September 14, 2005

Lecture 6: Dose/Response I (Overview of the Dose Response Relationship)

I. Summary from Lecture 3

A. In lecture 3, we discussed how the typical dose-response relationship is derived from a normally distributed population response.
   1. The response could be any biochemical, genetic, physiological, morphological, behavioral, etc. observation that we wish.
   2. In the normal distribution, we are interested first in the median numbers responding at a specific dose; we are also concerned with the variation in individuals responding across the full regime of tested doses.

B. We also discussed that examining the normal distribution as a percentage of population responding changes the bell shaped curve to a logistic or S-shaped curve.
   1. By definition the median response on the logistic curve is called the LD50 (if lethality is the toxicological endpoint or measured response and the dose is expressed on a body weight basis, usually employing the units mg/kg).
      a. If a concentration were used (such as it would be if aquatic organisms were being tested), than the median response would be the LC50.
   2. If a sublethal response is being measured, or alternatively, we are measuring a biochemical or physiological response, we could express the median response as an effective dose or concentration (ED50 or EC50).
   3. Note that we could examine any proportion or percentage of response;
      a. For example, if we were interested in 95% of the population responding, we would examine the dose-response relationship to estimate the LC95 (if a series of concentrations were being tested).
      b. Similarly, we might be interested in just the dose that gives 10% response (LD10).

C. In addition to expressing the magnitude of population response as a relationship to dose, we could express the response in relationship to time.
   1. In this case, we might use a fixed dose or concentration and determine the time it takes to kill or adversely affect 50% of the population (LT50). (See below **V**, example 2, "Time to Die")

II. How the Dose-Response Relationship Is Measured and Mathematically Deduced

A. Organisms reared under standard uniform conditions (to minimize inter individual variability) are divided into separate groups and then either dosed with a series of increasing concentrations or doses of toxicant (by feeding; by topical or dermal application; by exposure to vapors, etc.). One group is not exposed to toxicant
   1. Thus the dose or concentration the different groups are exposed to is considered the independent variable in the experiment. We have control over the independent variable and know its value (magnitude) prior to the start of the experiment.
   2. At each dose level, observations of mortality or any other biological response are made. These observations are the dependent variables. Their values are

unknown at the beginning of the experiment, but they are measured in response to the known independent variables.

B. The data, which are now expressed as number of organisms tested per dose, and the number responding, are fed into a computer program that can calculate one of two basic statistical techniques—probit analysis or logit analysis (logistical regression).
   1. The computer program will estimate the response at any percentage of population response.

C. Be aware that the resulting LD50 or LC50, for example, is just a statistical estimate of the median response of the population under the conditions of the experiment.
   1. The number generated is not a fixed solid characteristic of the toxicant's interaction with the population of test organisms.
      a. If the experiment was repeated again, a different estimate of LD50 or LC50 would be calculated owing to the natural variation in response from each group of individuals tested.
   2. Thus, in reality, if we kept on repeating the experiment, we would be measuring a population of potential responses of some specific level of response.
      a. Thus, to know the likelihood that we have captured in our measurements the "true" population response, the computer program also calculates confidence limits about each LD or LC estimate.
      b. In probit analysis, these confidence limits are called 95% fiducial limits (FL).

