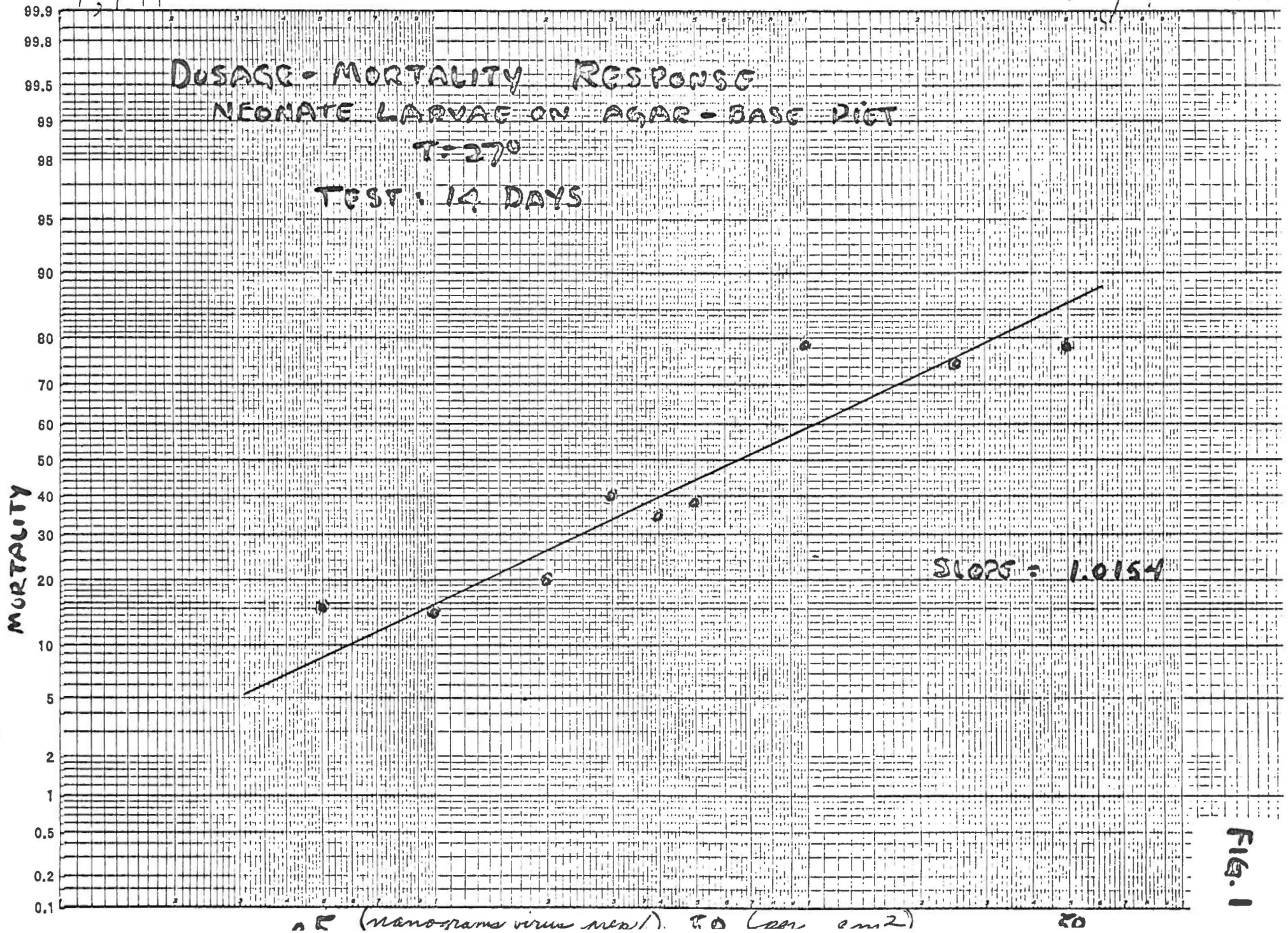


Fig. 1

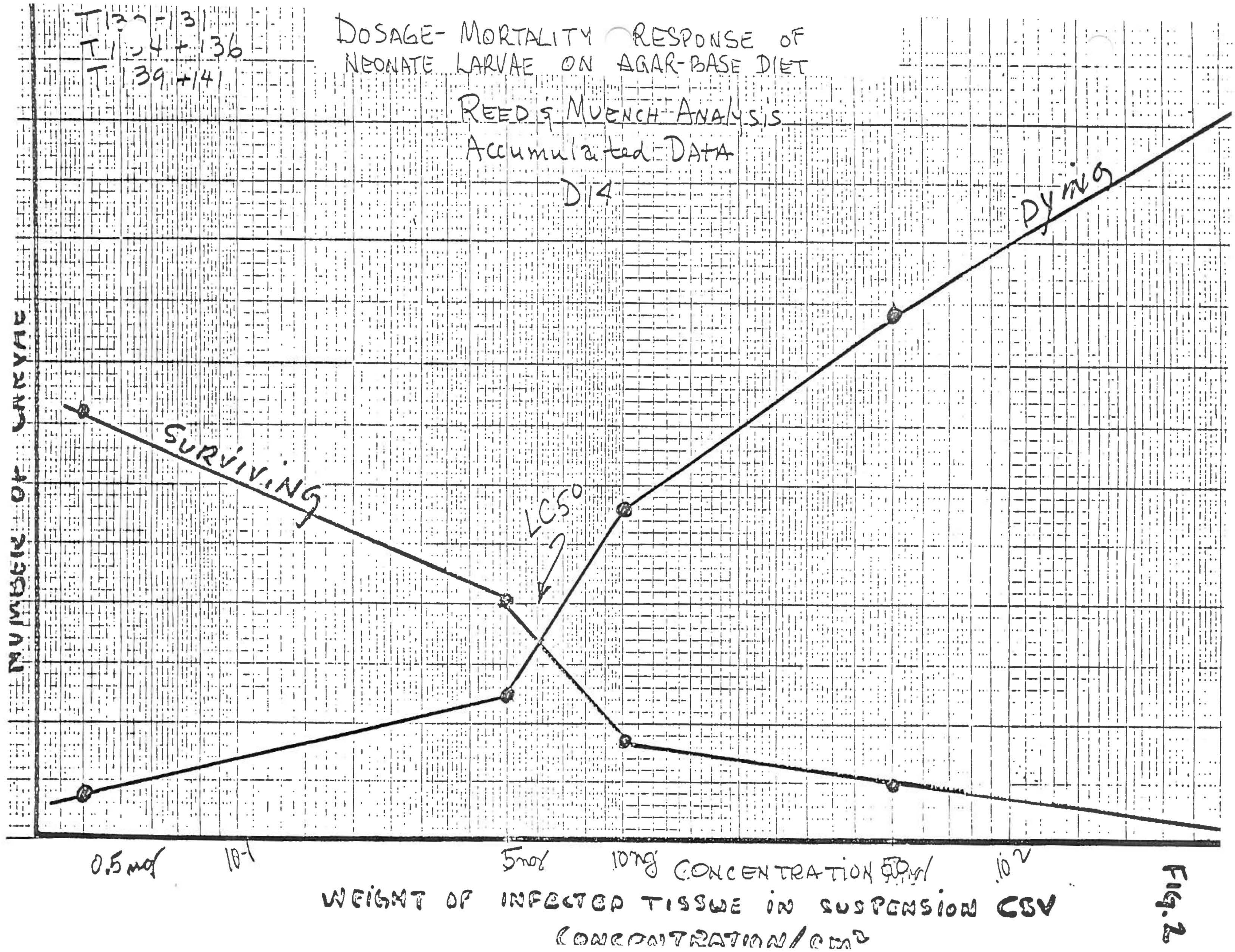

T13?-131
T1?4+136
T139+141
DOSAGE- MORTALITY RESPONSE OF
NEONATE LARVAE ON AGAR-BASE DIET
REED & MUENCH ANALYSIS
Accumulated DATA
D14
NUMBER OF LARVAE
SURVIVING
DYING
LC50
0.5 mg
10-1
5 mg
10 mg
50
10 2
CONCENTRATION
WEIGHT OF INFECTED TISSUE IN SUSPENSION CSV
CONCENTRATION/cm3

