

EXPERIMENT 5: LINKAGE MAPPING IN *DROSOPHILA MELANOGASTER*

Introduction to the laboratory: Mendel reported that all sets of genes that he studied assorted independently. Even early in the 20th century, it was probably clear to geneticists that organisms would tend to consist of thousands of genes. Could they all assort independently? It was discovered by Punnett and Bateson that indeed, some genes appeared linked together - were often transmitted as one. Further, Thomas Hunt Morgan observed that this linkage appeared to vary in frequency (e.g., some gene pairs might be linked 70%, and others 90%, of the time, while recombining their alleles the other 30% and 10%, respectively). Morgan's student, Alfred Sturtevant, proposed that the linkage and recombination could be used to determine the relative positions of genes on chromosomes. Sturtevant realized that the greater the distance between linked genes, the greater the chance that non-sister chromatids would cross over in the region between the loci. Sturtevant created the first linkage map using *Drosophila melanogaster*.

Since that time, *D. melanogaster* has become the most studied eukaryote on earth. It is known as a **model organism**: one used to elucidate (understand) principles that can then be applied to other less tractable (easily studied) species. *D. melanogaster* is a model for molecular and cellular biology, development, genetics, evolution, and behavior. In this laboratory you will learn skills needed to create a linkage map and develop basic familiarity with one of the most widely used organisms in biology.

DAY ONE: DROSOPHILA GENETICS AND RECOMBINATION

OBJECTIVES:

- ❑ Understand the nomenclature used in *Drosophila* genetics.
- ❑ Understand the principles of linkage and recombination and how they can be used identify the relative distance between genes.
- ❑ Be able to draw out the crosses that were performed by the laboratory technician that you will use to create a linkage map.

INTRODUCTION:

As Mendel demonstrated with pea plants, much can be learned about the basis for an organism's characteristics if the right kinds of controlled crosses are made. Thousands of mutant strains of *Drosophila* have been collected in the laboratory for genetic studies. A **mutant** fly differs from a normal (or **wild-type**) fly in one or more heritable characteristics. The characteristic exhibited by the mutant is due to a change in the normal DNA sequence or its location. Such a change is called a **mutation**. A mutation may affect the morphology, physiology, or behavior (or any combination of traits) of a fly. A list of common mutations, including line drawings and photographs of the more common ones, may be found in the *Drosophila* manual provided in the lab. A name and **symbol** are designated for each mutation in *Drosophila*, and this nomenclature differs somewhat from the standard nomenclature first developed by Mendel. **Recessive mutations** are expressed in **lower case** letters, while the name and symbol of **dominant mutations** begin with a **capital letter**. A symbol with a + **superscript** designates the **wild-type** allele of a given mutant gene; **sometimes just a +** is used to designate the wild-type allele. Note that if the mutant allele is recessive, then the wild-type allele must be dominant, and vice

versa. Note also that most mutant alleles are recessive, but dominant mutations do exist.

The following examples illustrate the difference between standard Mendelian nomenclature and the nomenclature used for *Drosophila*. The advantage of the *Drosophila* method is that the wild-type allele can be immediately identified, whereas the Mendelian nomenclature does not provide this information.

Mendelian nomenclature is used for identifying alleles in humans. In humans, the disease cystic fibrosis is under the control of a single gene: "Cf" = disease-free; "cf" = cystic fibrosis. Note that it is impossible to tell *from the nomenclature alone* which allele is wild-type and which represents a mutation. In *Drosophila*, the trait of wing curliness is also under the control of a single gene: "+" = straight; Cy = curly. In the *Drosophila* nomenclature, we can immediately see that the wild-type trait is non-curly wings, and that the Curly allele is dominant to the wild-type (non-curly) allele.

A fly carrying one identifiable mutation is called a **single mutant**. Those exhibiting two, three, or multiple mutant traits are **double**, **triple**, or **multiple mutants**, respectively. **Morphological mutations** usually involve color, size, or shape of the eyes, bristles, wings, or body. While some mutations are easily recognized with the naked eye, many others can only be identified by a careful comparison between wild-type and mutant flies under a dissecting microscope. You should practice the skills of mutant identification required for accurate and rapid phenotypic classification of large numbers of flies. While morphological mutations are convenient for use in student labs, many other kinds of mutants (e.g. behavioral, developmental, biochemical) have been studied to gain knowledge of these processes in lower animals.

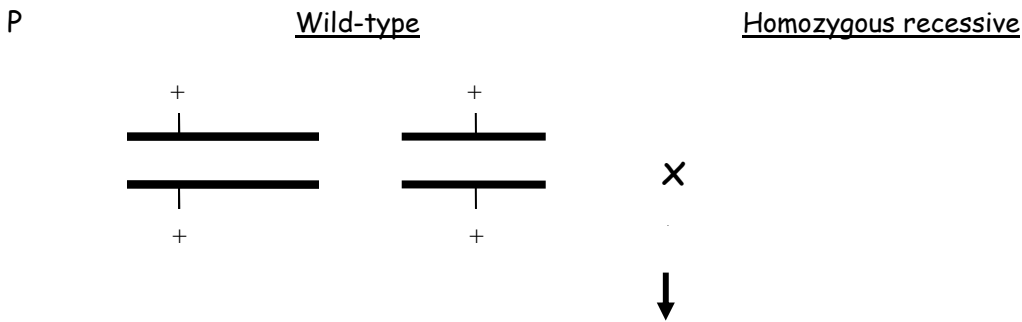
Pure-breeding or homozygous stocks are first made by inbreeding organisms with the desired form of a trait (or traits) for many generations, until only that form is observed in the offspring. The resulting stocks are then presumed to have just one form (allele) of genetic information for that trait. For convenience, we obtain such stocks from supply houses and then maintain them by inbreeding in our culture collection.

