AP Biology, Aquatic Science, Environmental Systems Morphometric Analysis of *Eurycea* in Austin, TX **Teacher Page**	***Enduring Understandings*** ***Vocabulary*** morphometric
Essential Questions:	***Topical Questions:***
Objectives	

Teacher Management

Estimated Time for Completion
2 class periods

Materials
Groundwater video "Austin Underground"
Computers
Salamander images file

Teacher Prep
This activity is designed to follow part 3 of the video "Austin Underground" and instruction on statistics.
Reserve computer lab or provide computers for students (or groups)
Follow instructions for ImageJ software download
Save salamander image files to computers

Safety Considerations
N/A

Vocabulary Building Strategies

Background Information for Teacher
<u>MORPHOMETRICS BACKGROUND</u>
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

Univariate analyses are very common in biology (and elsewhere), and you have undoubtedly conducted them yourself.

A. Descriptive statistics

Usually includes at least the following:

1. Observed limits (range)
2. Sample size (*n*)
3. Mean (average)
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):

B. 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.

C. Univariate analysis

The *t*-test is a common method used in evaluating differences in means between two groups.

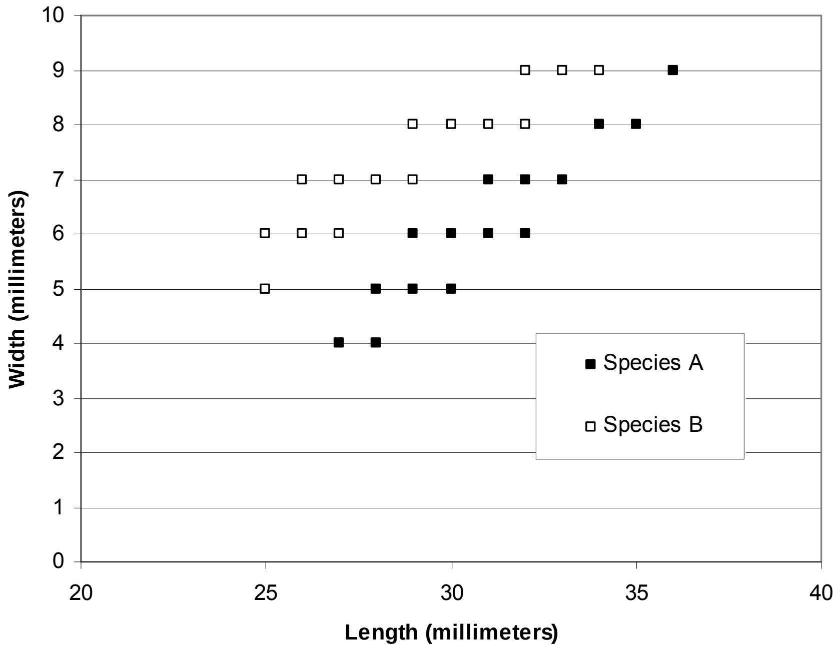
The *p*-level reported with a *t*-test represents the probability of error involved in accepting the hypothesis that there is a significant difference between two sets of data.

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 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.

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

Probing Questions

Focus Activity/Warm up

Show "Austin Underground" video segment about salamanders.

Engage

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. Use student sheet Morphometric Analysis.

Explore

Under construction

Explain
Under construction

Extend/Elaborate
Under construction

Evaluate
Under construction

Closure/Daily Assessment
Under construction

Differentiation
ELL
Special Education

ImageJ Software Download

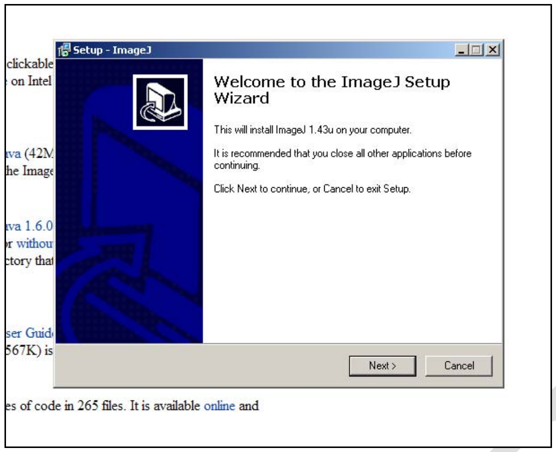
ImageJ is a free public source image processing and analysis program provided by the National Institute of Health. While the program has extensive application in the sciences, this introduction will cover only a few tools relevant to the lesson "Morphometrics of *Eurycea* in Austin, TX". Download from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

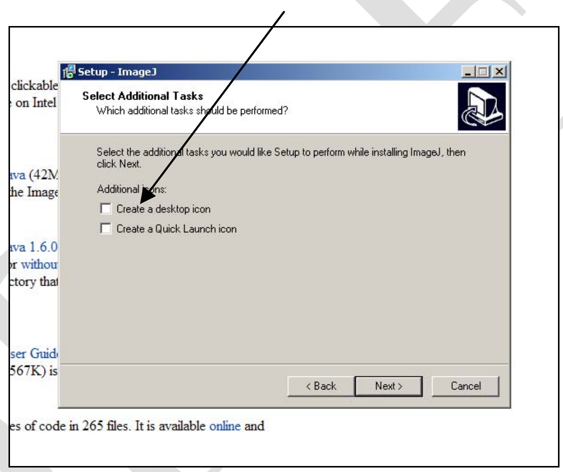
When prompted, click "Run".