Fig. 2

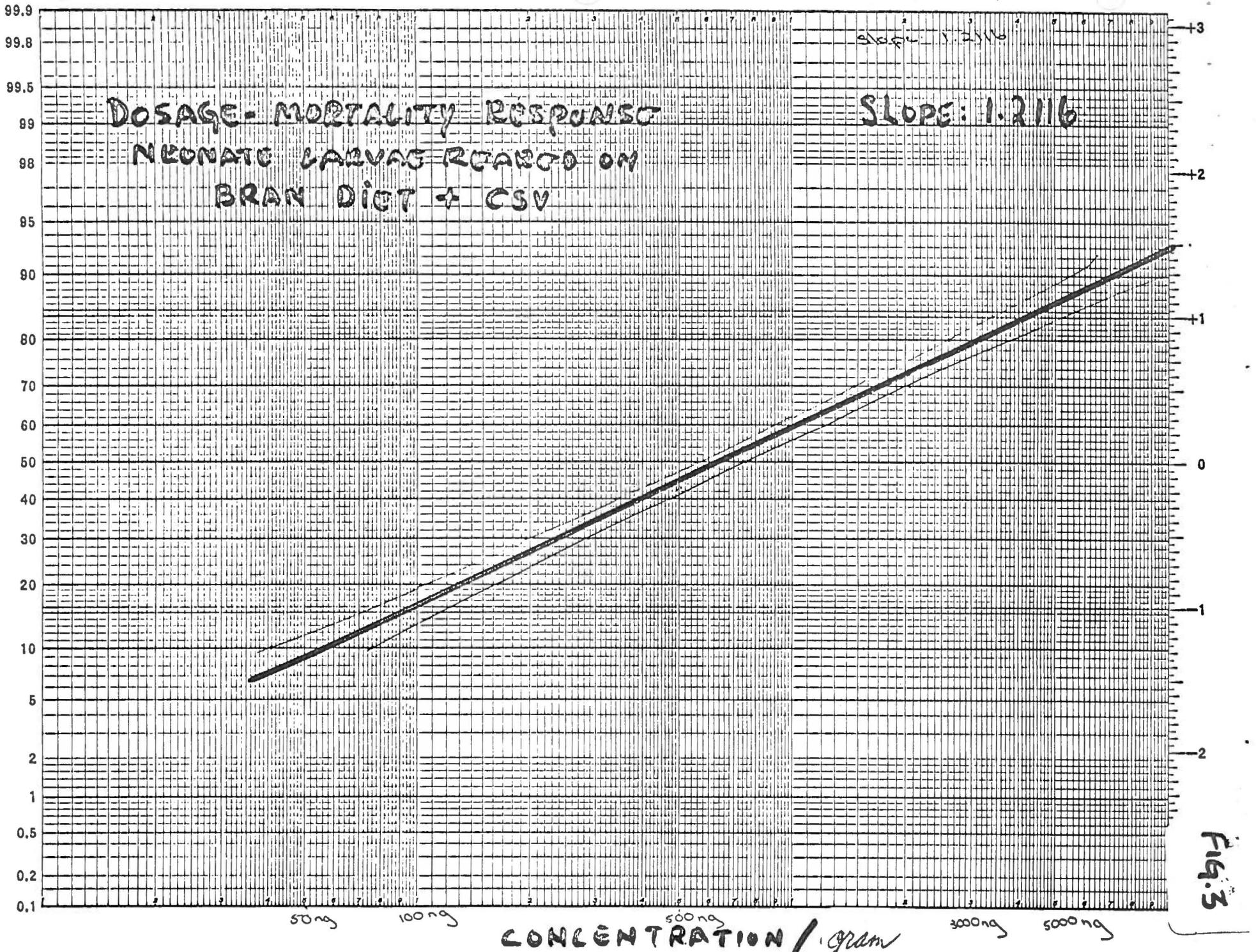

DOSAGE - MORTALITY RESPONSE
NEONATE LARVAE REARED ON
BRAN DIET + CSV
SLOPE: 1.2116
99.9
99.8
99.5
99
98
95
90
80
70
60
50
40
30
20
10
5
2
1
0.5
0.2
0.1
+3
+2
+1
0
−1
−2
50 mg
100 mg
500 mg
3000 mg
5000 mg
CONCENTRATION / gram

Fig. 3

STUNTING EFFECT – SURVIVORS
6 CONCENTRATIONS CSV

T109

NEONATES – BRAN DIET

D21

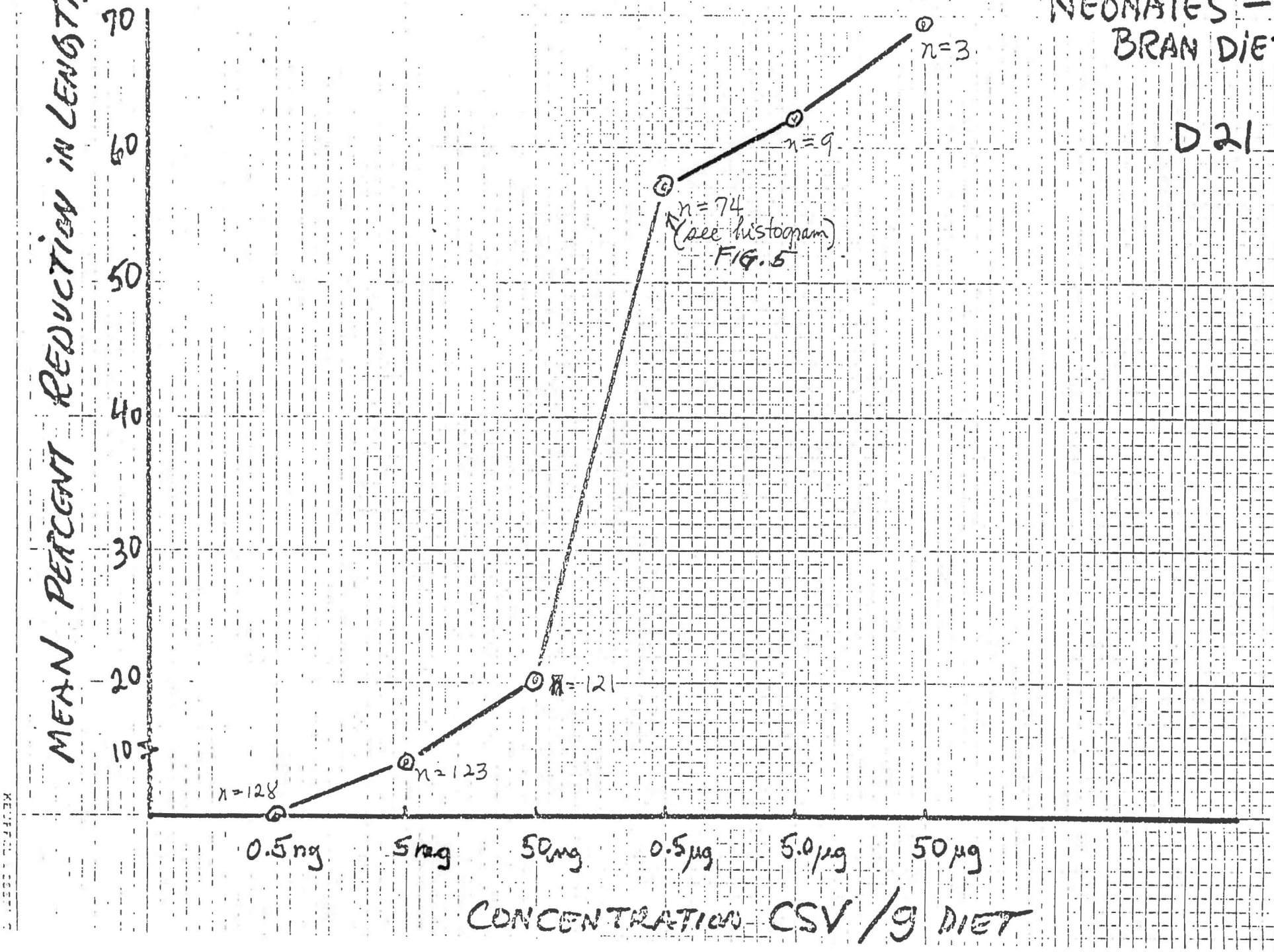
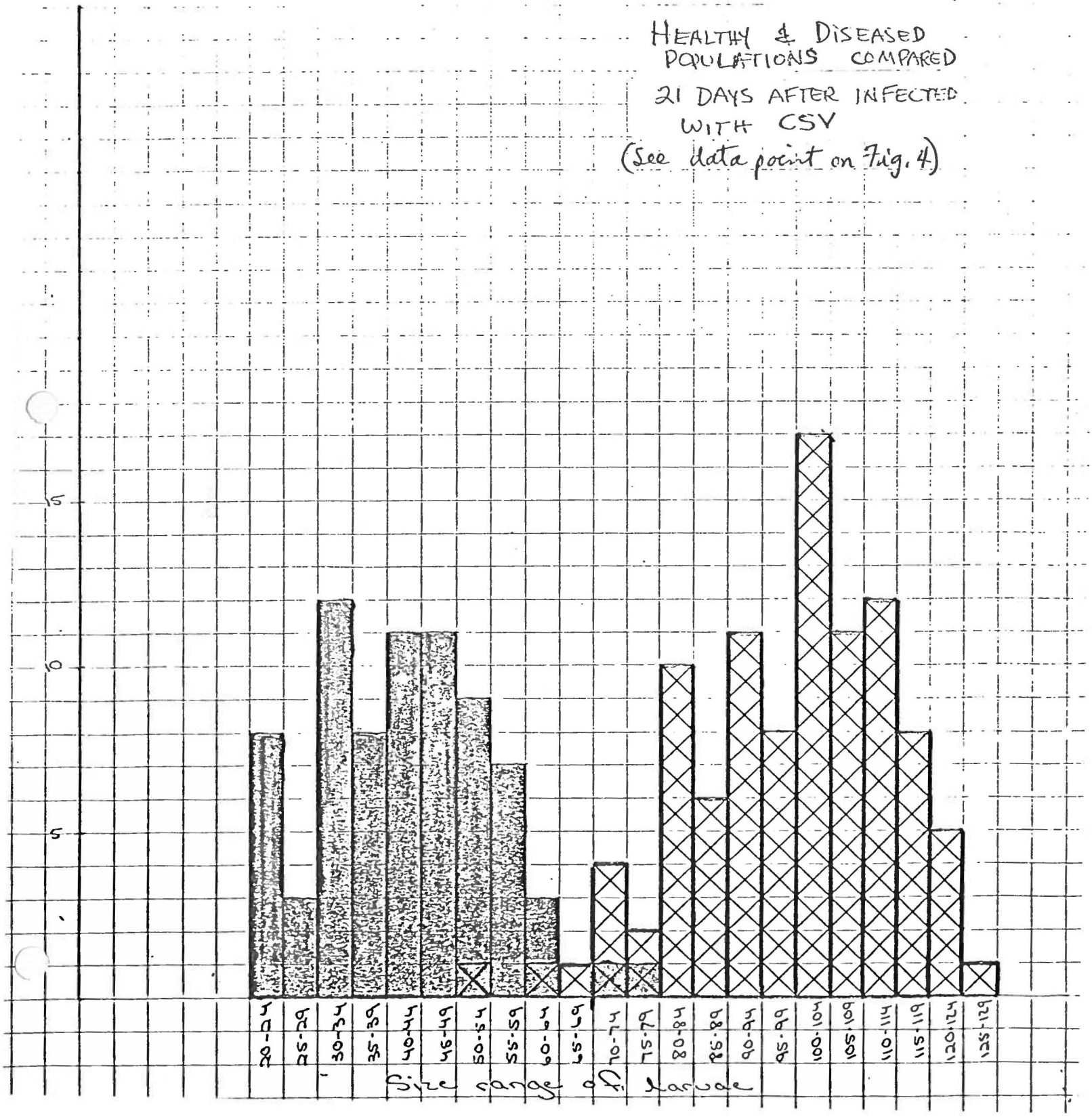


FIG. 4

T 129 C-1,2,3+4 combined ▩ D21 FIG.5
TR: 0.5µg n=74

T 129 CKs combined ⊠ n=98

HEALTHY & DISEASED POPULATIONS COMPARED
21 DAYS AFTER INFECTED WITH CSV
(See data point on Fig. 4)