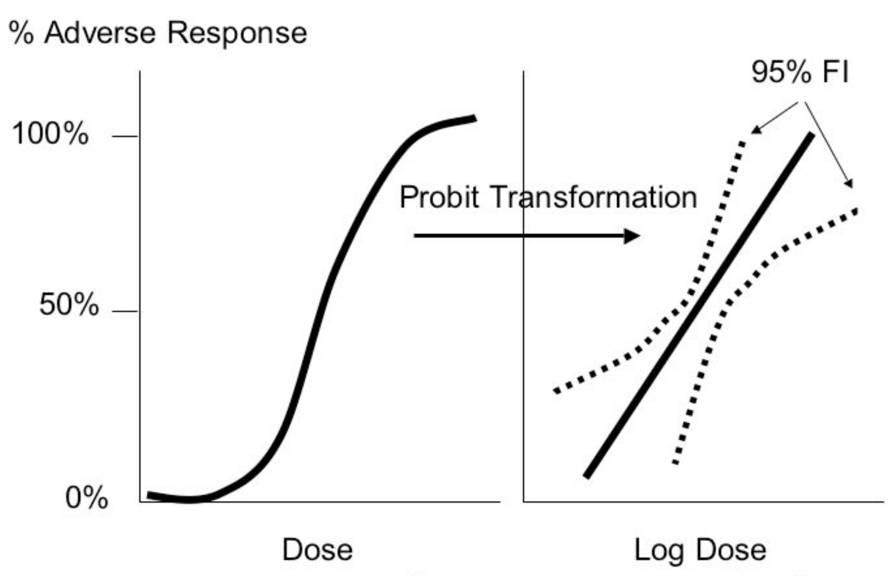

Figure 1. Dose-response function (arithmetic plot to show sigmoidal nature of curve, left side) and probit transformation (using logarithmic dose, right side) along with 95% fiducial limits).

c. The significance of a 95% fiducial limit: If the experiment was conducted 100 times, than the 95% FL is predicted to capture within its interval the true population response (at the specified dose) 95 times. Thus, there is a 5% probability that the true population response is outside of this interval.
d. Because the fiducial limits are narrower (meaning less variation in response) about the median response (i.e., the LD50), toxicologists usually rely on this parameter for expressing comparative toxicity.
e. Thus, at the lower and higher levels of response, a lot more variability is seen and the estimates of toxicity are less reliable.
   1. One can compare the toxicity of a toxicant to two or more populations by looking for overlap between the LC50 or LD50 of the tested populations.
   2. Similarly, one can compare the influence on toxicity response of any independent variable, for example temperature effect, pH effect, second chemical in a mixture, etc. One would conduct a dose-response experiment, statistically estimate the LD50 or LC50, and then observe whether overlap has occurred about the LD/LC50 for each independent variable tested.

D. The threshold for toxicity can be estimated by mathematically extrapolating the dose-response function through the dose at which no response has occurred or been measurable. This corresponding threshold dose is the NOAEL or NOAEC.
   1. Often, however, the NOAEL or NOAEC is estimated by visual observation of which dose in the testing regime caused no significant difference in response compared to the undoes group (i.e., the control group).

III. Using the Dose-Response Relationship to Deduce Genetic Variation in a Population and Track Changes over Time—The Value of the Slope of the Curve

A. For any single compound, the slope of the dose-response line helps determine the margin of safety (Figure 2).
   1. Shallow slope allows greater margin of safety; in other words, comparatively larger changes in dose result in small changes in response (Figure 2B,D).
   2. The slope also tells something about the variability in the population (Figure 2B, D);
      a. This variation is actually the variation in response, largely stemming from genetic variation leading to phenotypic variation within a given population.
      b. A steep slope indicates little variation in the population response;
      c. A comparatively shallower slope indicates that the response is much more variable over a greater dose range.

