



AP Biology, Aquatic Science, Environmental Systems



**Morphometric Analysis of Eurycea in Austin, TX
Teacher Page**

Enduring Understandings
Nature of Science and Data

Vocabulary
Morphometric Analysis - refers to the quantitative analysis of *form*, a concept that encompasses size and shape. Traditional morphometric data are measurements of size. Morphometric analyses are commonly performed on organisms for a number of reasons, one of which is estimating quantitative-genetic parameters of shape.

Essential Questions:

- Science uses models, specific processes/methodologies, and organization to help us make sense of our surroundings and new discoveries.
- How do scientists use data to determine the interdependence of variables?
- Why is it useful to use appropriate graphical analysis in the display and analysis of data?

Topical Questions:

Objectives

Students will complete a morphometric analysis of 3 salamander species unique to Austin:
1) Measure salamander variables using ImageJ (length, body width, eye width and head width)
2) Analyze data using univariate and bivariate analysis.

Teacher Management

Estimated Time for Completion

2 class periods

Materials

Groundwater video "Austin Underground"
Copies of "ImageJ Introduction to Basic Functions"
Computer with a mouse (much more difficult to use Image J with a touchpad)
Salamander images (Population A, B, C) file loaded on each computer
Excel Student Data Sheet, "Morphometric Analysis of Eurycea in Austin, TX" loaded on each computer (If you want students to create their own graphs, then use the data sheet "NoGraphs")

Teacher Prep

- 1) This activity is designed to follow part 3 of the video “Austin Underground” and instruction on statistics.
- 2) Reserve computer lab or provide computers for students (or groups)
- 3) Follow instructions for ImageJ software download
- 4) Save salamander image files to computers
- 5) Save Student Data Sheet (excel worksheet) to computer

Safety Considerations N/A

Vocabulary Building Strategies

Discuss terms from video

Define morphometrics

Background Information for Teacher

MORPHOMETRICS BACKGROUND

Usually, the first information that is used to differentiate between two species or populations is information on what the organisms look like. This includes qualitative differences (such as color patterns), as well as quantitative differences (often some aspect of shape). The methods that are used to describe and analyze shape are collectively known as morphometric analyses. Here our attention will be on quantitative differences.

ImageJ is a free public source image processing and analysis program provided by the National Institute of Health. While the program has extensive application in the sciences, this introduction will cover only a few tools relevant to the lesson “Morphometrics of *Eurycea* in Austin, TX”.

STATISTICS BACKGROUND

I. Univariate Analysis - One variable is analyzed, usually for descriptive purposes. Univariate analyses are very common in biology.

Descriptive statistics - aim to summarize a data set and usually includes at least the following:

- 1) Observed limits (range of your measurements)
- 2) Sample size (n), or how many things you measured
- 3) Mean (average-sum of all measurements, divided by the number of measurements)
- 4) Measures of variation around the mean, such as:

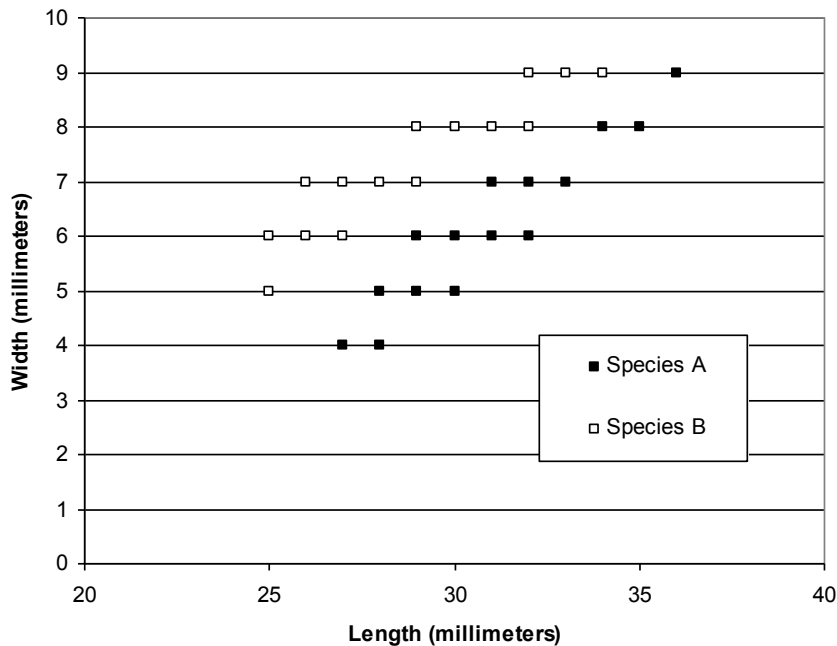
Standard deviation (also means dispersal of the individual observations around the mean, but in the same units as the original observations; standard deviation is the square root of the sample variance):

