**Biometry ANOVA**

**Objectives & topics**

* Writing hypotheses for ANOVA
* Assessing data for assumptions of ANOVA
* Conducting one-way and two-way ANOVA with Post-Hoc tests
* Using statistical methods to critique the primary literature
* Developing scientific writing skills that incorporate statistical methods

**Introduction to ANOVA**

Analysis of variance, or “ANOVA” for short, is a method for comparing three or more means. By using a single ANOVA rather than many t-tests, there are fewer opportunities for a type I error to occur. Differences between particular means can be investigated after the ANOVA (“*post hoc*”) with a multiple comparisons procedure such as Tukey’s Honest Significant Difference.

“Analysis of variance” refers to how this method partitions variation in the response variable according to multiple sources:

* In a **one-way ANOVA**, there is one factor with three or more groups. The only sources of variation are the groups and error.
* In a **two-way ANOVA**, there are two factors that are “crossed” so that every combination of levels from the two factors is represented. This configuration allows a test to be performed for interaction between the two factors. If no significant interaction is found, then the factors (or "main effects") can then be tested individually.  In this case the factors are *additive*.  If there is a significant interaction, then the factors are *not additive*, and the means for all the *combinations of the factors* are investigated. The sources of variation are groups for factor A, groups for factor B, interaction, and error.

**Directions for conducting a one-way ANOVA**

For this analysis in JMP, your data need to be “stacked”, meaning that there should be one column with the treatment names and a second column with the raw data (i.e. the data are not entered in separate columns for each treatment).

Here are the JMP manual instructions for a one-way ANOVA in JMP:

***F-test for the means in JMP***

Choose *Analyze/Fit Y by X.* Put the numerical/response variable in the *“Y, Response”* box, and put the categorical/explanatory variable Level” in the *“X, Factor”* window and click “OK.” You get side-by-side dotplots.

Click on the “Oneway Analysis of …” red triangle, and choose “Means and Std Dev.” This will give you a table of the means and standard deviations (SDs) of the treatment groups below the dotplots. (You also need this to test the “equal SD condition”)

To get the Analysis of Variance, or ANOVA, table, we will Click on the “Oneway Analysis of …” red triangle, and choose “Means/Anova.”

Once you’ve had JMP do the one-way ANOVA for you, interpret the results using the F-statistic and α=0.05. Relate this back to your hypothesis, and draw a conclusion.

To figure out which treatments are significantly different from each other, do a multiple comparisons test in which you test all possible combinations of the treatments. Here are the JMP manual instructions for doing those tests:

***Multiple comparisons in JMP***

Click on the “Oneway Analysis of …” red triangle, and choose *“Compare Means/All Pairs, Tukey HSD.”* You can set the *α* level to something other than 0.05 by clicking on the “Oneway Analysis of …” red triangle and choosing *“Set α Level”* to 0.01, say (but it does not have to match the *α* for the *F*-test).

Interpret your multiple comparisons results to clarify which means are different and are not different from each other. To do this, it helps to write out all the treatments in ascending order of their sample means, and then make a “chart with lines” where you connect the treatments that are not statistically significantly different with a line. By doing this, it’s easy to see which treatments are or aren’t different from one another. The middle table in the JMP output for this command shows this clearly as well, by putting the same letter next to treatments that are not significantly different.

**Directions for conducting a two-way ANOVA**

Before writing out your null and alternate hypotheses to test the main effects, you first have to test for an interaction between the two main effects. The hypotheses for ~~this~~ these tests are:

*H0 for the interaction*: there is **no** interaction between {*factor A*} and {*factor B*}, vs.

*Ha* *for the interaction:* there **is** an interaction between {*factor A*} and {*factor B*}

**IF there is NO interaction**, then we test both:

*H0*: There are **no** main effects due to {factor *A*}, vs.

*Ha*: There **are** main effects due to {factor *A*}

- and -

*H0*: There are **no** main effects due to {factor *B*}, vs.

*Ha*: There **are** main effects due to {factor *B*}

**IF there IS an interaction**, then we do not use the two-way ANOVA to test the factors *A* and *B*. There are two alternatives. **First**, you could perform two separate one-way ANOVAs, one for factor A and one for factor B. These tests will not be as powerful as the two-way ANOVA since there will be more “noise” due to the other factor that is unaccounted for, and the two tests are not independent of each other. **Second**, you could note that many statisticians feel that if there IS a significant interaction, the main effects of A or B are not interpretable! Therefore you should ask yourself if what you really want is the just the best treatment combination. If so you create a new categorical variable with *I*⋅*J* levels or treatments (what were the treatment combinations), and do the multiple comparisons on the *IxJ* treatment means (as if you did a one-way ANOVA on the *IxJ* treatments).