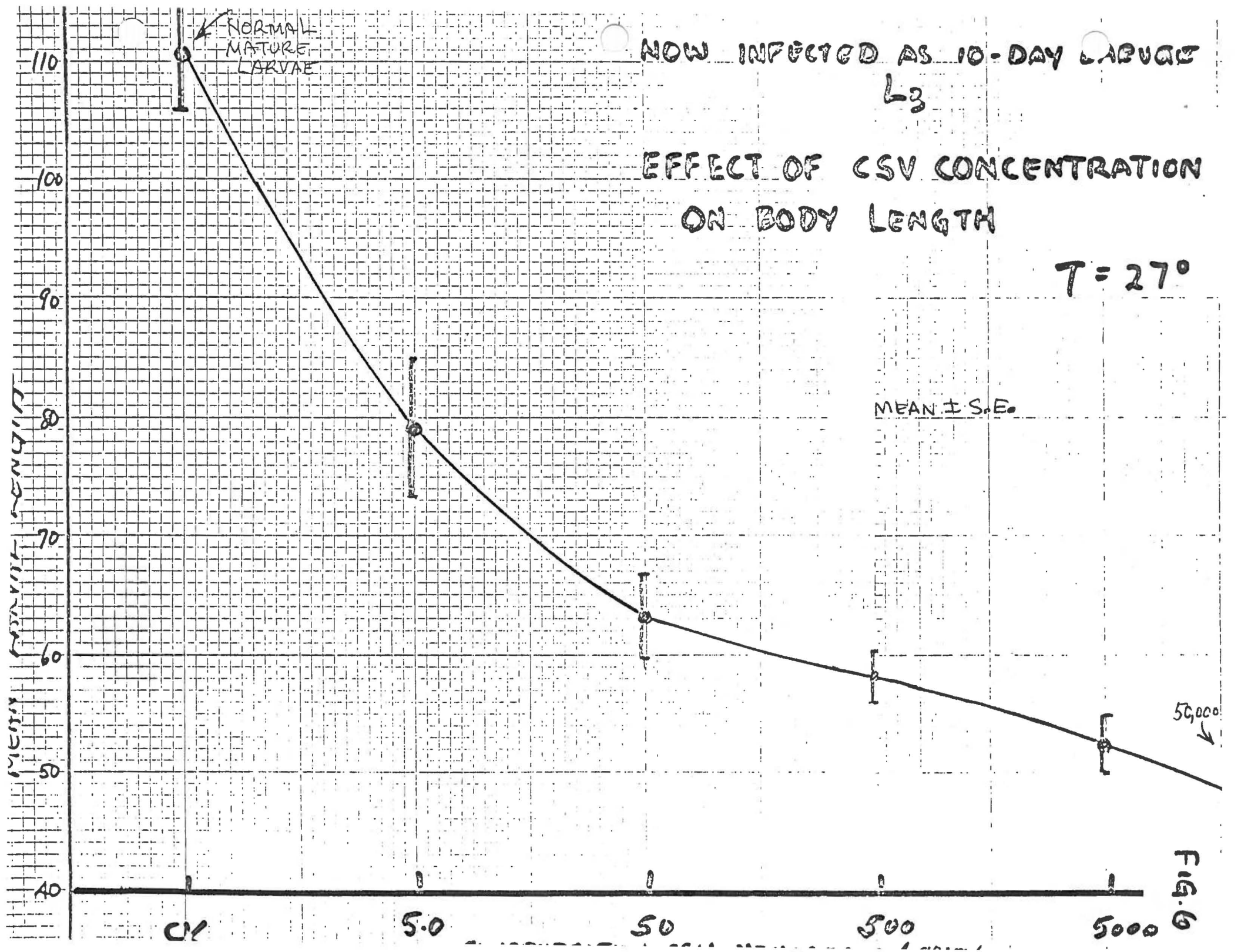

NORMAL MATURE LARVAE
NOW INFECTED AS 10-DAY LARVAE
L3
EFFECT OF CSV CONCENTRATION ON BODY LENGTH
T = 27°
MEAN ± S.E.
50,000
MEAN BODY LENGTH
110
100
90
80
70
60
50
40
0
5.0
50
500
5000

Fig. 6

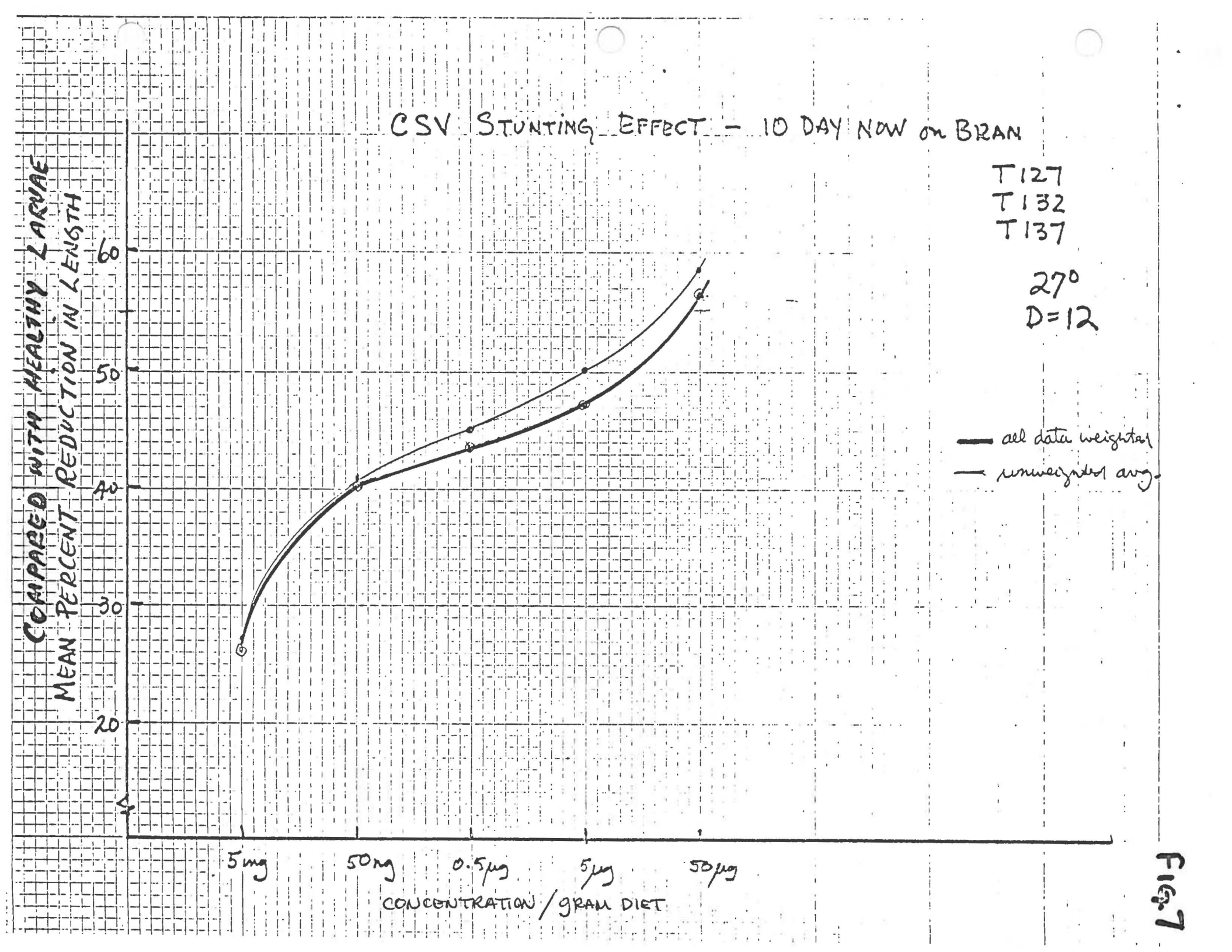

CSV STUNTING EFFECT – 10 DAY NOW on BRAN
T127
T132
T137
27°
D=12
COMPARED WITH HEALTHY LARVAE
MEAN PERCENT REDUCTION IN LENGTH
60
50
40
30
20
all data weighted
unweighted avg.
5mg
50mg
0.5µg
5µg
50µg
CONCENTRATION / GRAM DIET