Inferential statistics - Confidence limits: The range within which a given parameter (such as the true mean) is predicted to fall at a given confidence level. The conventional confidence levels are typically 95% or 99%, meaning that the Type I error rate is 5% or 1% respectively. The Type 1 error rate is the probability of falsely rejecting a true hypothesis. Therefore, if we specify the 95% confidence limits for the mean of a sample, it means that we estimate there is only a 5% chance that the true mean of population lies outside of the specified range.

Univariate analysis - The t -test is a common method used in evaluating differences in means between two groups. The p -level reported with a t -test represents the probability of error involved in accepting the hypothesis that there is a significant difference between two sets of data. If the p -level is ≥ 0.05 , there is no significant difference between each group. If the p -level is < 0.05 , there is a significant difference between each group.

II. Bivariate analysis - Often, two species are not differentiated on the basis of a single measurement (which is often a measure of size), but rather on the basis of the relationship between two or more measurements (which together constitute a measure of shape). For instance, individuals of most species tend to grow considerably throughout their lives, so any single measurement is more likely to reflect overall size (and hence age) than anything else.

For example, suppose we say that fish species A is laterally compressed (less wide, thinner) relative to fish species B (a statement about shape). If we measure the width of a large number of specimens of various ages of both species, we are likely to find that the ranges for width of the two species broadly overlap. Even though width is less for species A than for species B at a given size, small specimens of species B are probably not as wide as large specimens of species A. Thus, we need a way to standardize for size. This is often accomplished simply by a bivariate plot of two variables, such as width and length.



Notice that although the two species overlap broadly in both length and width, they are nonetheless easily distinguished by the relationship between these two variables. This difference may be expressed as a ratio of the two variables, such as width divided by length. Ex. While both species have individuals measuring 7 mm wide, those of species B are shorter in length than Species A. Similarly, both species have individuals that are 30 mm long, but species B of that category are much more narrow (5 and 6 mm wide) than species A (8 mm wide).

Bivariate plots are often the most effective way to describe a difference in shape. They are also useful to describe the relationship between a discontinuous variable (especially counts that vary with size) and a continuous variable, such as length or weight.

Misconceptions

Students might wonder why there is so much attention placed on the Barton Spring salamander since they occupy such a small niche and have a small population.

BS salamanders are indicator species or “the canary in the coal mine,” their relative health a sign of water quality. By protecting the salamanders, we, in turn, protect our aquifer and our city’s favorite swimming hole, Barton Springs Pool.

Probing Questions

TBD

Focus Activity/Warm up

Show "Austin Underground" video segment about salamanders.

Engage

You and your fellow classmates have visited two spring sites in Austin, Texas: Parthenia Spring (within Barton Springs Pool in Zilker Park) and Wheless Spring (within Wheless preserve in NW Austin near Lake Travis). Both of these sites are home to aquatic salamanders, but you would like to determine if they are the same species. What methods could you use to determine species?

At Parthenia Spring, you observe that some salamanders are distinctly different based on their eyes (both size and appearance). Could this be a natural variation with a single species?