***Two-way ANOVA in JMP***

The data must be “stacked” with the response variable in one column, a categorical column for factor *A* showing which level of factor *A* the response came from, and a categorical column for factor *B* showing which level of factor *B* the response came from.

Use *Analyze/Fit Model* and put the response variable in the *‘Y’* box, and the two factors in the *‘Construct Model Effects’* box using the *‘Add’* button. Then in the *‘Select Columns’* box, click on both factors so that they are highlighted at the same time (holding down the shift key). Click on the *‘Cross’* button from the *‘Construct Model Effects’* buttons, and you will see the cross-product or interaction term added to the model. Click on *‘Run Model.’*

*Analysis of interaction among treatments*

When you do a two-way ANOVA, you must test for any possible interactions among the treatments. Be sure the interaction term (e.g. Factor A \* Factor B) is built into your model. If you follow the JMP instructions above, you will do this by default.

Interpret the F-test for the interaction term. Note above (in the hypotheses) that if there IS a significant interaction term, you cannot interpret the results of this test and should use a one-way ANOVA approach.

In addition to the F-test on the interaction term, you can visually test for an interaction among the treatments by making “Interaction Profiles” in JMP:

**Interaction/profile plots in JMP**

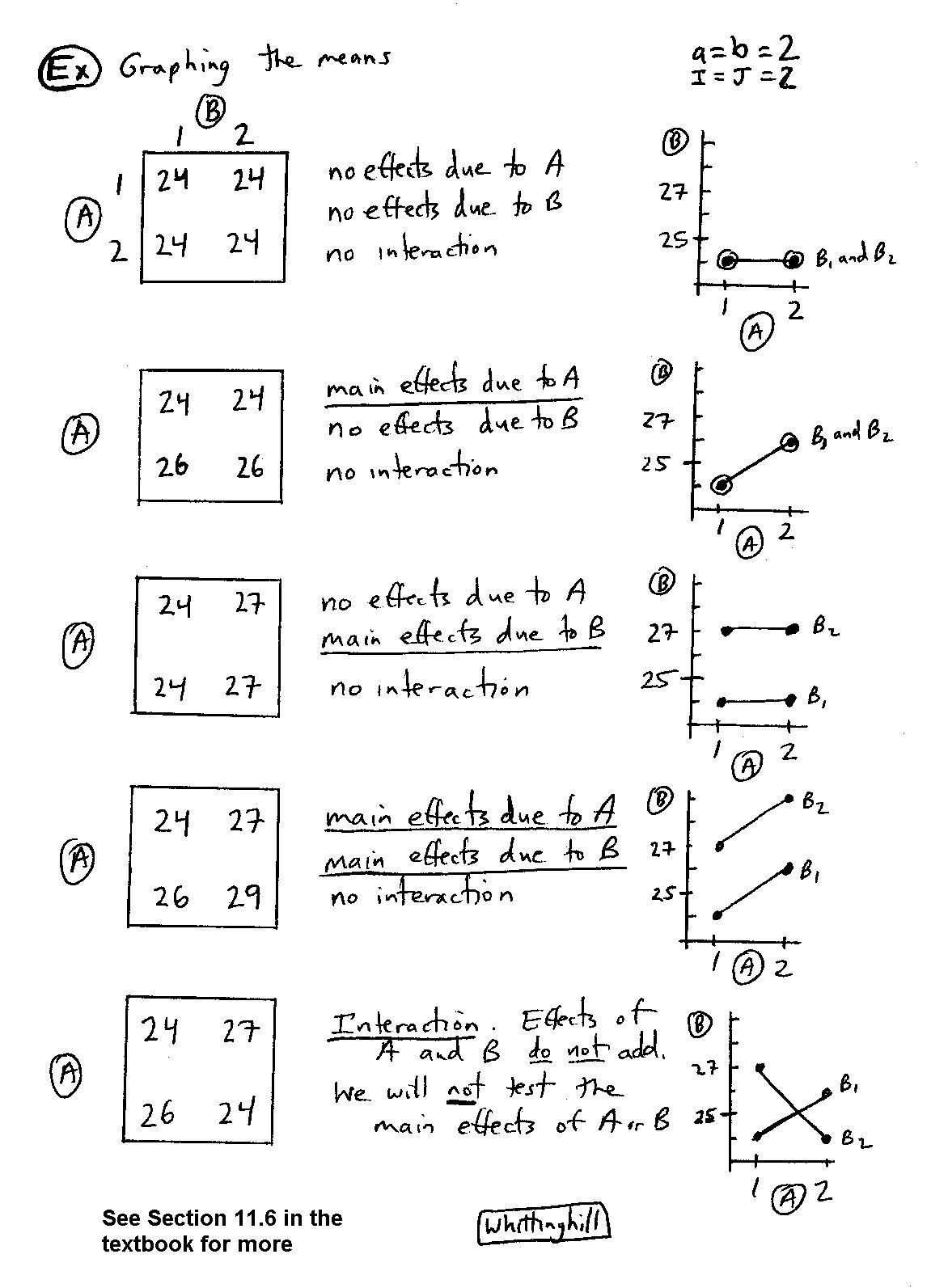
To get the **interaction/profile plots** go to the red triangle and select *‘Factor Profiling/Interaction Plots.’*