To get information about how the trait is determined, individuals homozygous for different forms of the trait must be crossed. Their offspring are known as **F1s** and should be **heterozygous** (with a different form of genetic information for the trait from each parent). Seeing which form of the trait is expressed in the F1s shows us which form of the information (or **allele**) is **dominant**. Moreover, crossing the F1 with another F1 or with a tester (homozygous recessive individual) will show us how many genes determine the trait, since we can predict what fraction of offspring should exhibit different forms of the trait if it is controlled by 1, 2 or more genes. When we are working with traits that have already been shown to be **simple traits** (controlled by one gene in a diploid organism), we can cross-multiply heterozygous F1s with other F1s or testers to see the numbers of offspring with different gene combinations passed on by the F1. This information, showing up in the combinations of traits exhibited by the offspring, tells us whether genes for the different traits are on different (**independently assorting**) chromosomes or whether they are **linked** on the same chromosome. If they are independently assorting (or **unlinked**), the genes for the different traits have an equal chance of being passed on in any possible combination.

For example, a cross between two pure-breeding parents, where one is (+/+, +/+) and the other is (ey/ey, eb/eb) would yield an F1 generation, all of whom are doubly heterozygous (+/ey, +/eb). (ey is a recessive mutation that renders the flies eyeless, and eb is a recessive mutation that causes an ebony,

or black, body color.) If these F1s are then crossed to a fly with the genotype (*ey/ey, eb/eb*), and the two genes assort **independently**, then the F2s should fall into the following four phenotypic categories *in equal numbers*: *eyeless, ebony*; *eyeless, wild*; *wild, ebony*; *wild, wild*. This is because the F1 parent will produce equal gametes of each possible type, and the doubly recessive mating partner (also called the **tester**) cannot contribute any alleles that would "cover up" the genotype contributed by the F1.

Assuming the genes are unlinked, draw the Parental (P) and F1 crosses described in the previous paragraph, showing the chromosomes, labeled with alleles, for each individual. A homozygous wild-type individual is shown below to help you start. Each line represents a relevant chromosome. Show the gametes produced by the F1 diheterozygous individual.



F1

F1 gametes

On the other hand, if the genes are linked, the same F2 phenotypes will be produced, *but they will not be produced in equal numbers*. Two of the classes will have fewer offspring than predicted and two will be enriched. The two smaller classes are the **recombinant offspring** because they represent offspring that received gametes from the F1 parent that recombined (underwent crossing over) during meiosis. For example, if the F1 parent carries *ey* linked to *eb* on one chromosome and *+* linked to *+* on the other, any gamete that has the genotype *ey,+* or *+,eb* must have arisen during a cross over event and would be deemed a **recombinant gamete**.

Assuming the genes are linked, draw the chromosomes as described in the previous paragraph (with linked genes), starting with the diheterozygous F1 individual and the tester, then show the F1 gametes that are produced by the diheterozygous F1, by showing the crossovers. Use different lines to distinguish the two chromosomes of the F1 (e.g., color, pen/pencil, texture, etc.) and retain those differences when drawing the gamete chromosomes.

DAY TWO: PROBABILITY

OBJECTIVES

Today you will be seeing the rules of probability in action. By the end of today's lab, you should be able to

- Understand the basic rules of probability.
- Understand how the rules of probability can be applied to predict the genotypes and phenotypes of offspring from a genetic cross.

INTRODUCTION:

When you made a prediction about the outcome of your F1 x F1 cross, based on certain models of how the genes for different traits were carried on the chromosomes, you might have listed all possible gene combinations, or organized them in a Punnett Square, or just used rules of probability. We can use rules of probability to predict an outcome if we have some idea of how the genes are being transmitted. What is incredible is that Mendel observed results agreeing with those predicted by probability and specific transmission patterns and that he deduced these patterns without knowing about chromosomes or DNA! In today's lab, we will be using tossed coins as examples of chance events, to see how the rules of probability used by geneticists can be observed in a simple system with which we are all familiar.

Chance events are **random** ones, occurring without any particular pattern. A **probability** is just the fraction of the time in the long run that a particular chance event will occur. One of the basic rules of probability is that the **probability of any chance event is the number of events in question divided by the total number of events possible**. For example, given a fair coin, with one side head and one side tails, the probability of a toss coming up heads is $1/2$ [the number of heads]/2 [the total number of sides possible]. This rule of probability is sometimes called the **Sampling Rule**.

Often times we are interested in the probability that two independent chance events will both occur, where independent events are those that do not influence each other's outcome. Here the rule is that the **probability of the independent events both occurring (prob of A and B) equals the product of their individual probabilities (prob of A x prob of B)**. For example, when tossing a fair coin twice in a row, the probability of getting a tail the first time *and* a tail the second time is $(1/2)(1/2) = 1/4$. This rule is called the **Product Rule**.