Fig 7

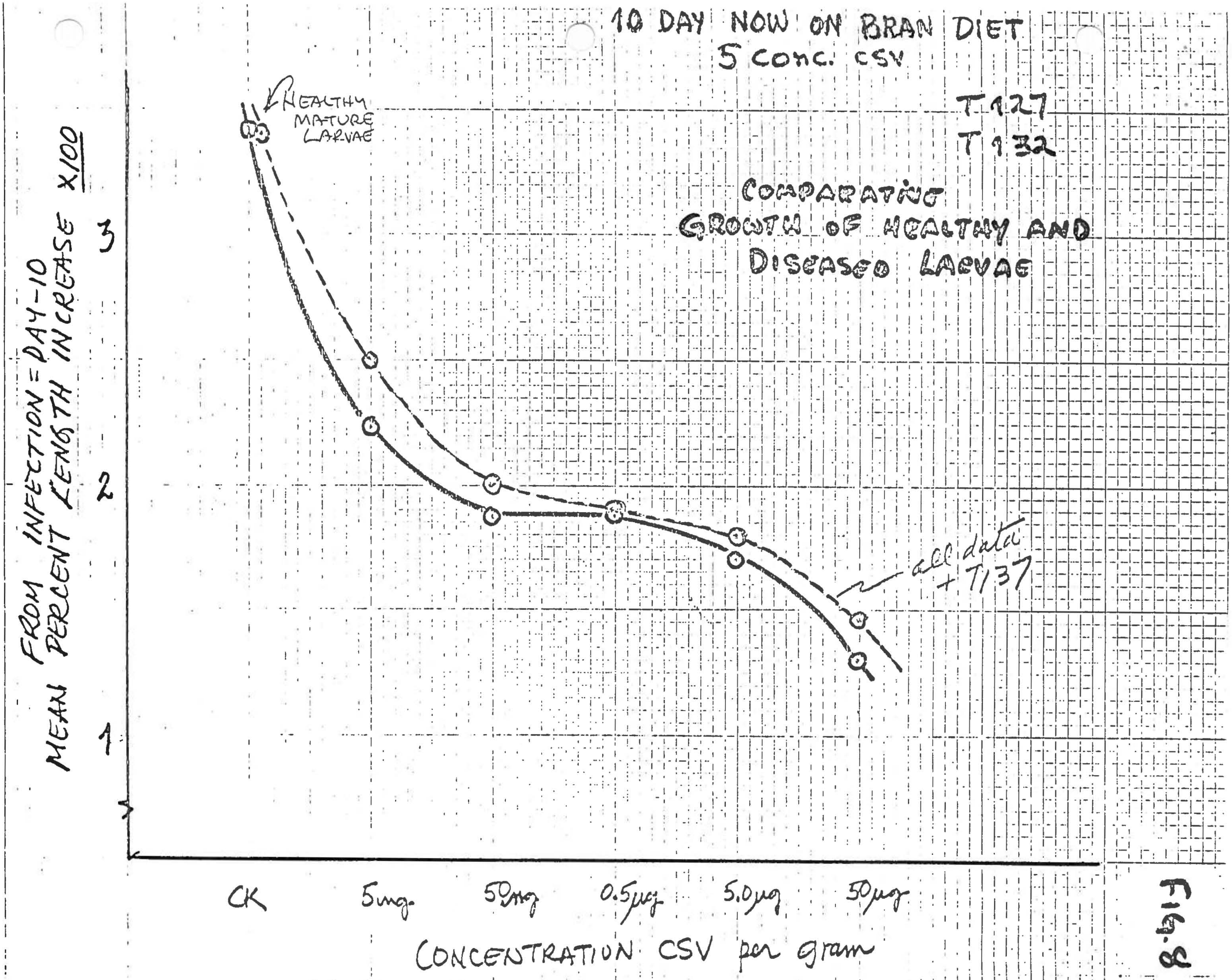

10 DAY NOW ON BRAN DIET
5 CONC. CSV
T 127
T 132
COMPARATING GROWTH OF HEALTHY AND DISEASED LARVAE
HEALTHY MATURE LARVAE
all data + T137
MEAN PERCENT LENGTH INCREASE X100
FROM INFECTION = DAY-10
3
2
1
CK
5mg.
50µg
0.5µg
5.0µg
50µg
CONCENTRATION CSV per gram

FIG. 8

FIG. 9

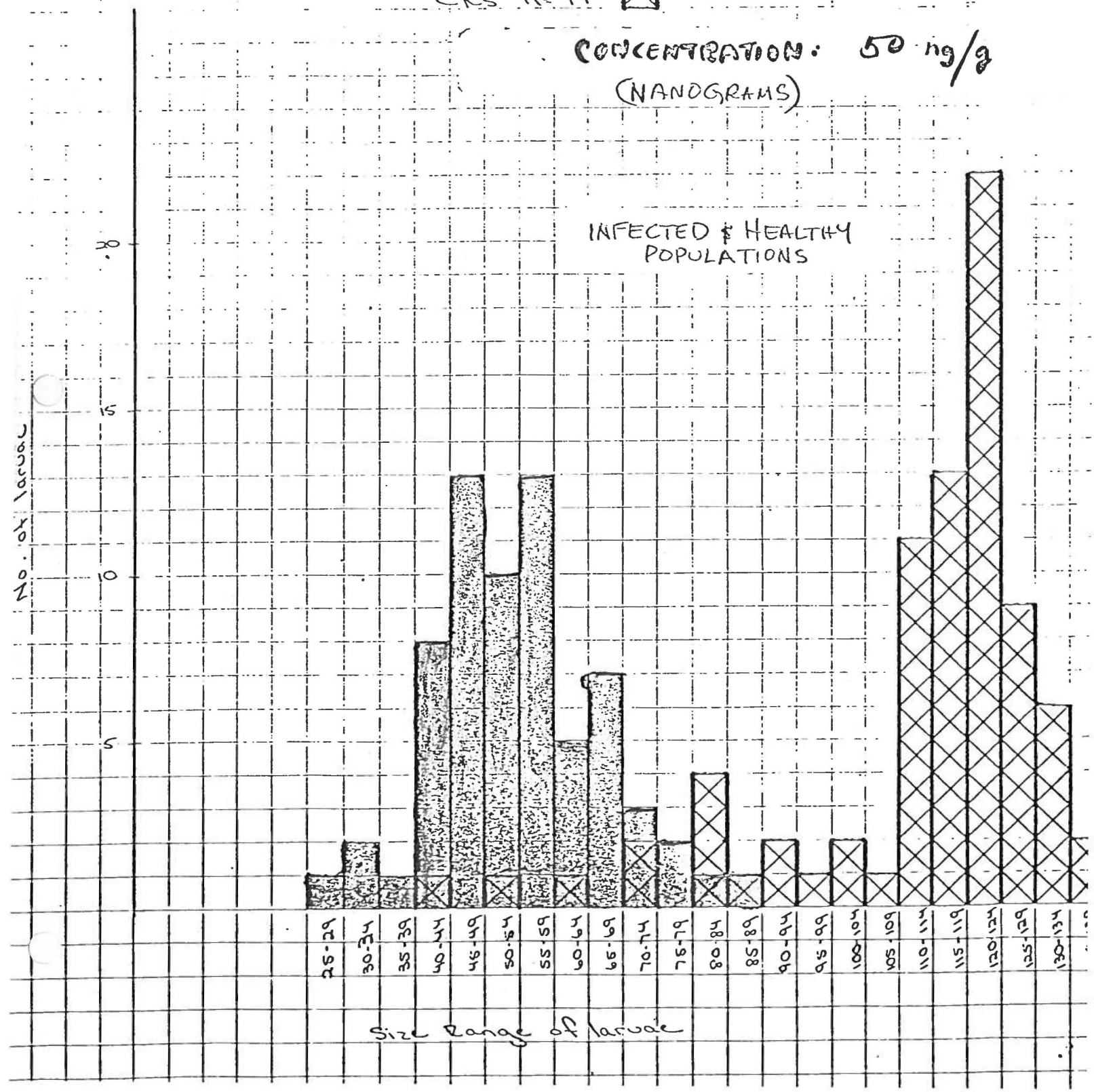
10 DAY NOW ON BRAN DIET D=14

T 132 D-1,2,3+4 combined

TRs n=69

CNs n=79

CONCENTRATION: 50 ng/g
(NANOGRAMS)



T 132

FIG. 10

TR A-1,2,3+4 50µg n=70

CKs n=79

CONCENTRATION = 50µg/g
(MICROGRAMS)