**Getting the treatment and interaction estimated means in JMP**

In a two-way ANOVA, to get the estimated means of the treatments and/or the interaction effects, go to the Least Squares Means Tables at the top of the ANOVA output (to the right). There should be separate tables for each treatment, and another for the interaction terms. Make sure the blue triangle is turned down to show you these tables, which list the estimated means next to the level of each factor.

**Interpreting interaction plots**

The scanned drawings on this page should help you with the interpretation of the interaction/profile plots. If the lines on these plots are not parallel, there is an interaction among the treatments. The F-test will help you to interpret the significance (or lack of significance) of any non-parallel pattern you see.



**Lab Activity**

In this lab you will utilize a published dataset from De Kort et al., 2020 to assess the experimental methods used in the study. The study by De Kort et al. seeks to understand plasticity in the phenotype (change in the appearance or function) of wild strawberry (*Fragaria vesca*) due to environmental conditions. The authors measured many different plant traits and environmental variables as part of their study and used a complex regression approach to understand the relationship between the variables. In order to conduct such a study, the authors needed to ensure equivalency across their treatment groups and sampling locations, ultimately measuring the initial growth of the plants as a proxy measurement for “maternal effects” like seed quality. While the authors report that they controlled for “maternal effects”, they do not provide any evidence that “maternal effects” were similar across sites or treatments. 

**Our goal in this lab activity is to test for the presence of maternal effects across sites and treatments in De Kort et al., 2020 by assessing whether the “Early Growth” variable differs between locations (“gradients”; see Figure 1 in the study) and treatments (dry, normal, wet).** You will present your results in the form of a written scientific report.

Main Publication:

De Kort H, Panis B, Helsen K, Douzet R, Janssens SB, and Honnay O. 2020. Pre-adaptation to climate change through topography-driven phenotypic plasticity. *Journal of Ecology*, 108:4, 1465-1474. <https://doi.org/10.1111/1365-2745.13365>

Published Data:

De Kort H, Panis B, Helsen K, Douzet R, Janssens SB, and Honnay O. 2020. Pre-adaptation to climate change through topography-driven phenotypic plasticity. *Dryad*, Dataset. <https://doi.org/10.5061/dryad.5tb2rbp17>

**Summary of Lab Procedure**

1. Familiarize yourself with De Kort et al., 2020 and write hypotheses
2. Make figures that visually summarize your hypotheses
3. Test the normality assumption in two-way ANOVA by conducting normality tests
4. Transform the data to achieve multivariate normality
5. Test homogeneity (equality) of variance assumption by conducting Levene tests.
6. Perform the two-way ANOVA
7. Interpret the two-way ANOVA
8. Consider the implications of the two-way ANOVA for De Kort et al., 2020 (this step includes conducting a one-way ANOVA)
9. Develop recommendations on how to treat the data in De Kort et al., 2020 (this step includes post-hoc tests from the one-way ANOVA and a second two-way ANOVA)
10. Write a report about your findings in the format of a scientific paper

**Detailed Instructions**

**Part 1: Getting organized**

1. Familiarize yourself with the De Kort et al. (2020) study and data available on canvas. The PDF of the article has been annotated with highlights to guide you. Do not try to read the entire paper now. Look at the highlighted areas and the data to get a sense of the structure of the experiment.
2. Develop your hypotheses regarding the Early Growth variable. Use the space below to write out both the null (H0) and alternate (Ha) hypotheses for this experiment (there are 6 hypotheses total; 3 sets of null and alternate!). Remember, our goal is to test for the presence of maternal effects across sites and treatments. Hint: we will be using a two-way ANOVA.