At other times, we may want to know the probability of one event or another **mutually exclusive** event occurring, where mutually exclusive means the two events *cannot both* occur. In this case, the **probability of one or the other occurring (prob of A or B) equals the sum of their individual probabilities (prob of A + prob of B)**. For example, when tossing a fair coin one time, the probability of getting *either* a head *or* a tail is $(1/2) + (1/2) = 1$ (or 100%). This rule is called the **Sum Rule** because the probabilities are added together rather than multiplied.

You can likely think of your own examples to illustrate these rules. We hope this lab exercise reinforces your understanding and allows you to become comfortable applying the rules of probability to genetics problems. One practice that might help is to remember your own examples and draw parallels. For example, you may know that the probability of rolling a one on one die and a one on the other (snake eyes!) is $1/36$; it might be helpful for you to think about all of the steps you automatically took in finding this:

- ◆ There are six sides on a die and only one of them has one dot, so the probability of randomly rolling a one is $1/6$.
- ◆ The way that one die comes up is independent of the way the other die comes up; so the probability of both coming up with ones is $1/6 \times 1/6$.

As another example, you know that the probability of drawing a face card from a deck of cards is $12/52$. Again, it may be helpful to remind yourself that this is because a face card is a king or a queen or a jack of each of 4 suits and drawing one of these is mutually exclusive of drawing another. Hence, there would be 12 different face cards you could draw from a total of 52 cards.

When you are confident working with two of the rules of probability, you can move on to using all three. It is always helpful to try to identify in words the independent and/or mutually exclusive events you are working with. For example, to find the probability that rolling two dice will produce a sum of 7, you first need to determine how many mutually exclusive ways this can happen:

1 on the first <u>and</u> 6 on the second = $(1/6)(1/6) = 1/36$	5 on the first <u>and</u> 2 on the second = $(1/6)(1/6) = 1/36$
3 on the first <u>and</u> 4 on the second = $(1/6)(1/6) = 1/36$	4 on the first <u>and</u> 3 on the second = $(1/6)(1/6) = 1/36$
6 on the first <u>and</u> 1 on the second = $(1/6)(1/6) = 1/36$	2 on the first <u>and</u> 5 on the second = $(1/6)(1/6) = 1/36$

You can then add these probabilities together to get the final answer of $6/36$ or $1/6$.

You can apply a similar method to finding the probabilities of getting offspring with certain genotypes and phenotypes, remembering that an offspring is a random sampling of one gene for each trait from each parent. The parents' contributions to the offspring are independent events; and a contribution of one allele for a trait is mutually exclusive of a contribution of another allele for the same trait.

THINGS TO DO:

The whole class will be participating in coin tossing trials and compiling their data to illustrate the rules of probability.

1. Each individual should start with a fair coin and toss it twice. Repeat this experiment 8 times and record the number of times the following outcomes arise:

Outcome	Your results	Pooled class results	Fraction expected	Number tosses expected
HH				
HT				
TH				
TT				
TOTALS				

We will then pool the results from the entire class. Write the pooled results in column three of the table.

- Use the rules of probability to calculate the *fraction* of pooled class experiments that should have fallen into each class. Multiply the pooled class results total by these fractions to determine the *exact number* of the experiments that should have fallen into each class. Write these numbers in the column labeled "Expected". We will look more closely at these numbers during lab next time.
- Now toss the coin 4 times in a row. Repeat this experiment 8 times, and record your outcomes. Note that when you record your data this time, you will not be keeping track of the *order* of the heads and tails - but simply the overall result - i.e. a toss of HHHT will be recorded in the same box as a toss of THHH, etc.

Outcome	Your results	Pooled class results	Expected
4 Tails and 0 Head			
3 Tails and 1 Head			
2 Tails and 2 Heads			
1 Tail and 3 Heads			
0 Tail and 4 Heads			
TOTALS			

- Use the rules of probability to calculate the *fraction* of experiments that should have fallen into each class. Multiply the pooled class results total by these fractions to determine the *exact number* of the experiments that should have fallen into each class. Write these numbers in the column labeled "Expected". We will look more closely at these numbers during lab next time.

DAY THREE CHI-SQUARE (χ^2) ANALYSIS

OBJECTIVES:

Today you will practice applying the Chi-Square test. By the end of today's lab you should

- Know how to apply the Chi-Square Test to test genetic hypotheses.
- Understand why the hypothesis that two genes are *unlinked* can easily be tested by Chi-Squared Analysis but the degree of linkage cannot.
- Distinguish between an experimental error and a sampling error.
- Know how to find the degrees of freedom for your data.
- Know how to convert a Chi-Square-value into a p-value and interpret the significance of a p-value.

INTRODUCTION:

Recall that one reason that the try-hybrid cross was made is to test hypotheses about how genes

are being transmitted. In particular, the cross will help you test hypotheses about

- Which form of information for each trait is dominant and recessive.
- Whether the 3 traits we are monitoring are simple traits.
- Whether the genes for the 3 traits are independently assorting or linked.

When we perform experiments, we generally predict the expected results based on a specific hypothesis. Then we see how closely our observed results match the expected. Of course, they are unlikely to match exactly. The 3 main reasons possible for the lack of a perfect match are:

- (1) you may have made some sort of **experimental error** (e.g. crossing the wrong flies),
- (2) your **hypothesis may be incorrect**, and/or
- (3) because you have a finite sample, by chance you got a sample that was not representative of all of the flies (This is called **sampling error** and is similar to what we observed with coin tossing in the last lab.)