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.

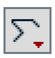
The pop-up screen should say Welcome to ImageJ. Click "OK". An ImageJ configuration pop-up should appear. Click "OK" and ImageJ should now appear as a toolbar at the top of your screen (similar to the one shown below). To start working with an image in ImageJ, drag and drop the image file (ie. jpeg)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

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.

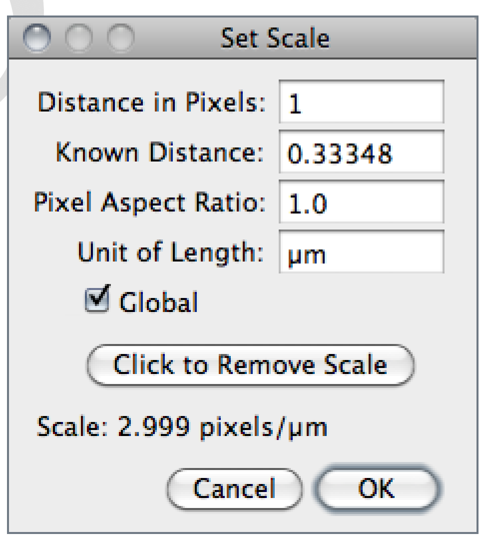


Segmented Line Selection Tool

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.

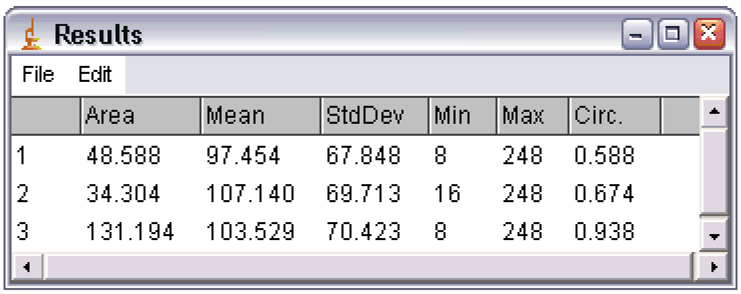
Set Scale

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:

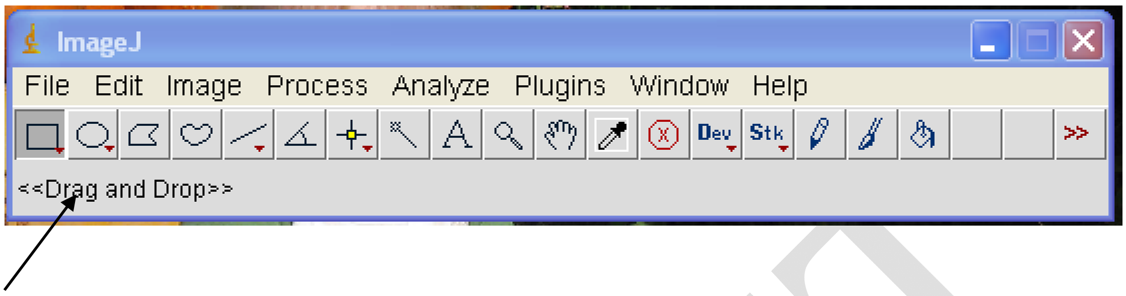
Population A – salamanders with larger eyes at Parthenia Spring
Population B – salamanders with smaller eyes at Parthenia Spring
Population C – salamanders at Wheless Spring

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

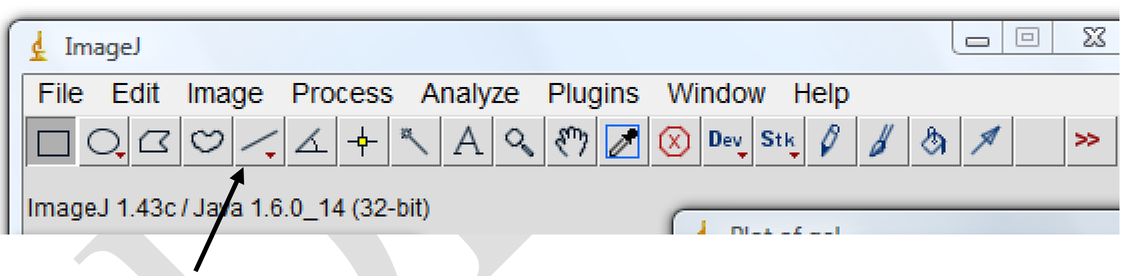
1. **Open a file**. On the toolbar, click on File, Open and navigate to a salamander image file (jpeg) from population A, B, or C. The salamander image will open in a separate window.