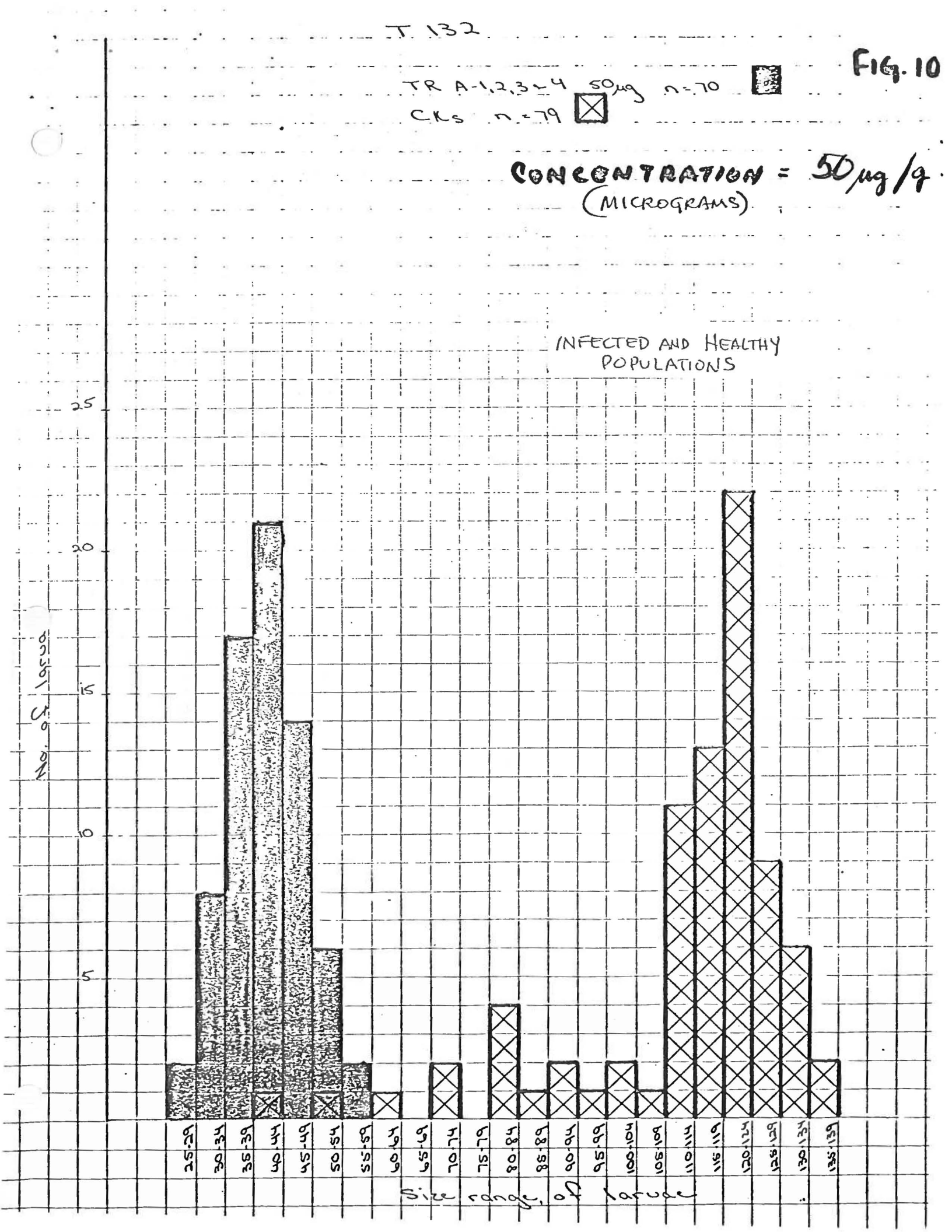


FIG 11

T 137
D14, T=2

EFFECT OF CSV INFECTION ON BODY WEIGHT

MEAN LARVAL WEIGHT
Milligrams/Larva

D=0

PERCENT

WEIGHT

PERCENT REDUCTION

MEAN WEIGHT

CK 5.0 50 500 5000 50,000

CSV Nanograms/Gram Diet

MEAN PERCENT REDUCTION IN LARVAL WEIGHT
MILLIGRAMS/LARVA

FIG 12

T 133

D = 7

TREATMENTS

CK

n = 108

n = 100

INFECTED POPULATION
7 DAYS