Your lab notes should tell you if experimental error is a major source of difference between observed and expected results. Once you can rule out errors in procedures, you need to apply a statistical test to determine whether your hypothesis is incorrect or you just have a sampling error. In analyzing the results of crosses, where there are offspring expected in discrete categories, we use the χ^2 test for goodness of fit.

Data from crosses are always *quantitative* and the sample size is always *finite*. Therefore, experimental results will never be "perfect". As a result, geneticists must use statistical tests to interpret data in order to properly evaluate the significance of any deviation between observations and the results predicted from the hypothesis being tested.

Consider the following example:

You wish to determine if the genes for *vestigial wings* and *sepia eyes* are linked in *Drosophila*. To do this, you start with the hypothesis that the genes are *not* linked and perform a test cross with a dihybrid to produce the offspring shown in the table below.

CROSS: +/vg, +/se X vg/vg, se/se

Phenotype	Observed #s	Expected #s
wild, wild	30	25
wild, sepia	20	25
vestigial, wild	20	25
vestigial, sepia	30	25
TOTALS	100	100

If the alleles for the genes assort independently, the expected ratios of gametes produced by the dihybrid would be 1:1:1:1. The question then becomes: *Do these data support your hypothesis that the two genes are unlinked or do they reject it?*

If you were to reject your hypothesis out-of-hand and find a map distance, you would analyze the data set as follows:

- Linkage phase in dihybrid is +,+ and vg,se since these are the two biggest classes in the offspring. Thus, the recombinant classes are +,se and vg/+.
- The map distance is calculated as:

$$\frac{(20 + 20)}{100} \times 100 = 40 \text{ cM}$$

However, can you really reject your hypothesis? Could the deviation you observed be simply caused by a sampling error? By eyeballing the data it is hard to tell for sure. The χ^2 test will allow you to make that determination in a statistically supportable manner.

A χ^2 test always starts with a **null hypothesis** that states that *there is no statistically significant difference between the observed data and the predicted data*. The experimenter then proceeds to test the null hypothesis by applying the following formula to the data set:

$$\chi^2 = \sum \left[\frac{(\text{observed} - \text{expected})^2}{\text{expected}} \right]$$

In the case of our example,

$$\chi^2 = \frac{(30-25)^2}{25} + \frac{(20-25)^2}{25} + \frac{(20-25)^2}{25} + \frac{(30-25)^2}{25} = 4.0$$

Note that the more the observed values differ from the expected values, the *higher* the value of χ^2 . Thus, high values of χ^2 indicate that the null hypothesis should be *rejected* (i.e. there is a statistically significant difference between the observed data and the predicted data) while low values indicate that the null hypothesis should *not* be rejected.

How high a χ^2 value is too high? To determine this, we use a standardized χ^2 table, as shown below.

	p-value (The probability that the observed results, or results more extreme, could have occurred by chance)									
df	0.95	0.90	0.70	0.50	0.30	0.20	0.10	0.05	0.01	0.001
1	0.004	0.016	0.15	0.46	1.07	1.64	2.71	3.84	6.64	10.83
2	0.10	0.21	0.71	1.39	2.41	3.22	4.61	5.99	9.21	13.82
3	0.35	0.58	1.42	2.37	3.67	4.64	6.25	7.82	11.35	16.27
4	0.71	1.06	2.20	3.36	4.88	5.99	7.78	9.49	13.28	18.47
5	1.15	1.61	3.00	4.35	6.06	7.29	9.24	11.07	15.09	20.52
6	1.64	2.20	3.83	5.35	7.23	8.56	10.65	12.59	16.81	22.46
7	2.17	2.83	4.67	6.35	8.38	9.80	12.02	14.07	18.48	24.32

To use the table, however, we need to know which "df" value to use and which "p" value to use.

Understanding "Degrees of Freedom"

The word, "freedom" in degrees of freedom refers to the freedom of a number to have possible values. Degrees of freedom are equal to the number of samples minus 1. For example, if you have four possible phenotypic classes, and you know the frequency of three of them, the fourth is not

independent (it must be equal to 1 minus the sum of the first three frequencies) In testing for linkage we generally calculate one test statistic and therefore, the degrees of freedom (df) are equal $n - 1$, where $n =$ the number of classes into which the data set falls. In our example, there are 4 phenotypic classes and therefore we use a df value of $4 - 1 = 3$.

Determining your number of observations

Determining the number of observations in a statistical test is critical to getting the most meaningful evaluation of your hypothesis. Consider the following experiment. Suppose that you wanted to determine if the chances of having a boy or a girl were 1:1 in your extended family. (Some families carry mutant alleles that skew this ratio.) You obtain the data below:

Family	Boys	Girls
Grandma Gertrude and Grandpa Bob	1	7
Aunt Emily and Uncle Ted	3	2
Aunt Kate and Uncle Eddy	2	1
My parents, Edith and Bill	4	3

There are several ways you could consider calculating the χ^2 test statistic:

1) The number of boys and girls could each be considered one observation, yielding:

$$\frac{(1-4)^2}{4} + \frac{(7-4)^2}{4} + \frac{(3-2.5)^2}{2.5} + \frac{(2-2.5)^2}{2.5} + \frac{(2-1.5)^2}{1.5} + \frac{(1-1.5)^2}{1.5} + \frac{(4-3.5)^2}{3.5} + \frac{(3-3.5)^2}{3.5}$$

$$= 2.25 + 2.25 + 0.1 + 0.1 + 0.17 + 0.17 + 0.071 + 0.071 = \mathbf{5.18}$$

2) Or we simply could pool all the data to place it in two classes: boy or girl (ignoring which family was involved). The totals are now 10 boys and 13 girls and the χ^2 value is:

$$\frac{(10-11.5)^2}{11.5} + \frac{(13-11.5)^2}{11.5} = 0.20 + 0.20 = \mathbf{0.40}$$

Is either of these correct? The answer is NO! Think about the hypothesis: "the chances of having a boy or a girl are 1:1 in your extended family". Given that hypothesis, each family unit (e.g., Aunt Emily and Uncle Ted) represents one observation. Therefore, we really have four observations with which to test our hypothesis:

$$\frac{(1-4)^2}{4} + \frac{(3-2.5)^2}{2.5} + \frac{(2-1.5)^2}{1.5} + \frac{(4-3.5)^2}{3.5}$$

$$= 2.25 + 0.10 + 0.17 + 0.071 = \mathbf{2.59}$$

In the first example, where both sexes were counted, we created *pseudo-replicates*. That is we counted boys *and* girls within each family when the hypothesis is about the *ratio* of boys to girls. This artificially gave us a greater sample size and inflates our chances of rejecting the null hypothesis falsely. In the second example, we under-represented our sample size by combining all children together from four families, artificially deflating our chances of rejecting the null hypothesis. If our

hypothesis was instead: "sex-ratio varies in humans due to genetic influences", then we might want to treat each extended family as one observation. We would then need to collect data on at least one more extended family before we could attempt a statistical test of this hypothesis.

Let us now discuss what the "p" value measures.

Understanding "p"-Values

The "p" value associated with your χ^2 value gives you the probability that the deviations you observed in your experiment are due to chance alone.

Another way to think about this is to consider the following:

Suppose that you performed a $+/ap \times ap/ap$ test cross in *Drosophila* and counted 100 offspring produced by the cross. You then repeated the cross many times, each time counting and scoring exactly 100 offspring. Mendel's first law predicts that equal numbers of wingless and winged offspring should be produced - in other words, the expected result would be 50 wingless and 50 winged. However, you would find, of course, that you got exactly 50 of each only some of the time. More often, due to sampling error, you saw something else: 47 wingless and 53 winged or 56 wingless and 44 winged, etc.

Further, suppose that as you accumulated more and more data sets, you graphed the data, with **the number of data sets falling into a particular distribution on the y axis and the p value associated with each data set on the x axis.**

As you added more and more data sets to your graph, you would notice that the data naturally fell into a bell curve, with the center and highest point on the curve being the expected data set, 50 wild, 50 apterous. However, notice that lots of data sets would fall on either side of the center. This is the natural result of working with populations of limited size. Due to sampling error, most of the data sets will not conform perfectly to the expected 50/50 values.

Since this is to be expected, how do we set a cut off point for when a data set is likely to have arisen by chance alone and when a data set truly displays a fundamentally different kind of behavior than is predicted?

There is no correct answer to the above question. All scientists can do is choose a value arbitrarily and be aware of what it means. Biologists have settled on a p-value of 0.05, which means, if the results of a statistical test would occur by chance more than 5% of the time, then the null hypothesis is not rejected. Conversely, if the results of statistical test would occur less than, or equal to, 5% of the time, by chance alone, then the null hypothesis is rejected and we accept the alternative hypothesis.

Realize, that 5% of the time we are **falsely** rejecting the null hypothesis when it is correct because of chance deviations from the expected. Likewise, we will sometimes fail to reject the null hypothesis when in fact it is incorrect, due to chance deviations. That is the price we pay for statistical inference - two types of error: falsely rejecting the null and falsely accepting the null. These two types of error are also known as type 1 and type 2, respectively.

In order to interpret your data set, you should find the χ^2 value that corresponds to a "p" value of 0.05 at the df value for the experiment. If that number is *greater than* the χ^2 value you calculated for the data set, then you fail to reject the null hypothesis (the deviation between the distribution in the

data set and an ideal distribution can be attributed simply to sampling error). However, if that number is *less than* the χ^2 value you calculated for the data set, then you reject the null hypothesis (the deviation you see cannot be attributed simply to sampling error; some other explanation is required).

Many scientists choose to specify the p-value they obtain in order to allow others to see for themselves how significant the data are (e.g., a p-value of 0.0001 is far more statistically persuasive than 0.04, yet both are called "significant").

A summary of statistical hypothesis testing: The Chi-square test is an example of a statistical test used in **significance testing**. It is used to determine the probability that a given result is due to chance. The value the statistical test must exceed to be **significant** is called the **critical value**. In other words, it is the value that a test statistic must exceed in order for the null hypothesis to be rejected. The critical value for a Chi-square test with 3 degrees of freedom is 7.82, because that is the Chi-square value that corresponds to a 0.05 probability of obtaining the result due to chance. When a p-value less than, or equal to, 0.05 is obtained, the results are said to be **statistically significant**. **Statistical significance** does not imply biologically interesting or important findings. The latter is an entirely different kind of judgment.

To determine if a data set passes the χ^2 test, follow the steps outlined below.