Could this be two distinct species? You will classify these two eye variations into two groups for your analyses.

The two most common ways of differentiating between species are using morphometrics and genetics. Without having a genetics lab, you will employ morphometrics to determine if the spring sites are host to the same species or different species.

Explore

The salamander image file contains 3 populations of salamanders (A, B, & C). Students will use the ImageJ program to measure salamanders and record total length, body width, head width and eye width. Students will enter and analyze data using the Excel Student Data Sheet, "*Morphometric Analysis of Eurycea in Austin, TX.*" If you have experience making graphs in Excel, then have the students create scatterplots on #7. If not, give the students the Excel worksheet with the graphs provided.

Explain

I. Morphometric Analysis - refers to the quantitative analysis of form.

Usually, the first information that is used to differentiate between two species or populations is information on what the organisms look like. This includes qualitative differences (such as color patterns), as well as quantitative differences (often some aspect of shape). In the activity, you analyzed quantitative differences. The methods that you used to describe and analyze shape are collectively known as morphometric analyses.

IA. Univariate Analysis - one variable is analyzed.

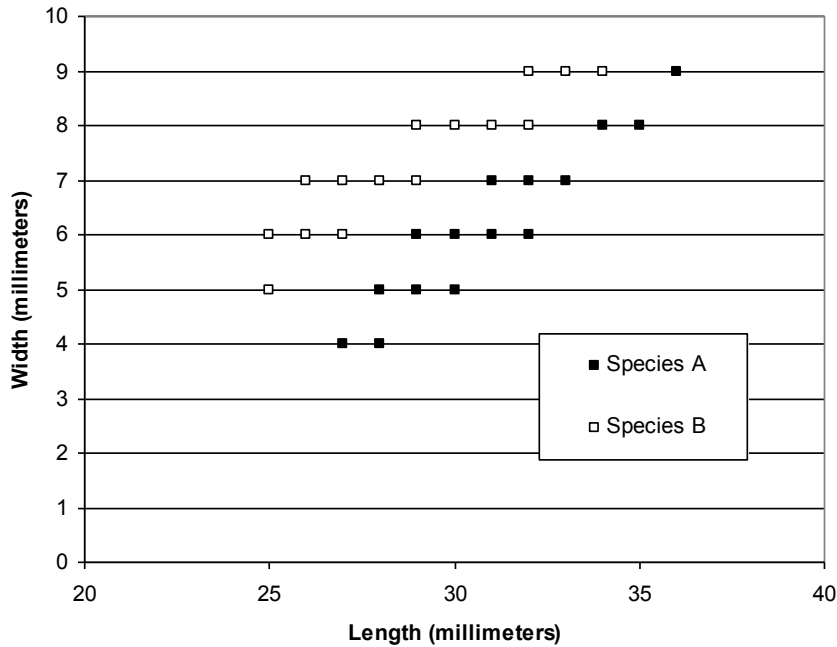
- The *t*-test is a common method used in evaluating differences in means between two groups.
- The *p*-level reported with a *t*-test represents the probability of error involved in accepting the hypothesis that there is a significant difference between two sets of data.
- If the *p*-level is ≥ 0.05 , there is no significant difference between each group.
- If the *p*-level is < 0.05 , there is a significant difference between each group.

Did the univariate analysis of the salamanders show significant differences (p -level < 0.05) among populations A, B and C?

IB. Bivariate analysis - Often, two species are not differentiated on the basis of a single measurement (which is often a measure of size), but rather on the basis of the relationship between two or more measurements (which together constitute a measure of shape). For

instance, individuals of most species tend to grow considerably throughout their lives, so any single measurement is more likely to reflect overall size (and hence age) than anything else.

Directions: Display graph below.