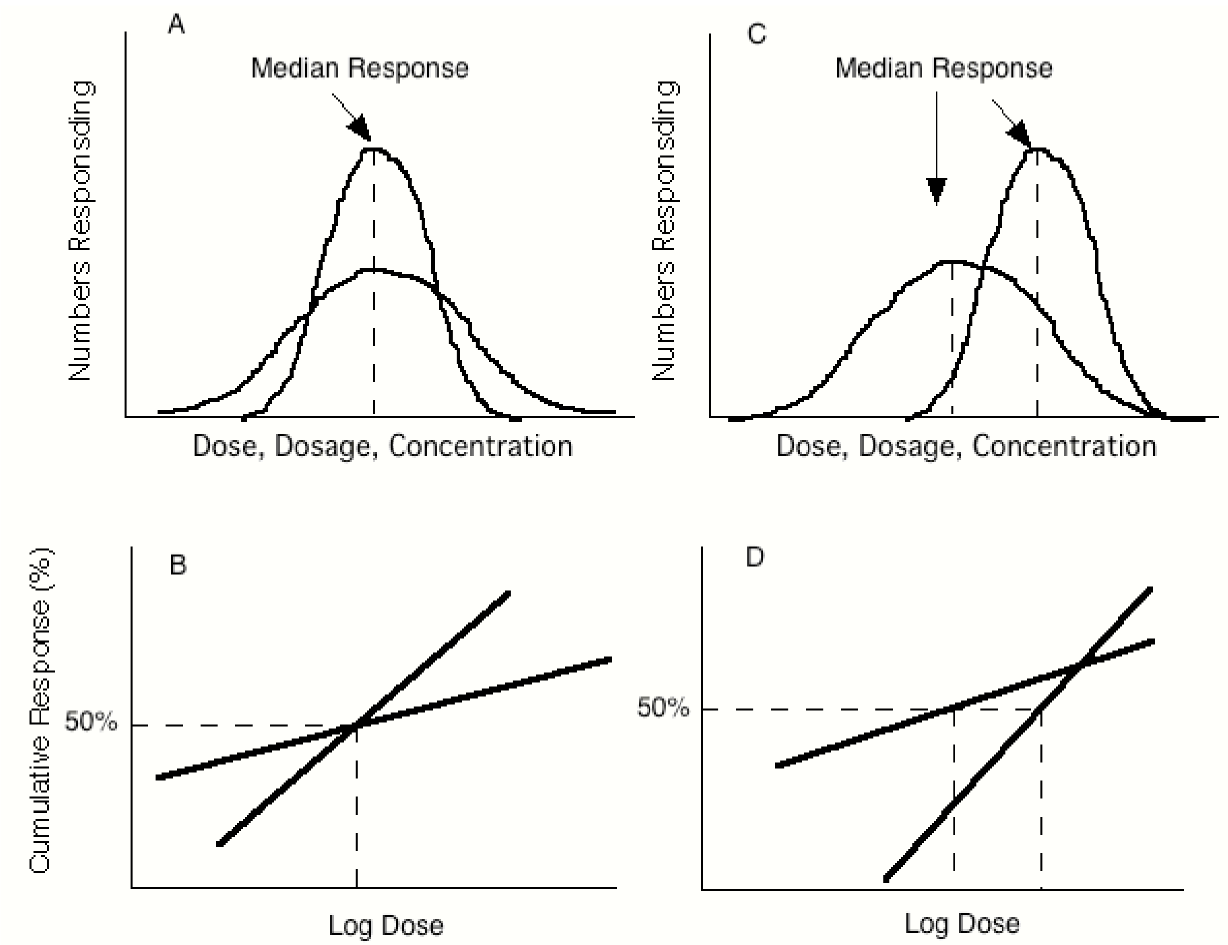

Figure 2. Relationship between slope and variability (distribution) of response of one or more populations to a single chemical, or response of a single population to two different chemicals; or response of two different species to a chemical.

B. Two different species might respond to a chemical with the same LD50/LC50, but the variation in susceptibility may differ substantially (Figure 2A,B). Alternatively, the LD50's may be substantially different, in addition to the variability being different (Figure 2C,D).
C. Note that the slope can also be used to assess the occurrence of resistance in a population. Populations naïve to a toxicant are fairly homogeneous in response. As a toxicant selects for resistant individuals, the variability in response increases (distribution flattens out), and as selection continues, most individuals will eventually become resistant, establishing a new, homogenous distribution but exhibiting a substantially higher LD50 (Figure 3).