Ho1:

Ha1:

Ho2:

Ha2:

Ho3:

Ha3:

**Part 2: Exploring the data**

1. After constructing your hypotheses, you should explore the data and create figures that help you visualize the hypotheses you are planning to test. Given what you wrote for the hypotheses above, ***how do you think you should show the data***? Make sure your answer makes sense in terms of the comparisons you made in your hypotheses.

*Paste your properly formatted figures here for reference*

**Part 3: Conducting Normality tests**

1. Conduct normality tests by going to analyzedistribution and putting the dependent variable into “y” and both independent variables into “by”. Fit a continuous probability distribution to each histogram and assess the fit of the probability distribution using a Shapiro-Wilk test. You should also create a QQ plot. Hint: hold down ctrl (command for Mac users) in order to do the same task across all histograms at once.

To fit a continuous probability distribution to each histogram, click on the red triangle at the top of the histogram and scroll down to “Continuous Fit”, and select a continuous fit. Once the distribution is shown for each histogram, use “Goodness of fit” to assess the fit of the probability distribution.

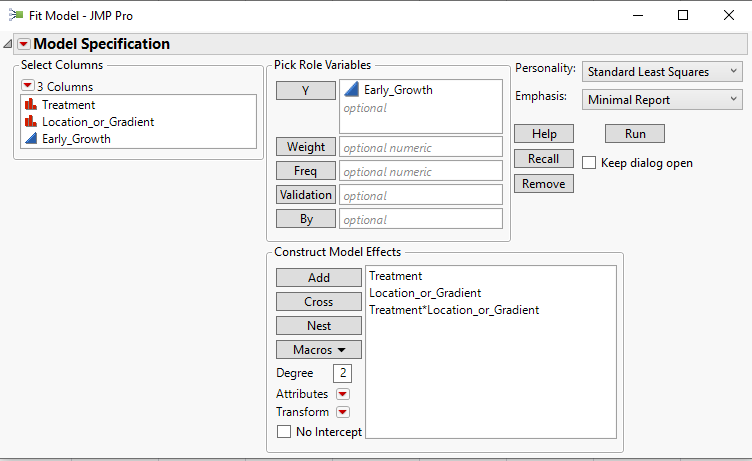
After these analyses are completed for each histogram, compare the distributions.Remember that the significance level for the Shapiro-Wilk test is 0.25 to protect against type II error from occurring. When p< 0.25, we can reject the null hypothesis that the data fit the distribution; if p> 0.25 then we cannot reject the null hypothesis, so normality can be assumed due to lack of evidence otherwise.

Make a note of which subsets of the distribution are normal and which are not normal. This is valuable information for your paper.