For example, let's look at two fish populations that have already been identified as two separate species. Suppose we say that fish species A is laterally compressed (less wide, thinner) relative to fish species B (a statement about shape). If we measure the width of a large number of specimens of various ages of both species, we are likely to find that the ranges for width of the two species broadly overlap. Even though width is less for species A than for species B at a given size, small specimens of species B are probably not as wide as large specimens of species A. To standardize for size, a bivariate plot of two variables, such as width and length, is used. Notice that although the two species overlap broadly in both length and width, they are nonetheless easily distinguished by the relationship between these two variables. This difference may be expressed as a ratio of the two variables, such as width divided by length. Ex. While both species have individuals measuring 7 mm wide, those of species B are shorter in length than Species A. Similarly, both species have individuals that are 30 mm long, but species B of that category are much more narrow (5 and 6 mm wide) than species A (8 mm wide).

Did your bivariate plots show differences between the salamander populations? Which populations showed the greatest differences? Which populations overlapped, but still showed distinguishable groups?

After calculating the ratios, what did you conclude about the salamander populations A, B and C? Are any of the Populations unique species?

CONCLUSION: The populations are in fact unique species. Population A is the Barton Springs Salamander, population B is the Austin Blind Salamander, and population C is the Jollyville Salamander .

Extend/Elaborate

To learn more about each of the three species identified in this lesson, visit <http://www.austintexas.gov/department/salamanders>

Evaluate

Student worksheet, "*Morphometric Analysis of Eurycea in Austin, TX*"

Closure/Daily Assessment

You discovered there are more than one species of salamander living in Austin's springs. Morphometrics can provide an inexpensive method for determining species.

Differentiation

ELL

Special Education

ImageJ Software Download

ImageJ is a free public source image processing and analysis program provided by the National Institute of Health. While the program has extensive application in the sciences, this introduction will cover only a few tools relevant to the lesson “Morphometrics of *Eurycea* in Austin, TX”. Download from <http://rsbweb.nih.gov/ij/download.html>

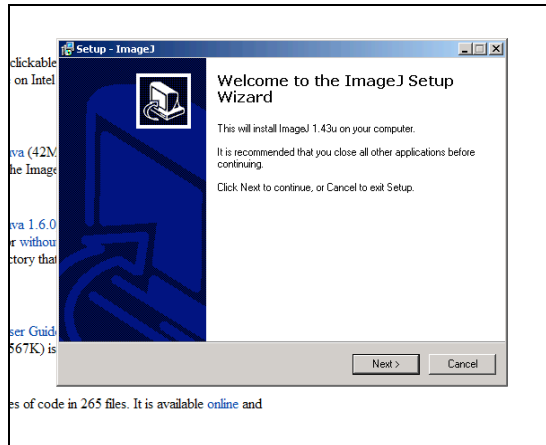
Installation for Windows

Under **Windows** select **bundled with 32-bit Java**. (For Mac users, select **ImageJ 1.43**)



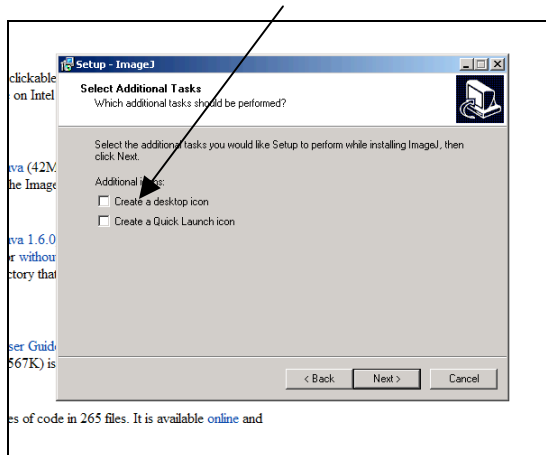
When prompted, click “Run”.

The ImageJ Setup Wizard should appear shortly.
When prompted, click “Next”.