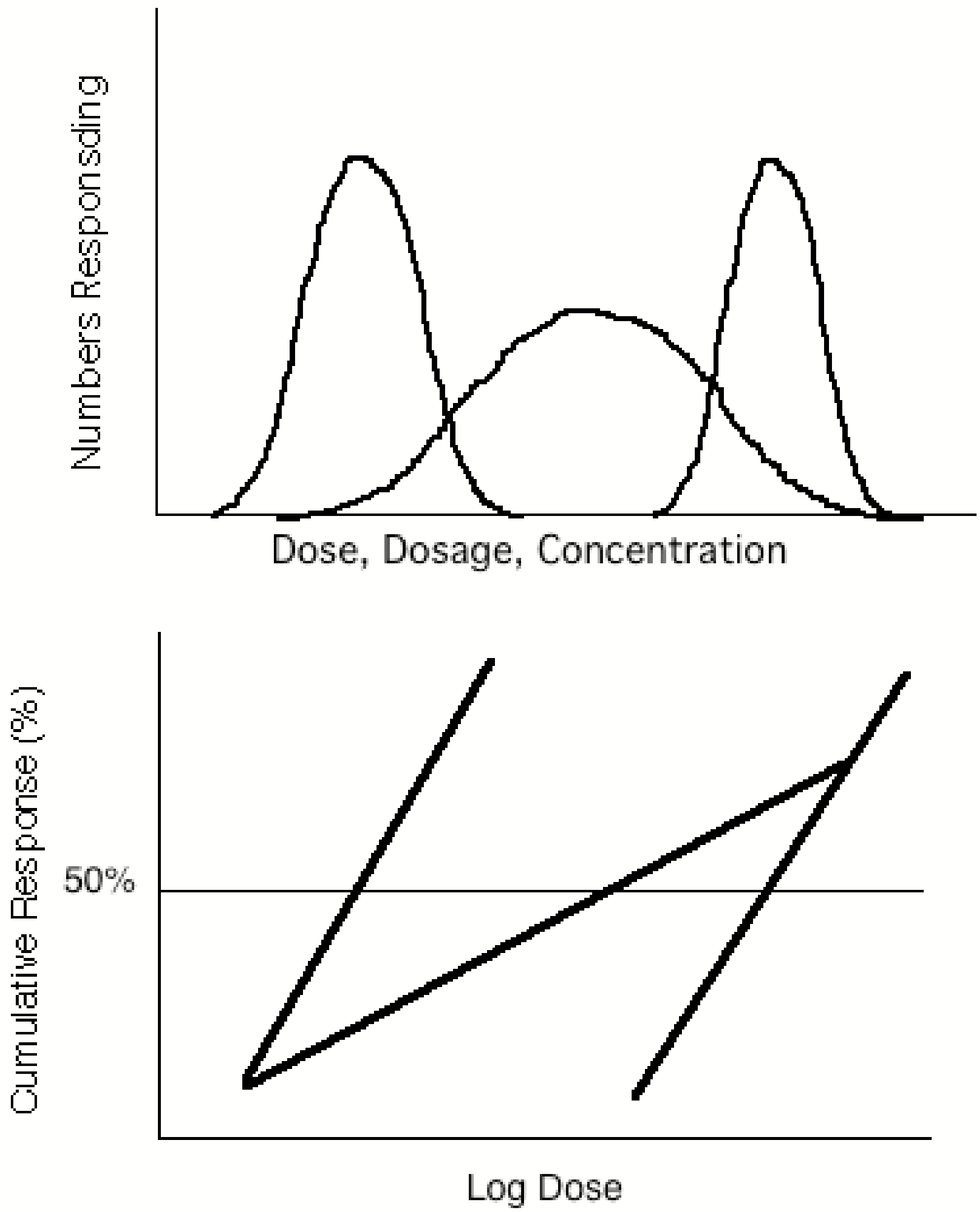

Figure 3. Change in susceptibility after repeated selection for resistant individuals.

IV. Example 1: Computer Program and Output for Estimating LC50

A. The following data represents the input and output to determine the LC50 for an organophosphate insecticide on codling moth neonate larvae.

B. Experimental Procedure
1. Insecticide was pipetted on leaf disks of known surface area
2. Neonate codling moth (n=5) were placed on replicate leaf disks per dose
3. 24 h and 48 h, dead larvae were counted
4. The data was transferred to an Excel spreadsheet and than imported in a statistical program called SAS (Statistical Analysis System)

5. After the toxicity parameters were estimated and printed out, the data for the probability of mortality was plotted in a graphing program.

A SAS (Statistical Analysis System) Program for Estimating the LC50 of an Insecticide on Treated Leaf Surfaces Against Codling Moth Neonate Larvae

<u>INPUT FILE:</u>

```
Data Guthion1;
      Input Dose N Dead;
      Observed=dead/N;
datalines;
0.0000   43    02
0.0099   42    13
0.0198   50    35
0.0296   36    28
0.0395   48    43
;

Proc Probit LOG10 OPTC INVERSECL;
   Model Dead/N=Dose;
run;
```

<u>OUTPUT FILE:</u>

```
                              Probit Procedure

Data Set        =WORK.GUTHION1
Dependent Variable=DEAD
Dependent Variable=N
Number of Observations=   5
Number of Events      =    121    Number of Trials =     219
Number of Events In Control Group =      2
Number of Trials In Control Group =     43


Log Likelihood for NORMAL -100.1627644

Probit Procedure

   Variable     DF   Estimate      Std Err    ChiSquare   Pr>Chi   Label/Value
   INTERCPT   1    5.33581127   0.87014    37.60266   0.0001     Intercept
   Log10(DOS) 1    2.92578526   0.5153     32.23771   0.0001     Slope
   _C_            1    0.04566056   0.0314                              Lower threshold

         Probit Model in Terms of Tolerance Distribution

                        MU        SIGMA
                   -1.82372     0.341789


                        Probit Procedure

        Estimated Covariance Matrix for Tolerance Parameters
                        MU              SIGMA                _C_
```