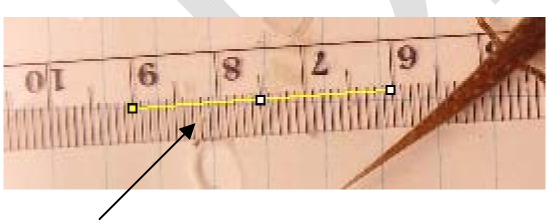


2. **Set the Scale**. The new window shows a salamander and a ruler. Before measuring the salamander you will need to set the scale. There are three different scales used in the photographs; ruler in centimeters, ruler in inches, and a grid of 4 squares = 1inch.

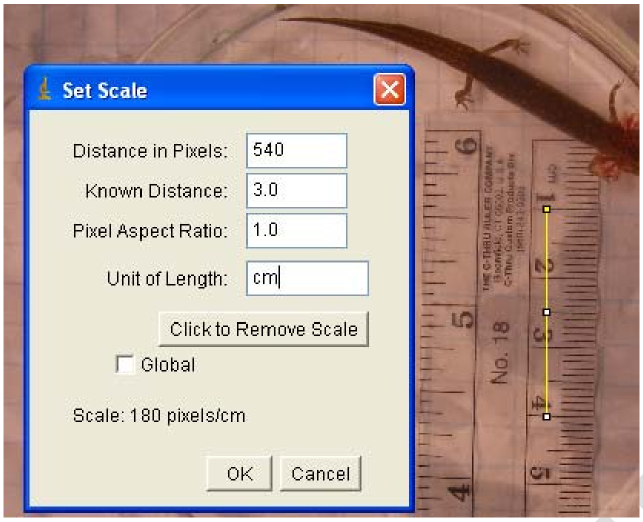
To set your scale:

- 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).



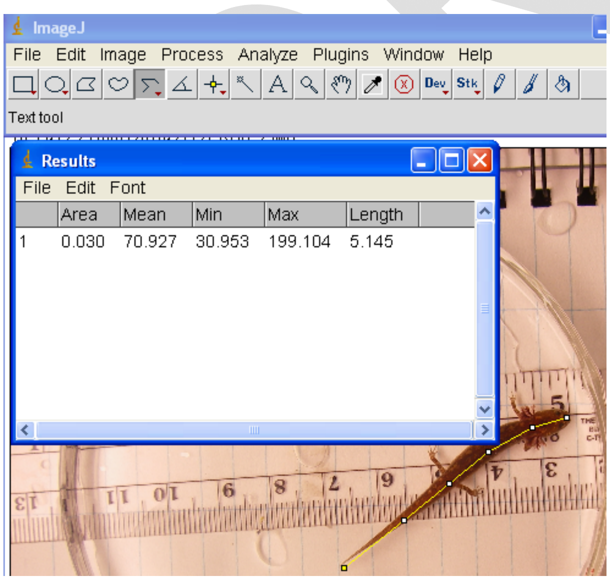


- On the toolbar, click on Analyze-Set Scale…change known distance to the distance you measured (ie. 3.0) and change unit of length to cm (or inches depending on the ruler units). Click OK.



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 curser 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 record total length (in mm*) of salamander on your data sheet. *convert cm to mm by multiplying 10. 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.



Once the data has been entered into an Excel spreadsheet, it is time to start the analyses. Below is a list of the analyses you should complete for each population and each measured variable (total length, body width, head width, and eye width). The excel formulas for each are given below and can also be observed in use in the "Sample Datasheet" worksheet:

Mean =AVERAGE(number1,number2,…)
Standard deviation =STDEV(number1, number2,…)
T-test =TTEST(array1,array2,tails,type)
 Array 1 is the measured values of one variable for population 1
 Array 2 is the measured values of the same variable for population 2
 Tails should be 2, referring to the probability distribution of the data
 Type should be 1, referring to the kind of t-test (1 represents "paired")
 * The value reported in the cell will be the p-value with 95% confidence limits

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 ____

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

Lastly, you will calculate the ratios for each point on you bivariate plots. This is done by dividing the value on X axis by the value on the Y axis. Create new columns in your Excel spreadsheet to display the ratios. Repeat the univariate analyses (mean, standard deviation, and t-test) for the ratios.

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 ______________________________

Based on the information obtained from the univariate analyses and the bivariate plots, would you conclude that Populations A, B, and C are the same or different species using morphometrics alone? Report supporting and refuting evidence within your conclusions.