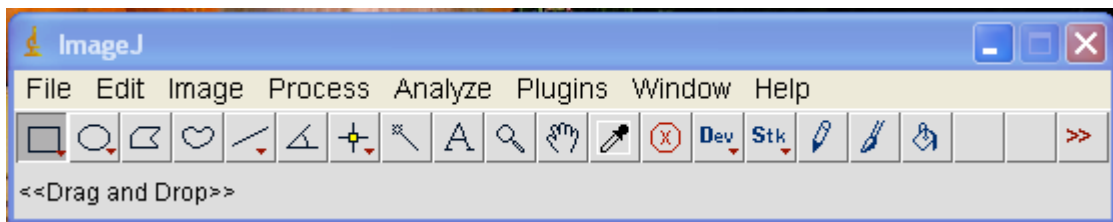
The next couple screens will have you select a location to install ImageJ and naming of the installed location. Click “Next” for the next 3 screens.

The next screen will ask about additional tasks. Ask your instructor if you should select the box for “create a desktop icon”. (The icon will appear as a microscope on the desktop.) When ready, click “Next”.



The following screen will prompt you to install the program. Click “Install”. You should now see the Completing ImageJ Setup Wizard screen. Click “Finish” which should automatically launch ImageJ.

The pop-up screen should say Welcome to ImageJ. Click “OK”. An ImageJ configuration pop-up should appear. Click “OK” and ImageJ should now appear as a toolbar at the top of your screen (similar to the one shown below). To start working with an image in ImageJ, drag and drop the image file (ie. jpeg) into the gray bar beneath the tool buttons. It will open in a separate window.



ImageJ Introduction to Basic Functions

Line Selection Tools

Use these tools to create line selections. The three line selection tools share the same toolbar slot. To switch to a different tool, right click on the current line tool and select the desired tool from the drop-down menu.



Straight Line Selection Tool

Select the straight line tool. Create a straight line by clicking on the starting point and holding down the left mouse button, dragging the mouse until you reach the end point. Release the mouse button and a straight line will remain.

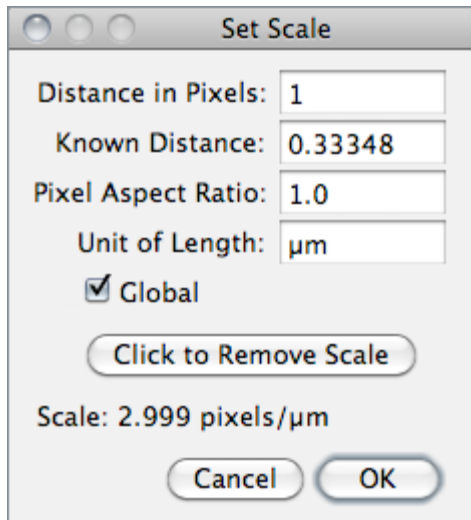


Segmented Line Selection Tool

Select the segmented line tool. Create a segmented line selection by repeatedly clicking with the mouse. Each click will define a new line segment. Double-click when finished. The points that define a segmented line selection can be moved or deleted, and new points can be added: Clicking on an existing point with the Shift key down adds a point. Clicking on an existing point with the Alt key down deletes it.

Set Scale

Use this dialog to define the spatial scale of the active image so measurement results can be presented in calibrated units, such as millimeters (mm). Before using this command, use the straight line selection tool to make a line selection that corresponds to a known distance. Then, bring up the dialog, enter the *Known Distance* and unit of measurement, then click 'OK'. ImageJ will have automatically filled in the *Distance in Pixels* field based on the length of the line selection. Leave the *Pixel Aspect Ratio* setting to a value of 1.0 for this lesson. When *Global* is checked, the scale defined in this dialog is used for all opened images during the current session instead of just the active image. Since the scale is different in each image, *Global* should not be checked.



Measure

Based on the selection type, calculates and displays either area statistics, line lengths and angles, or point coordinates. Calculates line length and angle if a line selection has been created using one of the three line selection tools. Records coordinates if one or more points have been defined using the point selection tool. Use the *Analyze>Set Measurements* command to specify what area statistics are recorded. For this lesson, be sure all boxes are unchecked. Once a line is created, use the *Analyze>Measure* command to measure the line length. Results table (similar to below) will pop up once a measurement is taken, but only line length should be reported.