MU	0.002272	-0.001592	0.000500
SIGMA	-0.001592	0.003624	-0.000277
_C_	0.000500	-0.000277	0.000991

Probit Procedure
Probit Analysis on Log10(DOSE)

Probability	Log10(DOSE)	95 Percent Fiducial Limits Lower	Upper
0.01	-2.61884	-3.12633	-2.36712
0.02	-2.52567	-2.98501	-2.29688
0.03	-2.46655	-2.89546	-2.25221
0.04	-2.42208	-2.82817	-2.21853
0.05	-2.38591	-2.77349	-2.19108
0.06	-2.35512	-2.72699	-2.16768
0.07	-2.32813	-2.68625	-2.14712
0.08	-2.30396	-2.64981	-2.12867
0.09	-2.28197	-2.61670	-2.11187
0.10	-2.26174	-2.58625	-2.09638
0.15	-2.17796	-2.46053	-2.03188
0.20	-2.11138	-2.36113	-1.98008
0.25	-2.05425	-2.27640	-1.93512
0.30	-2.00295	-2.20089	-1.89415
0.35	-1.95542	-2.13161	-1.85549
0.40	-1.91031	-2.06671	-1.81798
0.45	-1.86667	-2.00498	-1.78061
0.50	-1.82372	-1.94563	-1.74245
0.55	-1.78077	-1.88814	-1.70242
0.60	-1.73713	-1.83221	-1.65926
0.65	-1.69202	-1.77764	-1.61140
0.70	-1.64449	-1.72414	-1.55697
0.75	-1.59319	-1.67091	-1.49372
0.80	-1.53606	-1.61626	-1.41867
0.85	-1.46948	-1.55699	-1.32675
0.90	-1.38570	-1.48669	-1.20682
0.91	-1.36546	-1.47019	-1.17738
0.92	-1.34348	-1.45242	-1.14524
0.93	-1.31931	-1.43303	-1.10974
0.94	-1.29232	-1.41156	-1.06992
0.95	-1.26153	-1.38725	-1.02432
0.96	-1.22535	-1.35891	-0.97054
0.97	-1.18089	-1.32432	-0.90415
0.98	-1.12177	-1.27868	-0.81557
0.99	-1.02860	-1.20732	-0.67538

Probit Procedure
Probit Analysis on DOSE

Probability	DOSE	95 Percent Fiducial Limits Lower	Upper
0.01	0.00241	0.00075	0.00429
0.02	0.00298	0.00104	0.00505
0.03	0.00342	0.00127	0.00559

0.04	0.00378	0.00149	0.00605
0.05	0.00411	0.00168	0.00644
0.06	0.00441	0.00188	0.00680
0.07	0.00470	0.00206	0.00713
0.08	0.00497	0.00224	0.00744
0.09	0.00522	0.00242	0.00773
0.10	0.00547	0.00259	0.00801
0.15	0.00664	0.00346	0.00929
0.20	0.00774	0.00435	0.01047
0.25	0.00883	0.00529	0.01161
0.30	0.00993	0.00630	0.01276
0.35	0.01108	0.00739	0.01395
0.40	0.01229	0.00858	0.01521
0.45	0.01359	0.00989	0.01657
0.50	**0.01501**	**0.01133**	**0.01809**
0.55	0.01657	0.01294	0.01984
0.60	0.01832	0.01472	0.02192
0.65	0.02032	0.01669	0.02447
0.70	0.02267	0.01887	0.02774
0.75	0.02552	0.02133	0.03208
0.80	0.02910	0.02420	0.03814
0.85	0.03393	0.02773	0.04712
0.90	0.04114	0.03261	0.06211
0.91	0.04311	0.03387	0.06647
0.92	0.04534	0.03528	0.07157
0.93	0.04794	0.03689	0.07767
0.94	0.05101	0.03877	0.08513
0.95	0.05476	0.04100	0.09455
0.96	0.05952	0.04376	0.10702
0.97	0.06593	0.04739	0.12469
0.98	0.07555	0.05264	0.15291
0.99	0.09363	0.06204	0.21117

The first graph below (Figure 4) represents the plotted results (arithmetic data from the second table above) for Guthion (azinphos-methyl) insecticide.