Steps to Perform a Chi Square Test:

1. Calculate the χ^2 value for your data set, using the standard χ^2 formula.
2. Locate the appropriate degrees of freedom (df) on the vertical axis of the χ^2 table. (Remember that $df = n - 1$, where $n = \#$ classes in your data set.)
3. Determine the χ^2 table value corresponding to a "p" value of 0.05.
4. Compare the χ^2 value you calculated for your data set with the χ^2 table value. If the calculated value is *less* than the table value, you *fail to reject your null hypothesis*. If the calculated value is *greater* than the table value, you *reject your null hypothesis*.

We are now in a position to complete the analysis of our original data set, for which we calculated a χ^2 value of 4.0. The df will be 3, since there are 4 phenotypic classes in the data set. The χ^2 table value at $p = 0.05$ and $df = 3$ is **7.82**. Since $4.0 < 7.82$, **we fail to reject our null hypothesis**. Therefore, we determine that the data set does not deviate significantly from the 1:1:1:1 ratios predicted for independently assorting genes - i.e. there is no evidence for linkage.

THINGS TO DO:

χ^2 and Sample Size

It is important to realize that **sample size** has a profound effect on the interpretation of data sets by χ^2 analysis.

Suppose we repeat our original experiment but increase sample size to 1,000 progeny and happen to observe the same ratios. Now there are 300 wild, wild; 200 wild, sepia; 200 vestigial, wild; and 300 vestigial, sepia.

1. Perform a χ^2 test on this new data set.
2. What is the critical value? What is the p-value? Which of the two has changed and why?
3. Retrieve the pooled, coin toss data from last time. Work with your lab partner to perform χ^2 test on both data sets. Is the deviation we observed in each case likely to have resulted simply from sampling error?

DAY FOUR: HANDLING DROSOPHILA, IDENTIFYING MUTANTS, SCORING F2 FLIES

Today's laboratory will introduce you the common fruit fly, *Drosophila melanogaster*, as an experimental organism. You will learn to identify mutations and begin scoring the results of your linkage experiment. After today's experiment, you should be able to:

OBJECTIVES:

- Properly anesthetize and handle *Drosophila*.
- Properly adjust a stereo dissecting scope and light source for optimal viewing of *Drosophila*.
- Know how to accurately identify the three mutant characteristics being followed in the cross.
- Be familiar with the stages of the *Drosophila* life cycle.
- Be able to identify the stages in a culture.
- Understand why it is important to know the life cycles of organisms used in genetic studies.
- Begin counting your F2 offspring and recording how many flies of each phenotype, you obtained. Use the table at the end of this lab exercise to gather your data throughout the remainder of the experiment. Simply add to this data sheet each day that you count and score the phenotypes of your F2s.

INTRODUCTION:

Drosophila melanogaster is used extensively in genetic breeding experiments because it has a short life cycle, exhibits great variability in inherited characteristics, and may be conveniently raised in the laboratory to produce large numbers of offspring. The organism is useful for demonstrating the principles of genetic mapping, which you will be exploring in this first experiment. To do this, you will be analyzing a cross with real flies to demonstrate the principles behind genetic mapping. Your success in these experiments will depend greatly on your ability to properly handle the flies and to accurately observe their characteristics.

Techniques for Handling *Drosophila*

Many characteristics of *Drosophila* must be examined under a stereo dissecting microscope. Flies are immobilized by anesthesia when they are examined or being transferred for mating. You must master the techniques of anesthetizing, handling, and examining the flies before attempting to make a cross.

Traditionally, flies were anesthetized with **ether**. Now, because of potential health hazards, student laboratories use carbon dioxide or **FlyNap** to render flies unconscious. Both of these have the advantage over ether of being relatively harmless to humans and free from overdosing dangers to the flies. Carbon dioxide gas must be continually applied to flies (through a diffusion plate that sits beneath the microscope). While this is the most common anesthesia in fly laboratories it requires a fair amount of hardware and plumbing that is not very portable. In this lab we will be using FlyNap. While the package directions tell you how quickly the FlyNap will work, you must watch the bottle to see when all of the flies become still. You will also notice a distinctive odor of the FlyNap; this causes adverse physical symptoms in some people. Hence, used wands should be placed in a capped vial and the use of FlyNap should be minimized. If you find yourself reacting unfavorably to the odor, just step outside for some fresh air.

Unconscious flies should be placed on a **counting plate** or **index card** for sorting and examination. When a **dissecting scope** is used, the light source should be adjusted so that the rays of light fall directly on the fly. Adjust the eyepieces of the microscope to the correct interpupillary distance of your eyes; this is important to obtain stereo vision. Use **low magnification for scanning and sorting**, and high magnification for checking the detailed structural features. To manipulate the flies while viewing under the microscope, a **camel hair brush** should be used. Through practice you will be able to turn the flies over and move them about freely. Learn to manipulate the fly gently so that it will not be injured or killed. You can push the fly with the brush or lift it with a slightly moistened brush.

When unconscious flies are introduced into a container, do not drop them directly on the food or any moist surface. The flies will stick to the moist surface and usually cannot escape. Place the flies on the **dry** inside side of the bottle and keep the bottle lying on its side until the flies awaken. If flies get stuck on the food, tap the **side** of the bottle against the bench to dislodge them. In general, this is a useful practice just prior to returning flies to the incubator.