	Area	Mean	StdDev	Min	Max	Circ.
1	48.588	97.454	67.848	8	248	0.588
2	34.304	107.140	69.713	16	248	0.674
3	131.194	103.529	70.423	8	248	0.938



Morphometric Analysis of *Eurycea* in Austin, TX Student Sheet

You and your fellow classmates have visited two spring sites in Austin, Texas: Parthenia Spring (within Barton Springs Pool in Zilker Park) and Wheless Spring (within Wheless preserve in NW Austin near Lake Travis). Both of these sites are home to aquatic salamanders, but you would like to determine if they are the same species. At Parthenia Spring, you observe that some salamanders are distinctly different based on their eyes (both size and appearance). This leads you to wonder whether this is natural variation of a single species or if there are two distinct species at this site; therefore, you have classified these two eye variations into two groups for your analyses.

The two most common ways of differentiating between species are using morphometrics and genetics. Without having a genetics lab, you decide to employ morphometrics to determine if the spring sites are host to the same species or different species.

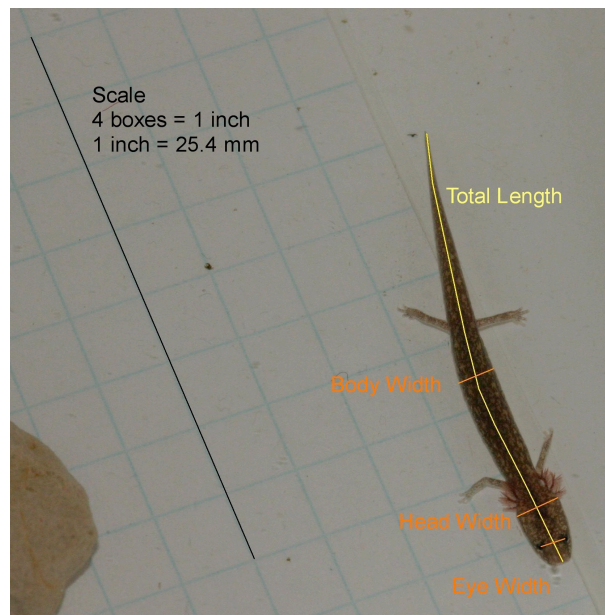
Methods

A subset of each population is captured, photographed, and released back to the site of capture. The populations and associated photographs have been split into three categories:

- Population A – salamanders with larger eyes at Parthenia Spring
- Population B – salamanders with smaller eyes at Parthenia Spring
- Population C – salamanders at Wheless Spring

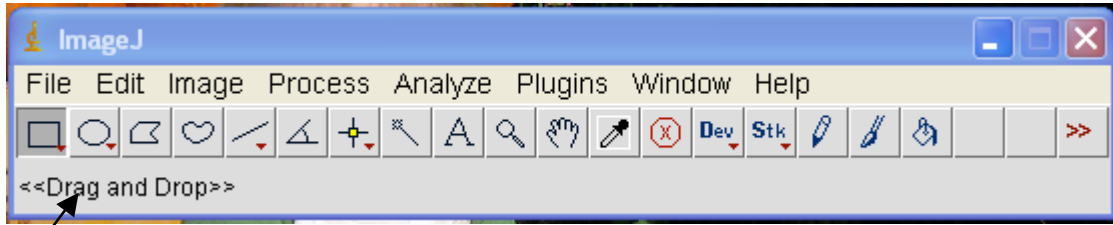
Photographs were taken using a scale for measuring back in the classroom. There are three different scales used in the photographs; ruler in centimeters, ruler in inches, and a grid of 4 squares = 1 inch. Be sure to use the correct scale for each image and **convert all measurements to millimeters for analyses**. Using the program ImageJ, you measure (as accurately as possible) a few quantitative variables of each individual that will be used for comparison:

- Total length (tip of snout to tip of tail, following the center line of the body)
 - Body width (width at the widest part of the torso – use your best judgement)
 - Head width (width at the widest part of the head – use your best judgement)
 - Eye width (distance between two eye spots, center of spot to center of spot)
- * Refer to the ImageJ worksheet to provide you with instructions on installation of the program (if applicable) and how to use ImageJ.