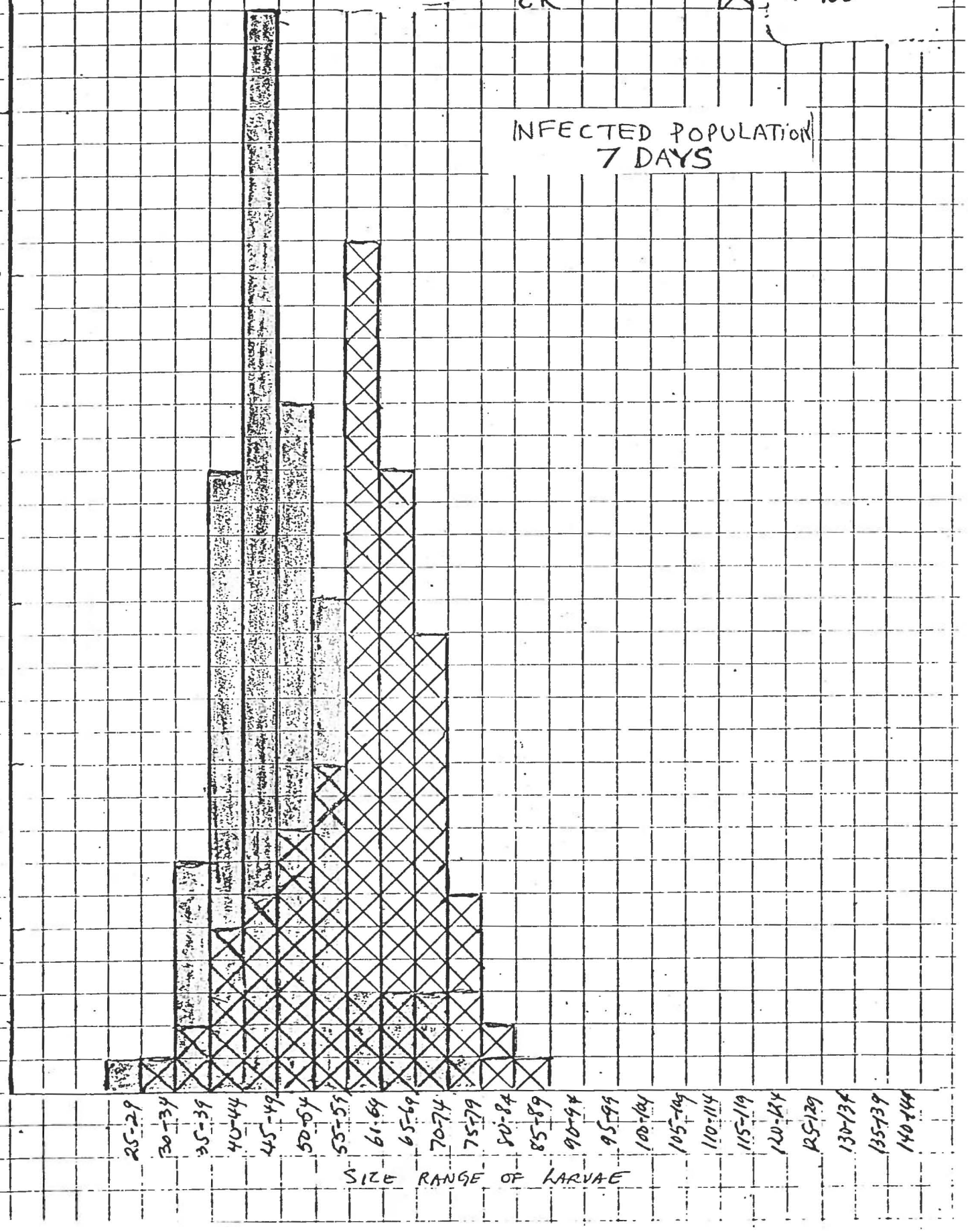


FIG. 13

T 133

D = 10

TREATMENTS

CK

n = 121

n = 73

INFECTED POPULATION
10 DAYS

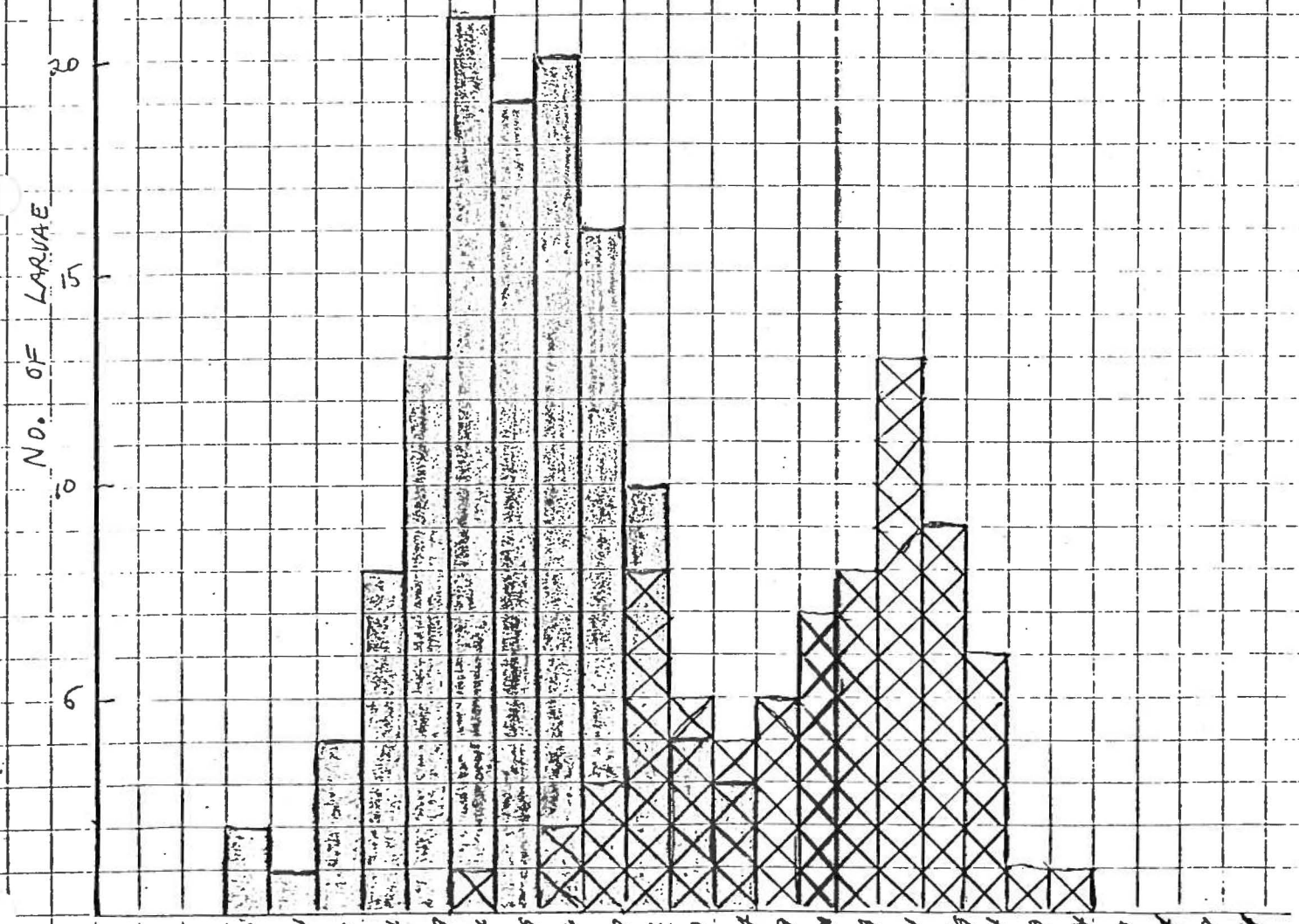


FIG. 14

T 133

D = 13

TREATMENTS

CK

n = 103

96

INFECTED POPULATION

13 DAYS

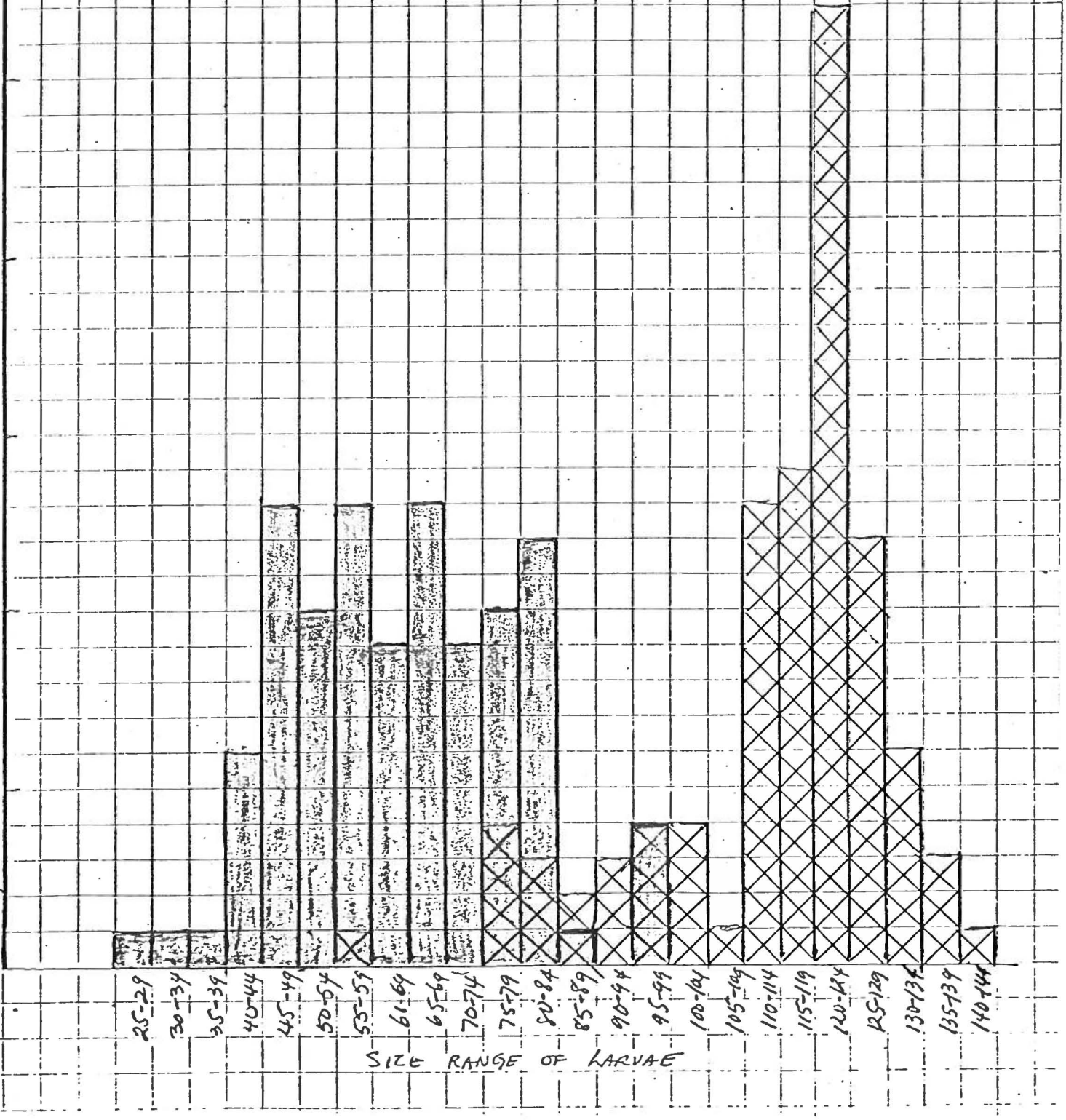
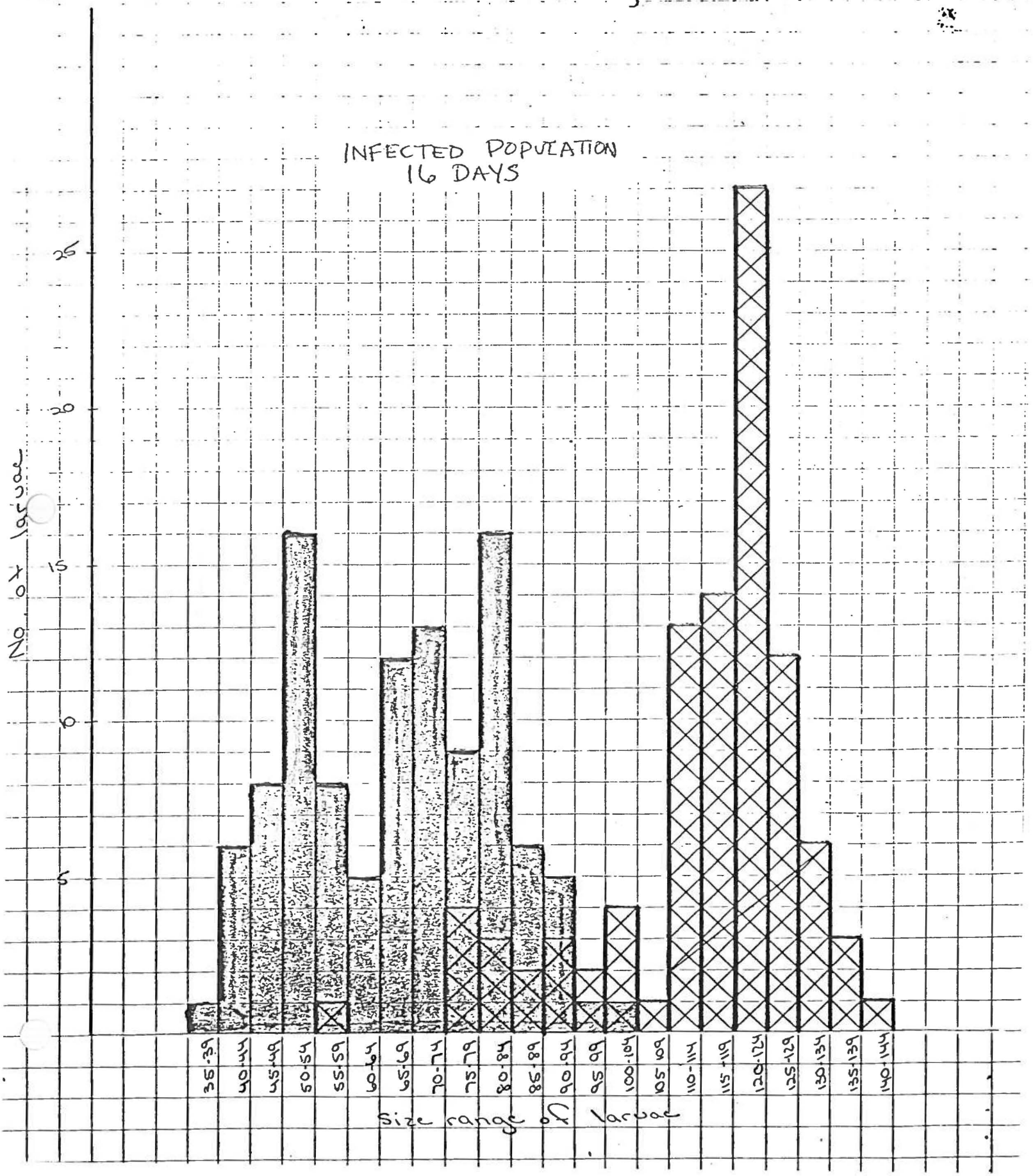


FIG. 15

T 133 D=16 TRs n=107

T 133 D=13 CRs n=96 (mature larvae)