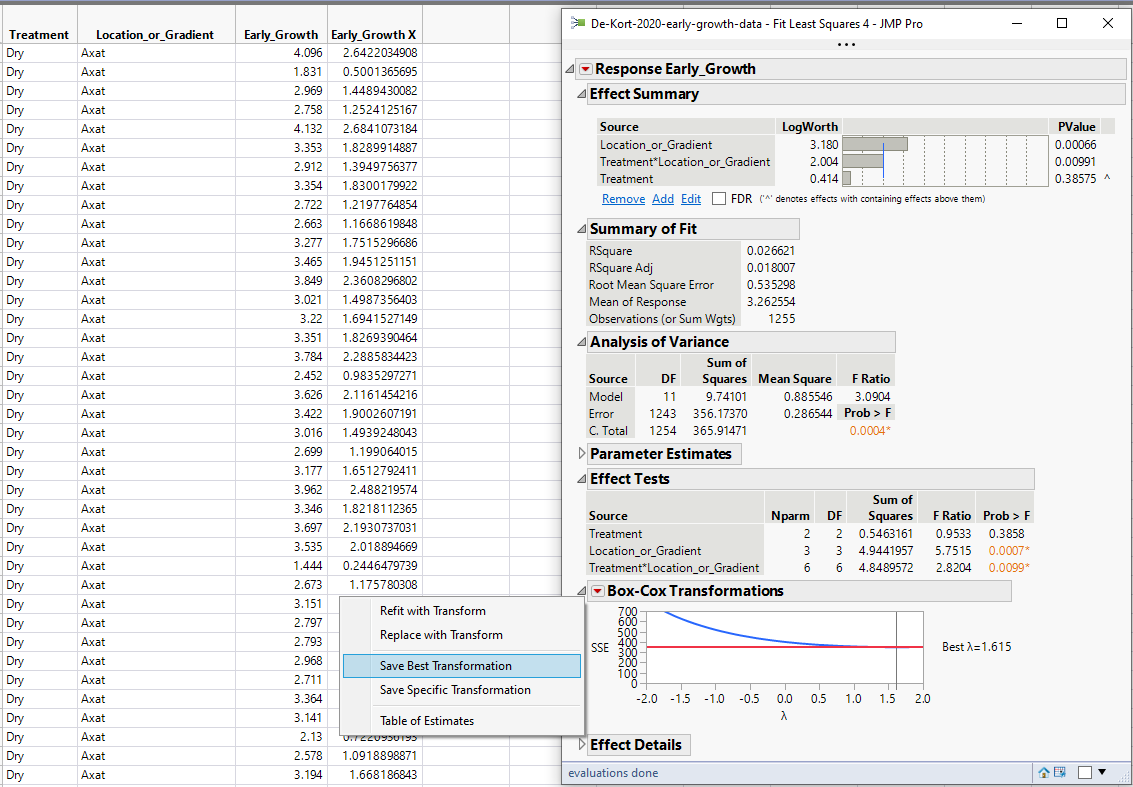
*Write notes here*

**Part 4: Transforming the data to achieve multivariate normality**

1. You should have discovered in part 3 (above) that some subsets of the data are normal, but there are many subsets that are not. We will conduct a Box-Cox transformation on the data to try and meet the assumptions of ANOVA. The Box-Cox transform is very similar to the log transform but performs better in multivariate analyses such as the one we are conducting here. The procedure for the Box-Cox transform is as follows.
   1. AnalyzeFit Model
   2. Highlight both independent variables, select “macros”, and click “full factorial”. It should look like this when you are done:



* 1. Click “run”. In the new window, click the little red triangle, and select: factor profilingBox-Cox Y Transform
  2. A new graph for the Box-Cox transform will appear at the bottom
  3. From the new graph, click the red triangle and “save best transform”. This will create a new variable in your spreadsheet containing the transformed variable. It should look like this:



1. Conduct the normality tests from Part 3 (above) again by going to analyzedistribution and putting the new transformed dependent variable into “y” and both independent variables into “by”. Fit a continuous probability distribution to each histogram and assess the fit of the probability distribution using a Shapiro-Wilk test. You should also create a QQ plot for each distribution. Hint: hold down ctrl (command for Mac users) in order to do the same task across all histograms at once.

Compare your results in F to those in Part 3. Did the Box-Cox Y transform improve the multivariate normality of the data?