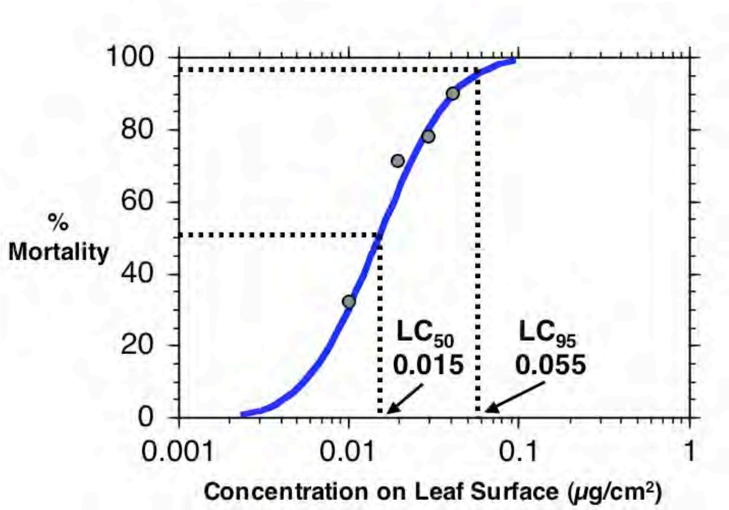

Figure 4. Concentration-response function for neonate codling moth on leaf disks treated with Guthion insecticide.

The second graph below (Figure 5) represents analysis of data for a bioassay with Intrepid (methoxyfenozide). Note that not only is methoxyfenozide less toxic to neonate codling moth larvae than azinphos-methyl, but the slope of the line is somewhat flatter. Methoxyfenozide has an entirely different pharmacodynamics action than azinphosmethyl. Also, the slope of the line suggests greater genetic variability in susceptibility to methoxyfenozide than to azinphos-methyl.

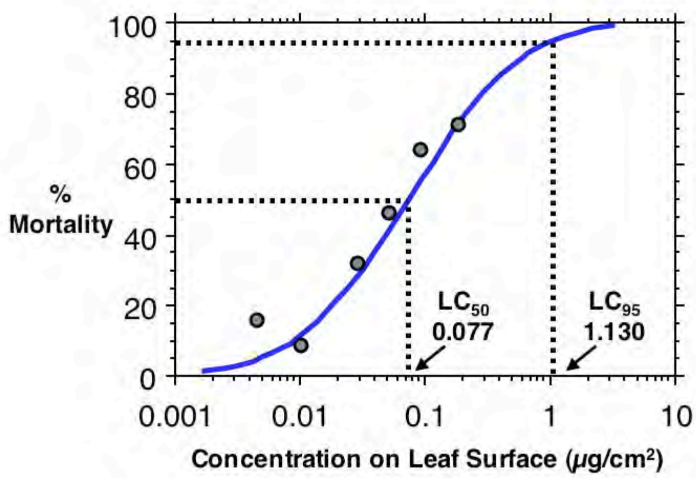

Figure 5. Concentration-response function for neonate codling moth on leaf disks treated with methoxyfenozide.

V. Example 2: "Time to Die", the LT50 (time of exposure before 50% of population respond).

Apple trees were sprayed with Guthion insecticide on both sides or only on one side to test the hypothesis that sufficient spray moves through a canopy to be lethal to codling moth larvae. The rationale for this experiment is that perhaps only one side of a tree needs to be sprayed, and therefore growers can use less insecticide (and save money!).

After the trees were sprayed leaves were collected and neonate codling moth larvae were exposed for various time intervals over 120 minutes. Numbers of dead larvae were counted after specified time intervals (15, 30, 60, 90, 120 minutes). Untreated leaves were also assayed.

The first step is to make a table of the data. Note that there was mortality in the untreated controls at some time intervals, so the mortality in the treatments had to be corrected for this "natural" background. To correct for control mortality, Abbott's formula is a commonly used function. (Abbott, W.S. 1925. A method of computing the effectiveness of an insecticide. J. Econ. Entomol.; ***18*** : 265-267.)