T. 133 D = 20 TRs n = 101

T. 133 D = 13 CRs n = 96
(mature larvae)

Fig. 16

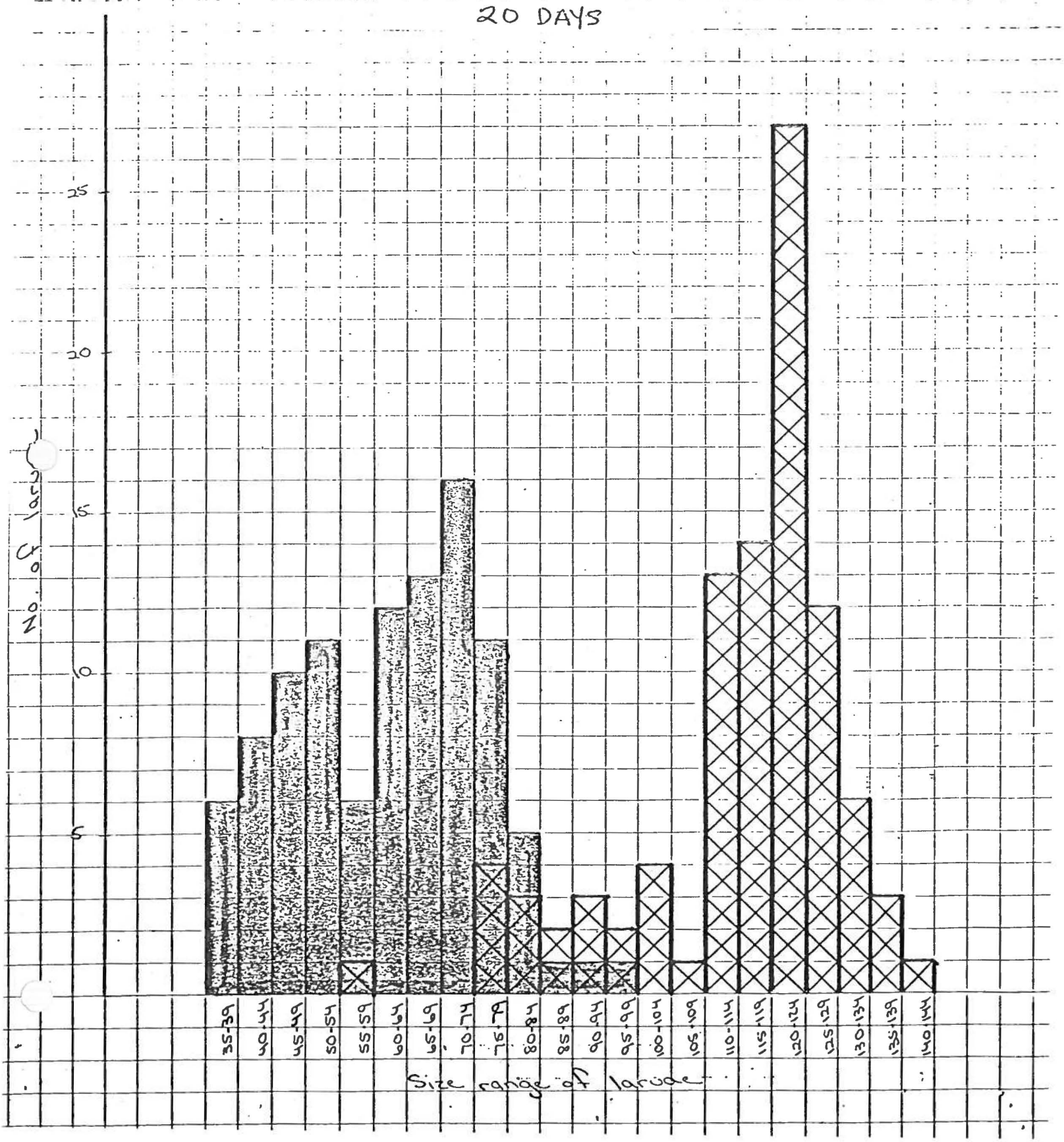
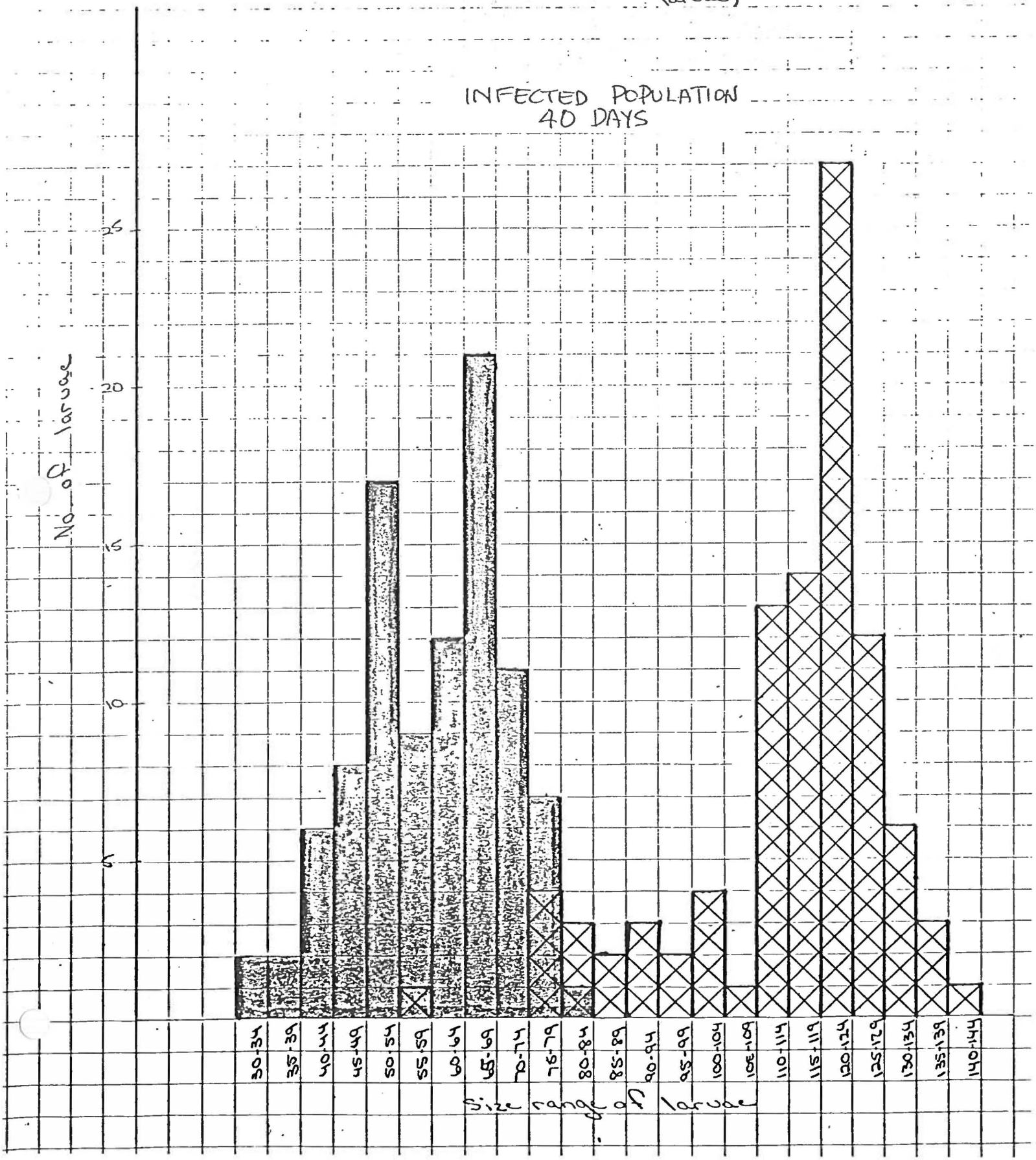


FIG. 17

T 133 D = 40 Treatments n = 96

T 133 D : 13 Checks n = 96 (mature larvae)