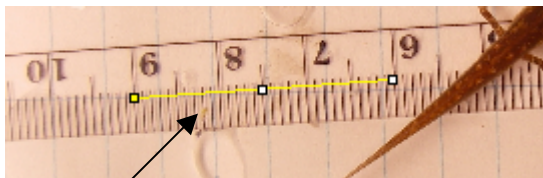
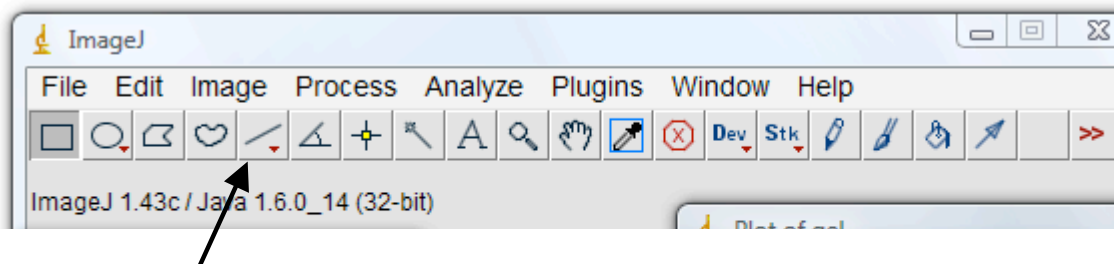
ImageJ Student Sheet

1. **Open a file.** On the toolbar, click on File, Open and navigate to a salamander image file (jpeg) from population A, B, or C. The salamander image will open in a separate window.

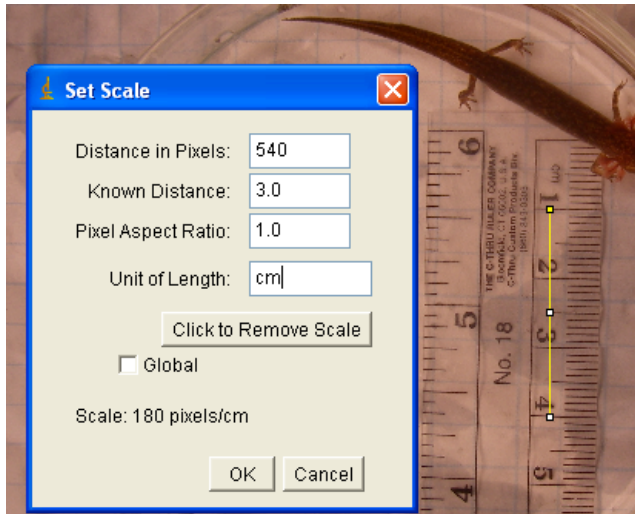


2. **Set the Scale.** The new window shows a salamander and a ruler. Before measuring the salamander you will need to set the scale. There are three different scales used in the photographs; ruler in centimeters, ruler in inches, and a grid of 4 squares = 1 inch. To set your scale:


- Click on the straight line tool . Click on a major line segment on the ruler and holding down the left mouse button drag the mouse until you reach another major line on the ruler. Release the mouse button and a straight line will remain (see example below).

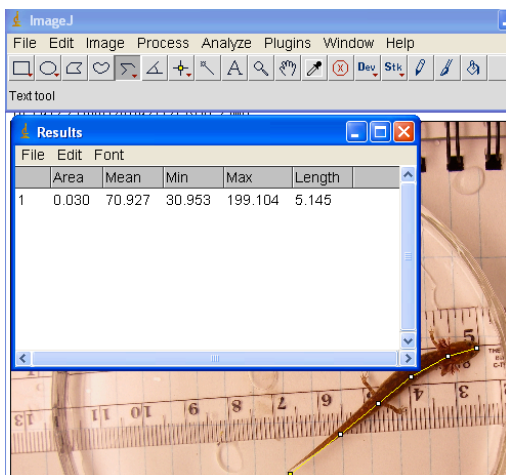


- On the toolbar, click on Analyze-Set Scale...change known distance to the distance you measured (ie. 3.0) and change unit of length to cm (or inches depending on the ruler units). Click OK.