Identification of Sex (optional)

Male and female adult flies (Fig. 1-1) can be distinguished from each other by the following criteria:

- **Sex comb:** Male flies have sex combs; female flies do not have them. The sex comb (Fig. 1-2) is located on the proximal tarsal joint of each of the forelegs of the male. It consists of a row of about ten stout bristles. The sex combs are used by a male to separate a female's wings and facilitate genital contact. Under the dissecting scope, however, it appears as a tiny black spot.
- **Abdominal structure:** From a dorsal viewpoint, the male has heavy pigmentation at the posterior part of the abdomen with two pigmented bands anterior, while the female has five pigmented bands along the entire abdomen. The posterior end of the abdomen is rounded in the male, but somewhat pointed in the female, for ovipositing. For those adults that have emerged from their pupal case within the last hour, pigmentation is very light and more difficult to use in distinguishing sexes (flies will look very pale and their wings may be wrinkled).

- **Genital region:** The genital organs are visible from the ventral side. The penis of the male is surrounded by dark and heavily bristled genital arches, while the vaginal opening of the female is covered by a light colored ovipositor plate.

The fastest, most commonly used method of identifying flies by sex is a combination of abdominal structure and genital region. Both are easily visible with a scope, and with a little practice can allow identification with minimal manipulation. The sex-comb criterion is also frequently used for sex identification because of its reliability and the ease of observing the sex combs. Since the sex combs are visible through the pupal case in the mature pupa, it is possible to identify the sex of a fly before its emergence from the pupal case. As you become more experienced you may be able to distinguish the sex of the flies with your naked eyes by viewing the abdominal bands.

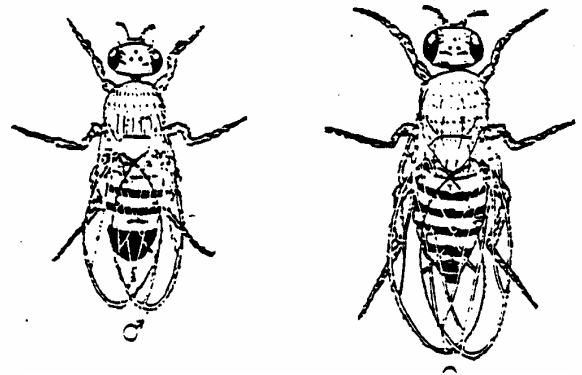


Figure 5-1. Male (left) and female (right) adults of *Drosophila melanogaster*.

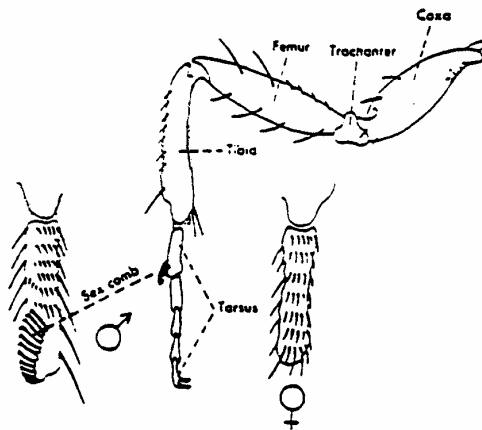


Figure 5-2. Foreleg of male *Drosophila*, showing sex comb. The proximal tarsal segment is enlarged to show the presence of the sex comb in the male and its absence in the female. Sex combs are used to displace the females wings during copulation (they do it doggie style).

The Life Cycle of *Drosophila*

When organisms are used in genetic studies, it is important to know their life cycles. One reason is that you want to know how long you must wait for offspring in order to plan wise use of your time. An elephant cross, for example, would require that you fill your time for 2 years before having offspring to analyze! A second reason for being familiar with the life cycle is so that you can monitor the cross and know that it is progressing normally. If you know the timing of the developmental stages before the offspring emerge, you can look for the expected stages and note any irregularities. Still a third reason to know about the life cycle is to facilitate the handling of materials. For instance, knowing that recently emerged adults show sexual characteristics, but are unable to mate, allows the early isolation of virgin females for controlled crosses.