T 133 D=7 Treatments n=106

HEAD CAPSULE WIDTH SIZE DISTRIBUTION

D7

INFECTED POPULATION
AFTER 7 DAYS

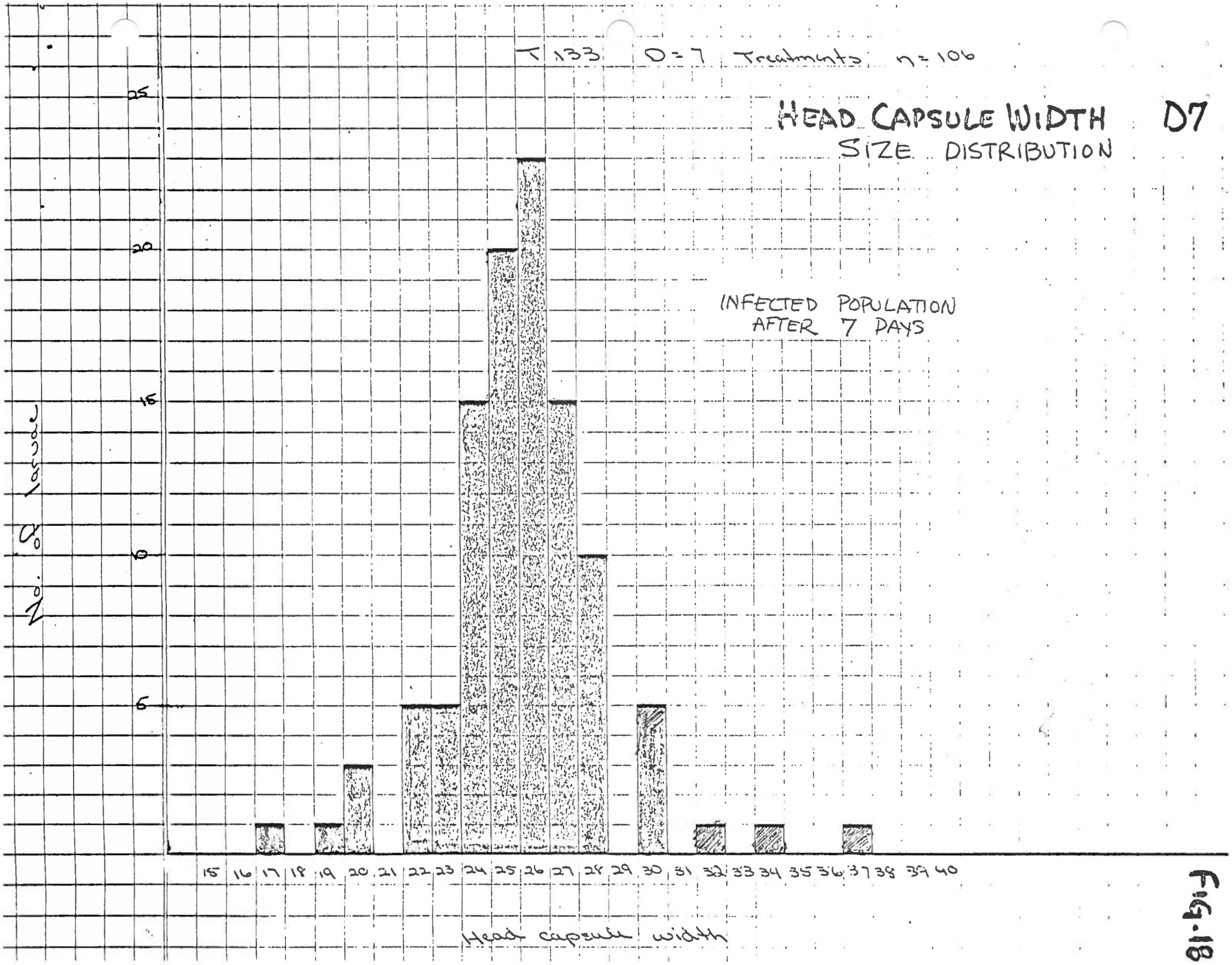


Fig. 18

T 133 D=13 Treatments n=104

HEAD CAPSULE WIDTH D:13

SIZE DISTRIBUTION

INFECTED POPULATION
AFTER 13 DAYS

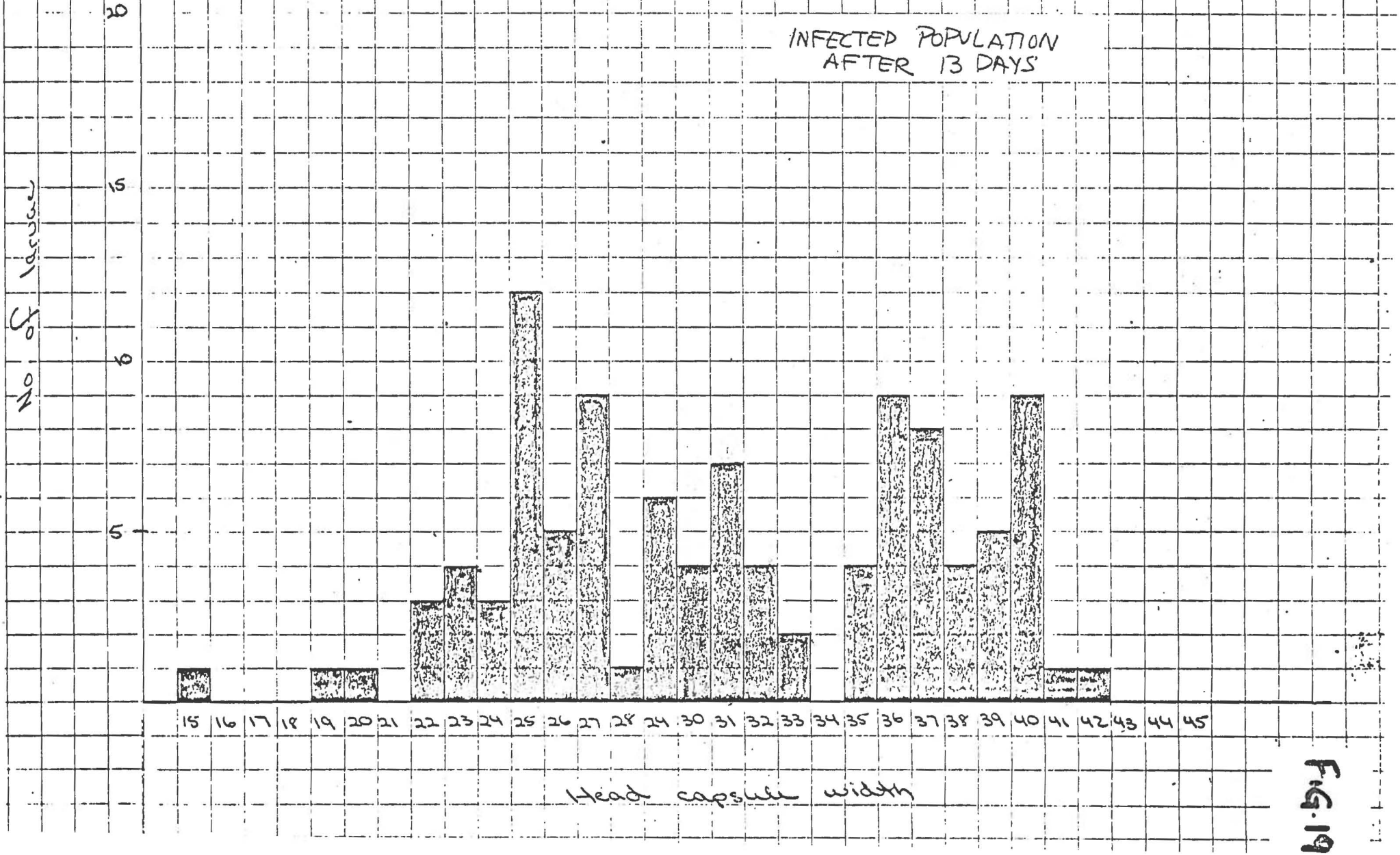


Fig. 19

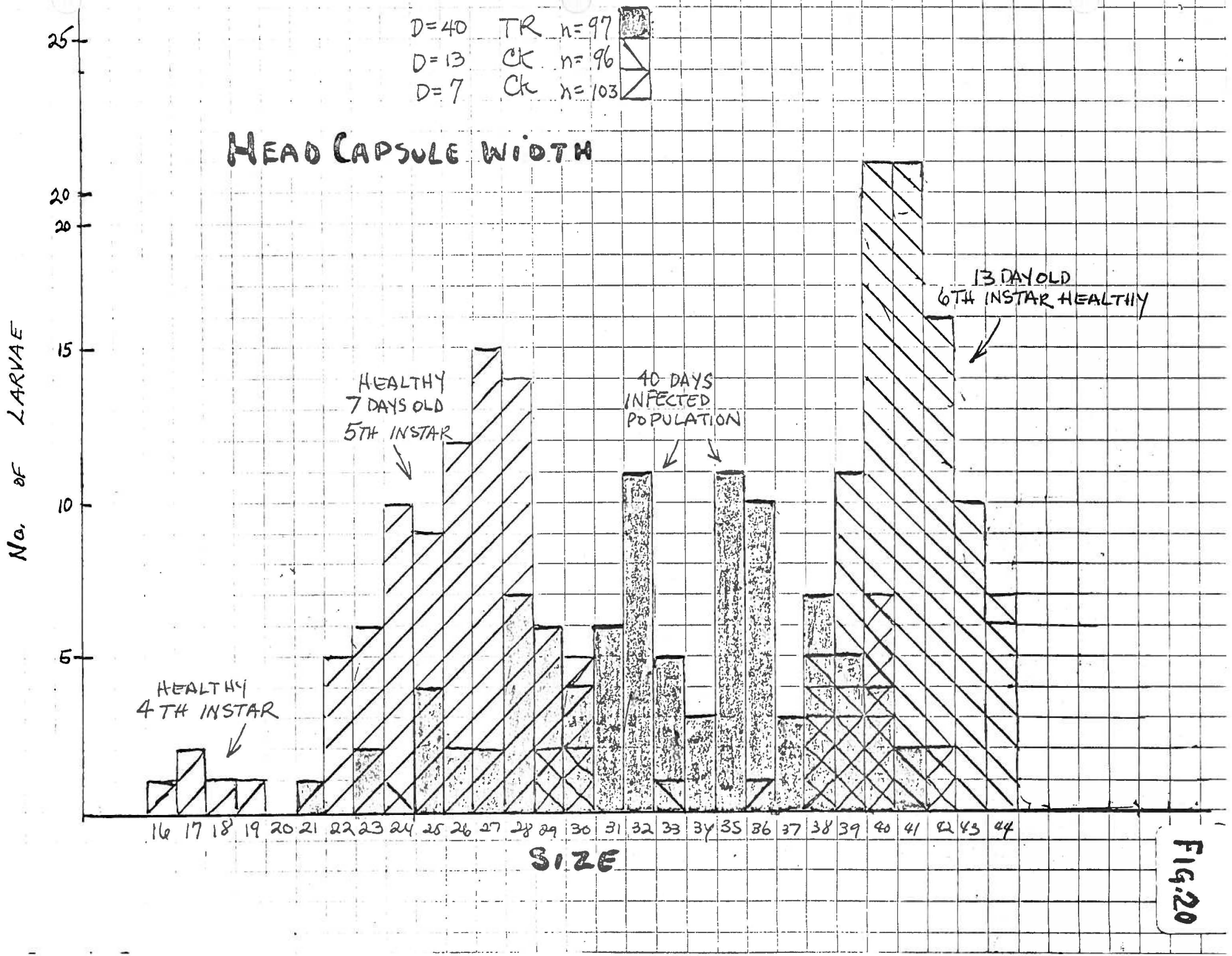

D=40 TR n=97
D=13 Ck n=96
D=7 Ck n=103
HEAD CAPSULE WIDTH
No. OF LARVAE
25
20
20
15
10
5
HEALTHY
4TH INSTAR
HEALTHY
7 DAYS OLD
5TH INSTAR
40 DAYS
INFECTED
POPULATION
13 DAY OLD
6TH INSTAR HEALTHY
16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44
SIZE

FIG. 20

BIMODAL DISTRIBUTION OF VIRUS-INFECTED NOW LARVAE FROM MAINTENANCE CULTURE

n= 1038

FIG. 21

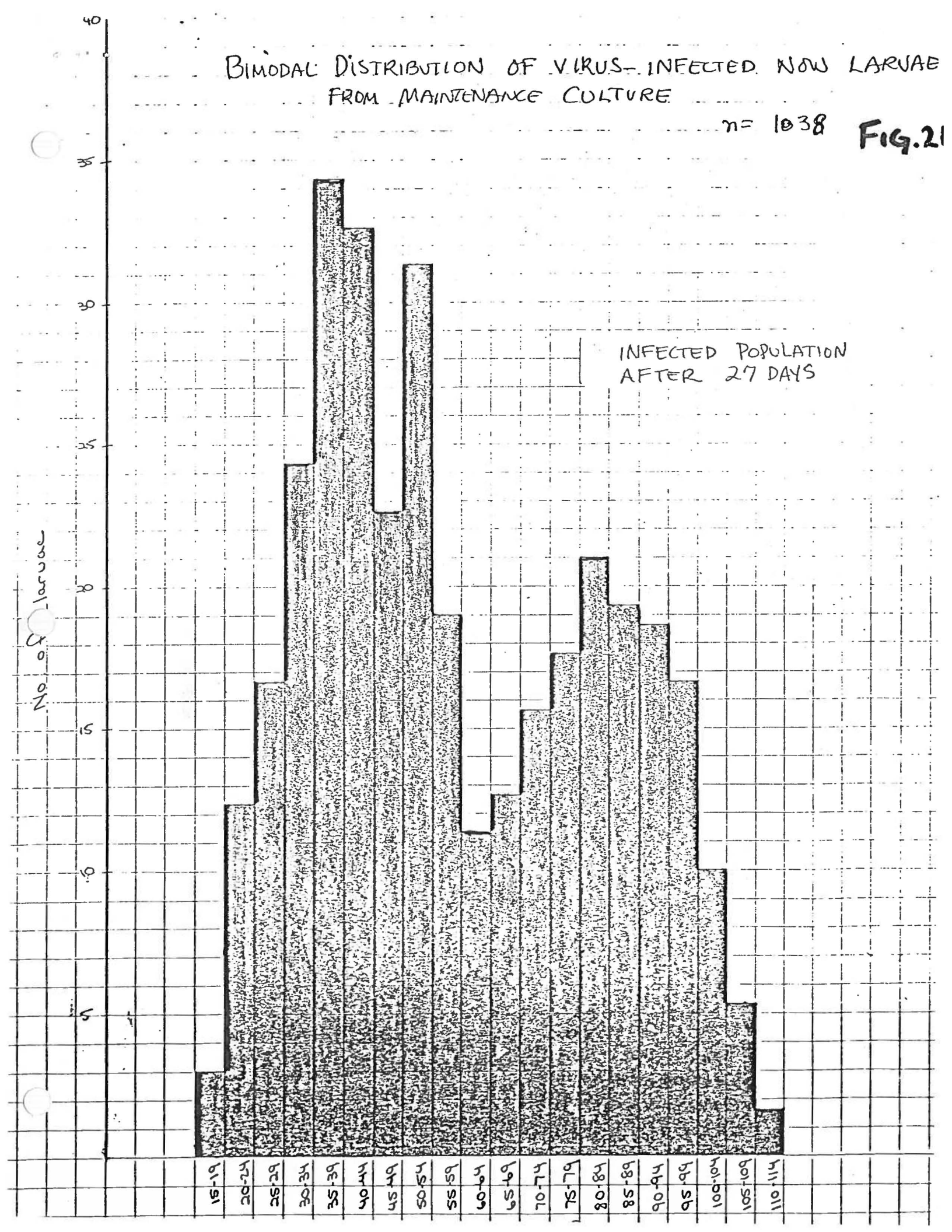


FIG. 22

T 138 n = 1039

CS T 138 MAINTENANCE COLONY
BIMODAL DISTRIBUTION
n = 1039

HEALTHY POPULATION
n = 902

VIRUS INFECTED POPULATION
n = 1038

PROBIT ACCUMULATED PERCENT OF POPULATION

SIZE