3. Measure the salamander.

- On the toolbar, **right click** on the line selection tool to open the drop down menu and click on Segmented line .
- To measure the total length of the salamander, move the cursor to the tip of the salamander tail and double click. Drag and click the line along the middle of the salamander body (more clicks=more accuracy) until you reach the snout of the salamander. Double click to end line.
- On the toolbar, click on Analyze, Measure (see example below). Look for length and convert cm to mm by multiplying by 10. Open the excel document titled Student Data Sheet. Record total length (in mm) of salamander in the correct cell on your data sheet. In this example the total length = 51.5 mm.
- Next measure body width (width at the widest part of the torso-use your best judgement) and record body width (in mm) on your data sheet.



4. Repeat #1-3 for all images in A, B and C.

5. Once all the all the measurement data has been entered into the Excel “Student Data Sheet,” follow the formulas below to complete the calculations for analysis.

Mean: Excel formula

=AVERAGE(and the range of cells in parenthesis) e.g. (C2:C8)

Standard deviation: Excel formula

=STDEV(and the range of cells in parenthesis) e.g. (C2:C8)

T-test: Excel formula

=TTEST(array1,array2,tails,type) e.g. (C2:C8,C9:C15,2,1)

- Array 1 is the measured values of one variable for population A (select the range of cells with this data e.g. C2:C8)
- Array 2 is the measured values of the same variable for population B (select the range of cells with this data e.g. C9:C15)
- For ‘tails’, enter 2. This refers to the probability distribution of the data
- For ‘type’ enter 1, referring to the kind of t-test (1 represents “paired”)
- The result of a TTest is called a p-value (with 95% confidence limits).

p-value ≥ 0.05 means the null hypothesis is true (differences b/w the two groups of data is by chance).

p-level is < 0.05 , the difference between the two groups is not chance, but due to specific factors.

6. Using univariate analysis (t-tests), are there significant differences between the following and how do you know:

Population A and B

Total Length _____

Body Width _____

Head Width _____

Eye Width _____

Population A and C

Total Length _____

Body Width _____

Head Width _____

Eye Width _____

Population B and C

Total Length _____

Body Width _____

Head Width _____

Eye Width _____

7. Now it is time to take a bivariate approach. Using excel graphs, make three scatter plots with data from all three populations. Each population, A, B, and C, should be its own series in each plot.

8. Describe any trends you observe in each of the following three plots and discuss how plotting the data either supports or refutes the univariate analyses above:

Total Length vs. Body Width

Head Width vs. Eye Width

Total Length vs. Head Width

9. Lastly, you will calculate the ratios for each point on each scatter plot. This is done by dividing the value on X axis (horizontal) by the value on the Y axis (vertical). (HINT: Look at your data sheet and use the exact recorded measurements to do the math). Insert the formula for the ratios in your Excel spreadsheet to calculate the ratios.

Division: Excel formula =cell/cell e.g. (Total Length/Body Width on the Student Worksheet is = D3/E3)

Repeat the univariate analyses (mean, standard deviation, and t-test) for the ratios.

10. Using univariate analysis (t-tests) of variable ratios, are there significant differences between the following and how do you know:

Population A and B

Total Length/Body Width _____

Head Width/Eye Width _____

Total Length/Head Width _____

Population A and C

Total Length/Body Width _____

Head Width/Eye Width _____

Total Length/Head Width _____

Population B and C

Total Length/Body Width _____

Head Width/Eye Width _____

Total Length/Head Width _____