% Corrected Mortality =
[(% Treatment Mortality - % Control Mortality)/(100% - Control Mortality)]*100

Raw Data Entered Into an Excel Spreadsheet for Calculation of % Mortality and Correction for Mortality in the Control (Untreated Leaves)

Treatment	Time	Number Dead	Total Number	% Mortality	% Corrected Mortality
Untreated	15	0	48	0.0	
Untreated	30	0	50	0.0	
Untreated	60	2	46	4.3	
Untreated	90	19	46	41.3	
Untreated	120	22	46	47.8	
Both Sides	15	1	49	2.0	2.0
Both Sides	30	20	49	40.8	40.8
Both Sides	60	36	49	73.5	72.3
Both Sides	90	42	48	87.5	78.7
Both Sides	120	45	49	91.8	84.4
One Side	15	5	49	10.2	10.2
One Side	30	13	49	26.5	26.5
One Side	60	25	49	51.0	48.8
One Side	90	34	47	72.3	52.9
One Side	120	40	43	93.0	86.6

Note that when preparing data for statistical analysis, all observation for each replicate treatment should appear on the same line. This is the most common format that modern statistical programs receive and handle data.

A probit analysis on the above data was run with the following results (only the "One Side" treatment is shown in the output table below.

Calculated Data from Probit Analysis of Time to Mortality Experiment with Neonate Codling Moth Larvae Exposed to Trees Sprayed on One Side with Guthion Insecticide.

Probability	% Mortality	TIME	95% Lower FL.	95% Upper FL
0.01	1	-52.3	-94.6	-27.0
0.02	2	-37.9	-75.3	-15.4
0.03	3	-28.7	-63.0	-8.0
0.04	4	-21.9	-53.8	-2.4
0.05	5	-16.3	-46.3	2.2
0.06	6	-11.5	-40.0	6.1
0.07	7	-7.3	-34.4	9.5
0.08	8	-3.6	-29.5	12.6
0.09	9	-0.1	-25.0	15.4
0.10	10	3.0	-20.8	18.0
0.15	15	16.0	-3.8	28.9
0.20	20	26.3	9.5	37.7

0.25	25	35.1	20.7	45.5
0.30	30	43.1	30.5	52.7
0.35	35	50.4	39.3	59.8
0.40	40	57.4	47.3	66.8
0.45	45	64.2	54.7	73.9
0.50	50	70.9	61.6	81.3
0.55	55	77.5	68.1	89.0
0.60	60	84.3	74.5	97.2
0.65	65	91.3	80.9	105.8
0.70	70	98.6	87.4	115.1
0.75	75	106.6	94.2	125.4
0.80	80	115.4	101.7	136.9
0.85	85	125.7	110.3	150.4
0.90	90	138.7	120.9	167.6
0.91	91	141.9	123.5	171.8
0.92	92	145.3	126.3	176.4
0.93	93	149.0	129.3	181.3
0.94	94	153.2	132.7	186.9
0.95	95	158.0	136.6	193.3
0.96	96	163.6	141.1	200.8
0.97	97	170.5	146.7	210.1
0.98	98	179.6	154.0	222.3
0.99	99	194.0	165.6	241.8

Note that probability represents the proportion of the tested population (of insect larvae) that has been estimated to die after the indicated time. The probability was transformed to % Mortality (% M) by multiplying by 100. 95% FL are fiducial limits (analogous to confidence intervals) that represent intervals likely to capture the "true" population mortality/time response 95 per 100 times the experiment was conducted (i.e., the probability of not capturing the true population response at any time interval would be 5%).

The calculations data along with the actual observed data points were graphed in a program called DeltaGraph (Red Rock Software; PC and MAC compatible) and then edited for presentation using Corel Draw (PC and MAC compatible) (Figures 6 and 7).

From Figure 6, we can conclude that the LT50 for neonate larvae exposed to leaves from the unsprayed side of a tree is about 71 minutes. This time to death for 50% of the population is about 17 minutes greater than the LT50 for larvae exposed to leaves from the sprayed side of the tree (LT50 ~54 minutes) (Figure 7). However, be aware of overlapping 95% Fiducial Limits. At a probability of 5%, we cannot resolve the difference between treatments in the observed distribution of larval responses.