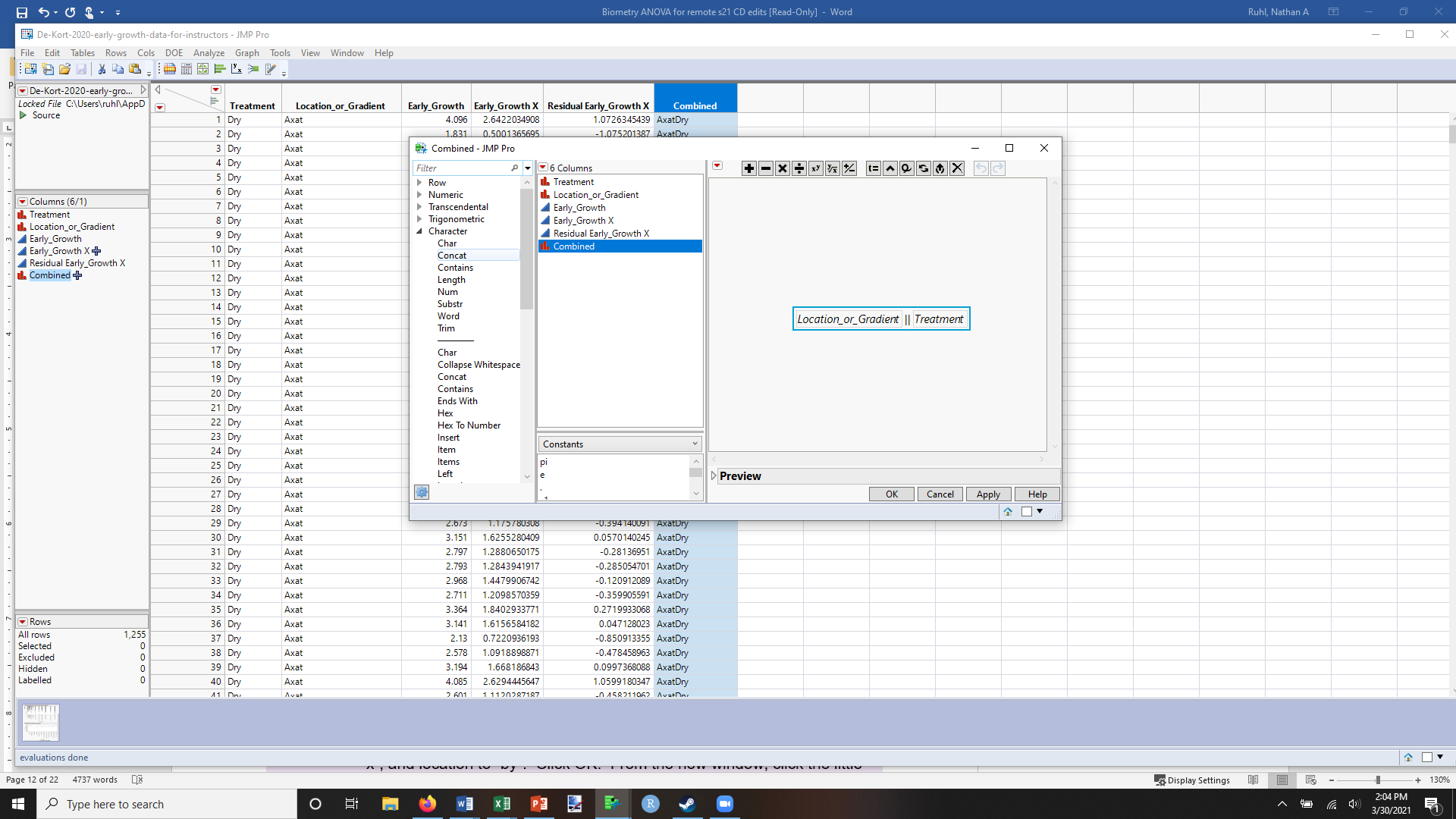
*Write notes here about the effectiveness of the transform*

**Part 5: Testing for Equality of Variance**

1. Now we will assess the transformed dependent variable (Early Growth X) for homogeneity or equality of variance using a Levene Test. The Levene Test is used to test the null hypothesis that the distributions being compared have equal variance. The significance level of the Levene test is 0.05, so values lower than 0.05 will cause us to reject the null and we will have failed to meet the assumption of homogeneity of variance. If our values are greater than 0.05, then we may proceed with our planned ANOVA since no compelling evidence of unequal variance was found.

To conduct a Levene test you will need to do two procedures:

* 1. First, create a new blank variable, go to column info, define that new variable as categorical, and name the column “Combined”. Now, write a formula for that variable that “concatenates” or combines our two factors (Treatment and Locations) into a single factor with 12 levels (3 treatments x 4 locations = 12). The “Concat” function is in the “character” menu. It should look like this:



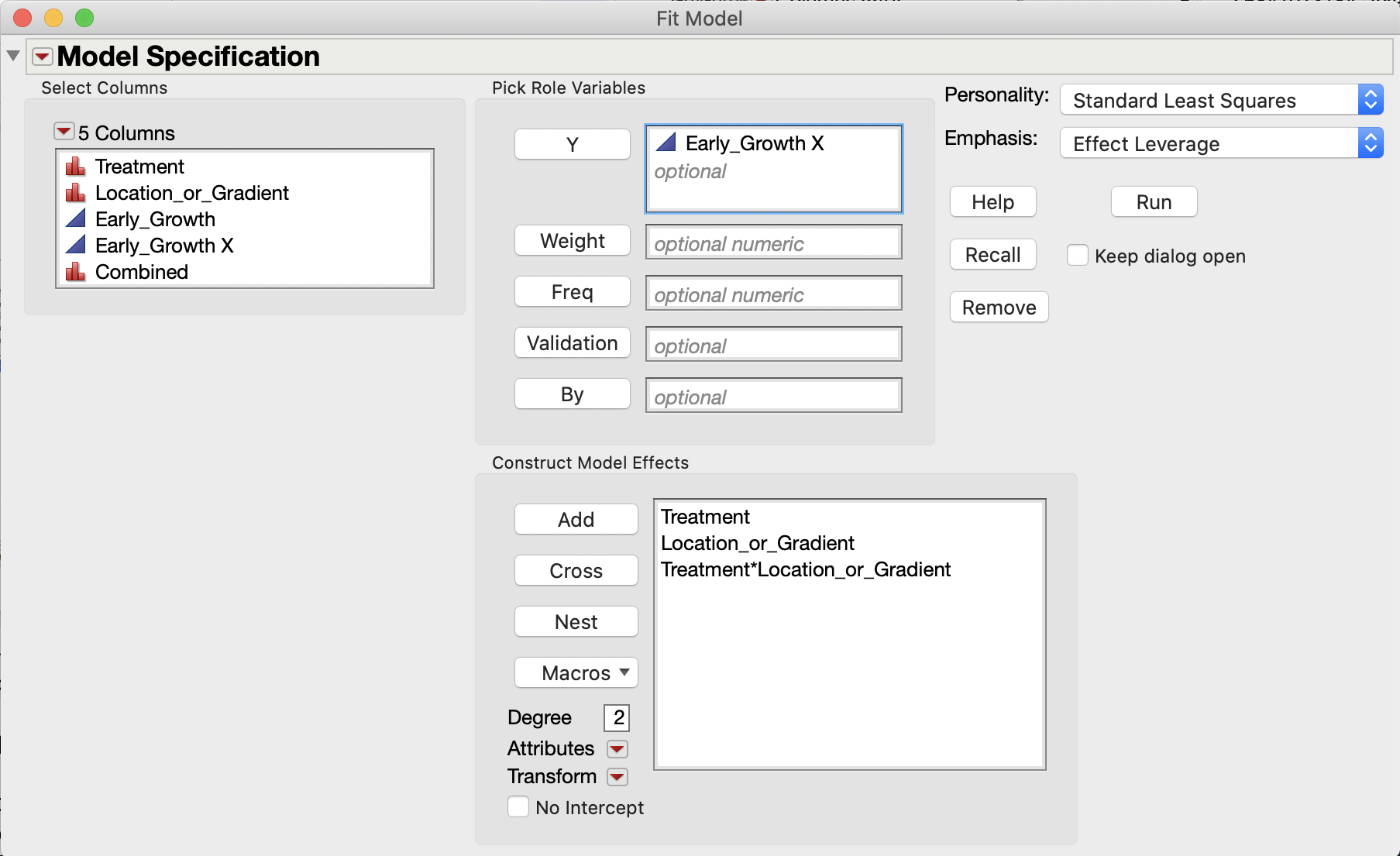
* 1. Go to Analyzefit y by x. Drag “Early Growth X” to “y” and “Combined” to “x”. Click OK. From the new window, click the little red triangle, and select “unequal variance”. Do your results suggest that you should reject or fail to reject the null hypothesis that we have equality of variance?

The Levene test provides us with insight on whether or not it is acceptable to proceed with an ANOVA, but it also suggests potential problems and/or ways that De Kort et al. could have altered their data analysis.

*Make note of any relevant observations about the structure of the data and whether the data passes the Levene test here.*

**Part 6: Conducting the two-way ANOVA**

1. Having passed both the normality tests and the tests of homogeneity of variance, we can now proceed to building our two-way ANOVA. Follow this procedure:
   1. AnalyzeFit Model
   2. Highlight both independent variables, select “macros”, and click “full factorial”. It should look like this when you are done:



* 1. When you are done, click “Run”. Your setup of the ANOVA should look the same as it did before in #5b (above)

1. We have one more normality test to conduct. This normality test is on the residual variation in the data that is not described by our model. If our data is properly transformed for our model structure (seen in the “model effects” box above), then the residual variation will be normal. We will extract the residual variation from our model and test those residuals to see if they are normal. To extract the residuals, click the little red triangle at the top left next to “Response Early\_Growth X”save columnsresiduals. This procedure saves a new column of data in your data sheet.
2. Test the “Residual Early\_Growth X” variable for normality. You will not need any independent variables in the “by” boxes. Make sure you use a Shapiro-Wilk test with an appropriate significance level and also interpret the QQ plot.

*Record your result here. Did your residuals pass this last normality test?*

**Part 7: Interpreting the two-way ANOVA**

1. You should have found that your residuals pass the normality test. Now we can move on to looking at the results of the ANOVA. Identify the ANOVA table in JMP. The ANOVA table includes a description of the parameters of your analysis and indicates whether there is a significant effect somewhere in your analysis. You should include the ANOVA table in your final paper. Reconstruct the ANOVA table from JMP as a properly formatted table here below.

*Construct the ANOVA table here*

1. Now interpret your hypotheses using the “Effect Test” box. There is a line for each of your hypothesis sets from #2 above. Interpret your hypotheses using the p-value and a significance level of 0.05. Note that interpreting your hypotheses gets more complicated if you have a significant interaction (see background materials above)

H1:

H2:

H3:

1. The DF, F-statistics, and p-values from the “Effect Test” box should be reported in your study. You can do this in sentence format or as a table.