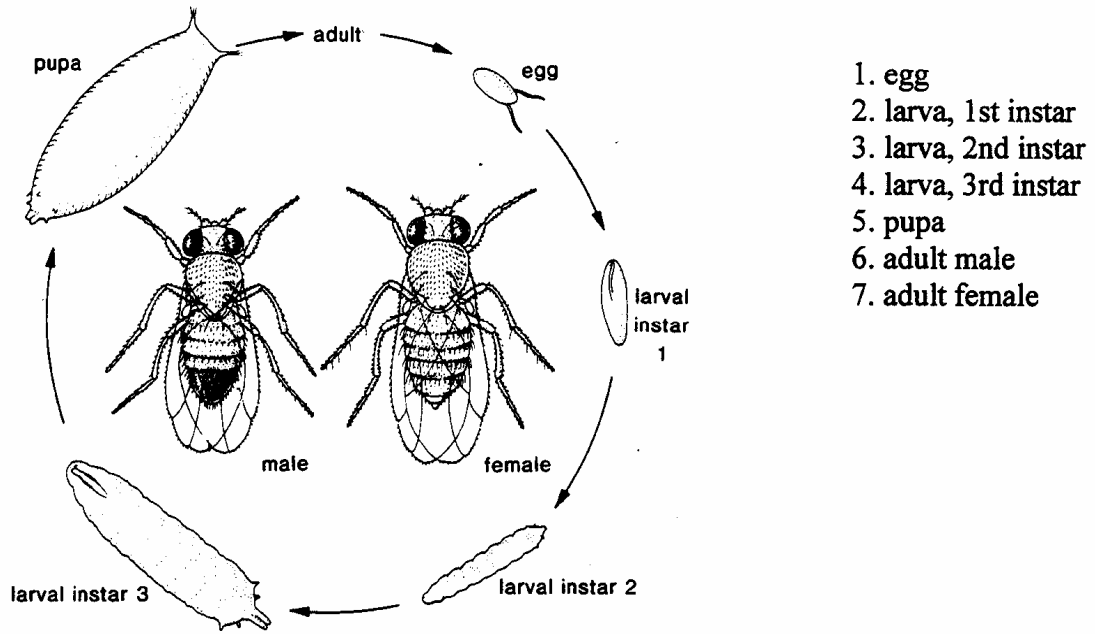


Figure 5-3. The Life Cycle of *Drosophila*

The life cycle of *Drosophila* is shown in Figure 1-3. The fertilized **egg** is laid by the adult female shortly following fertilization. The eggs hatch about one day after they are laid and produce small white **larvae**. The larvae feed upon food and grow rapidly. During its development, the larva undergoes two molts and three instars. At 25°C, a **first instar** will become a mature third instar in about four days. The **third instar** larvae crawl up out of the medium, adhere to a dry surface, and develop into the **pupae**. Pupation takes about three days, during which the larva within the pupal case undergoes metamorphosis and transforms into the **imago or adult** form. The adult emerges by forcing its way through the anterior end of the pupal case. The newly emerged fly is slender, light-colored, and has folded wings. After about two hours, the wings will expand and the body will become darker and more compact. The male adults are capable of fertilizing the females ten hours after their emergence. When the flies are cultured at 25°C under optimal conditions, the entire life cycle from egg to adult is completed in about ten days. At room temperature, the life cycle may be completed in two weeks.

Culturing and Crossing *Drosophila*

In the laboratory, fruit flies are raised in culture bottles containing cooked food. A variety of recipes are available for preparing culture media. The popular molasses-cornmeal medium is used in our laboratory for most experiments. The medium provides a balanced diet for the flies and contains mold inhibitors to maintain its freshness. A few granules of yeast have been added to the medium in each bottle to promote the active growth of the flies. Sometimes contamination of the medium by mold and/or bacteria occurs. Contamination should be noted in your laboratory records, in case it affects the outcome of your cross. Additionally, some bacterial contamination leads to softening of the medium and the entrapment of flies when you invert the bottle. Any time you see soft medium, report it to your lab instructor and harden the medium by the addition of instant medium.

Drosophila cultures should be incubated at 25° C. If an incubator at that temperature is not available, the culture will do well at room temperature (20-24° C). Extremes of temperature should be avoided. Male flies exposed to 31° C or higher temperatures for several hours may become sterile; thus, *Drosophila* cultures should be kept away from direct sunlight to avoid a rise of temperature inside the bottle.

In making a cross, 3 to 5 pairs of selected male and female flies are placed in a fresh vial (about 1/5 the area of the bottles you are using). Unconscious flies must be placed on a dry surface; not on the medium. In a pinch, a single male can be used to inseminate several females. The sex and phenotype of each fly used in the cross should be carefully checked and recorded, and the bottle should be properly labeled. Virgin females are required in many cases to make a cross. After emerging from their pupal case (eclosion) adults remain virgin for at least 10 hours, during which time the sexes can be separated and held until crosses are made

THINGS TO DO:

1. Find your equipment for working with the flies: **Fly manual, index cards, sorting tray, camel hair brush, FlyNap, dissecting microscope and light (in back cabinet), fly morgue.**
2. Practice the techniques of anesthetizing and handling flies as demonstrated by your instructor.
3. Isolate several flies from a wild-type stock bottle and identify the sex of each. Also, make note of the wild-type color, size, and shape of the eyes, bristles, wings, and body.
4. Lab pairs will be assigned specific mutants from demonstration stock bottles for the identification of various mutations exhibited by single, double, or multiple mutants. Once you have set up your observation scope and can identify your mutant, move about the lab and examine other mutants. Focus particularly on colors, shapes, and sizes of eyes, wings, bristles, and body. From your notes, you should be able to identify these mutants when you see them again. You will have about 20 minutes to make your observations.
5. To avoid contamination of the stock, do not return the unwanted flies to the stock bottle; reservoir bottles will be available. To discard adults (read before doing): 1) Place your funnel over the fly morgue, (2) while tapping the bottom of your bottle against your bench (to keep flies away from the opening) remove the plug and immediately turn the bottle upside down and against the funnel. Holding the funnel against your bottle, raise both and tap them against the morgue briskly until the adults are all in the morgue (where they die immediately).
6. Begin counting your F2 offspring and recording how many flies of each phenotype, you obtained. Use the table at the end of this lab exercise to gather your data throughout the remainder of Experiment 3. Simply add to this data sheet each day that you count and score the phenotypes of your F2s.

DAY FIVE: FINISH SCORING F2 FLIES; CREATE A LINKAGE MAP

OBJECTIVES:

Today you will finish counting and scoring your F2 flies. During the last part of the lab period, we