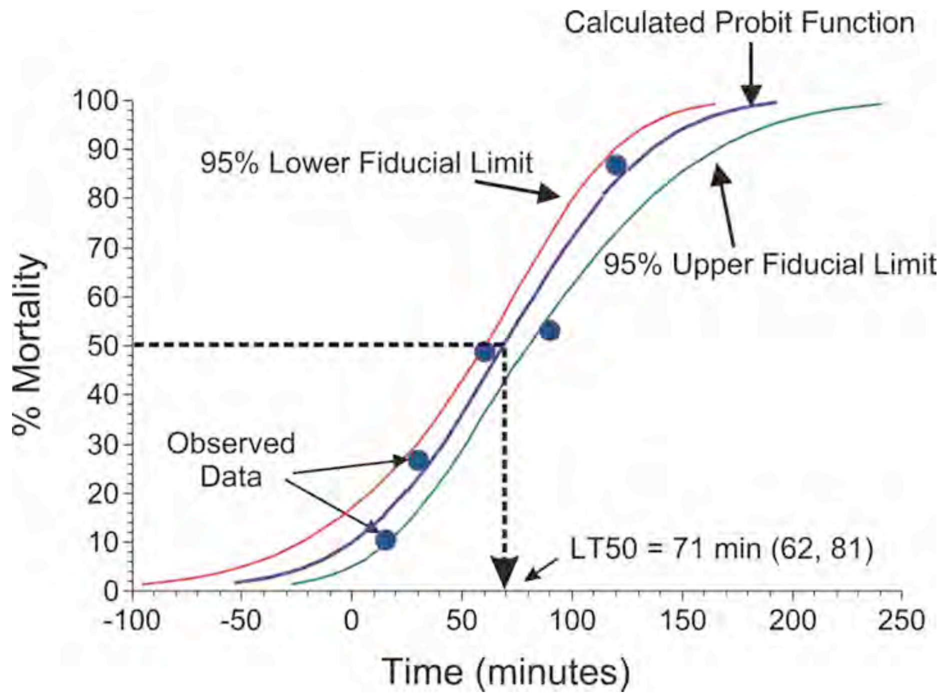

Figure 6. The dose-response (actually the time-response function) and associated 95% fiducial limits for neonate codling moth larvae exposed to leaves collected from the unsprayed side of an apple tree. The values for the 95% fiducial limits are shown in parentheses adjacent to the LT50 of 71 minutes.

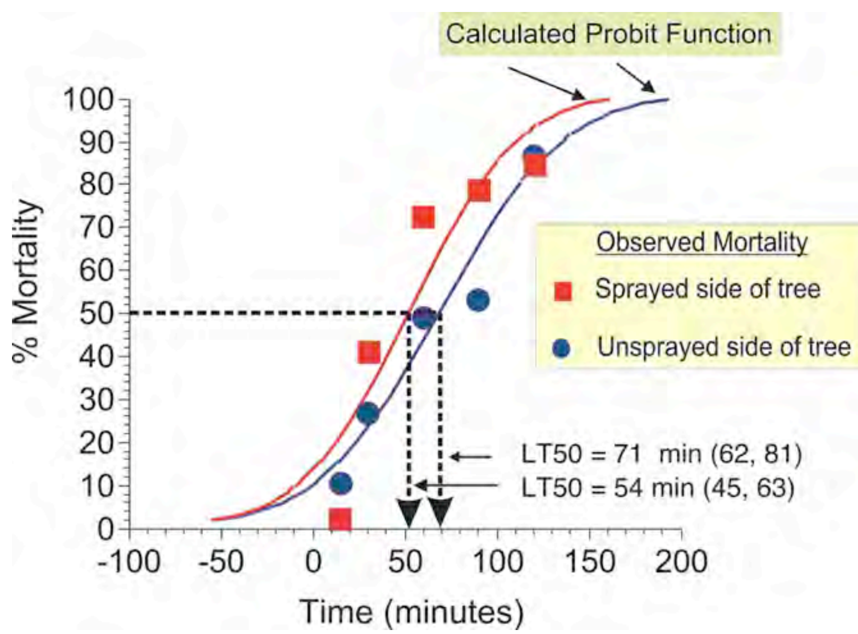

Figure 7. Comparison of the time-response function for treatment "one side" sprayed and treatment "both sides" sprayed. Note the relative position of each function, but beware of the overlapping fiducial limits.