*Make a note to yourself about the values from JMP or construct a table*

**Part 8: Considering the big-picture and next-steps**

1. Our analysis has revealed a significant interaction between treatment and location for the “maternal effects” variable, meaning that maternal effects are not controlled in De Kort et al., 2020. Remember that in this case, where we are testing whether the authors have successfully controlled for maternal effects, we “want” p-values that are greater than the significance level and values below the significance level (0.05) are problematic. A significant result for location is not that problematic taken individually (the point of the study is that the plant responds to differences in environment) but the interaction is problematic because it interferes with the ability of the authors to learn about the effect of their treatment (soil moisture level) on the plant, so we can interpret that hypothesis anyway.

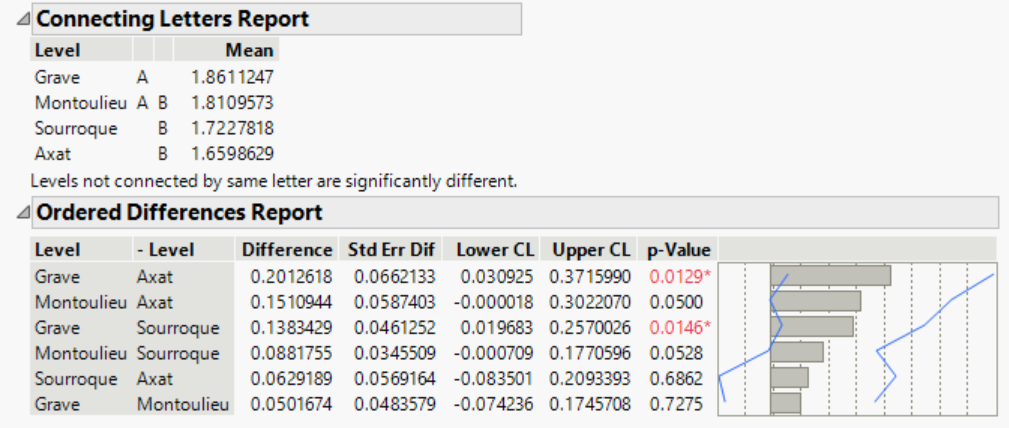
We have shown that De Kort et al. (2020) might have some problems with their analysis. The next step is to see if we can fix the problem using post-hoc tests to guide us. Our treatment hypothesis was not significant (which is good), so we will focus on the location variable.

Conduct a one-way ANOVA on the Early\_Growth X variable by going to Analyze fit y by x and assigning the dependent variable to y and Location to the x axis. Click OK, then conduct a one-way ANOVA by clicking the little red triangle and then “means/ANOVA”. You should include the ANOVA table for this one-way ANOVA in your paper.

*Construct the one-way ANOVA table here*

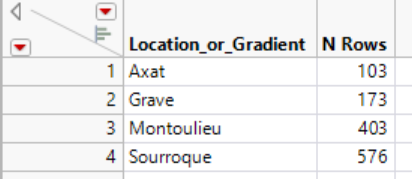
**Part 9: Removing data and testing an alternative dataset**

1. As expected, the one-way ANOVA for Early\_Growth X by Location is significant, so we should do a post-hoc test in order to determine where those significant differences lie. Click the little red triangle and select compare means 🡪 all pairs, Tukey HSD. The output should include this information:



These results suggest three options.

* 1. Remove Grave (173 rows of data removed)
  2. Remove Grave and Montoulieu (576 rows of data removed)
  3. Remove Sourroque and Axat (679 rows of data removed)



1. For your paper, you will report on the effectiveness of one of these options. Sort the data by location, highlight the rows that need to be removed from the analysis, and Hide/Exclude them. You will need to conduct a new Box-Cox y-transform with the appropriate locations excluded, reassess the ANOVA assumptions (normality and equality of variance), and conduct a new two-way ANOVA. Your paper should report the ANOVA table and effects tests (as in #11 and #13 above).

*Construct your tables and/or make notes on your results here*

**Part 10: Writing your paper --- you will write your paper as a group**

* **Use the grading rubric posted on Canvas AND this document as a guide for your report.**
* This is a group assignment and all members of the group should be contributing equally (comparably) to developing and finishing the paper. Group members may contribute differently to sections, but not in overall effort. All group members should read a complete draft of the paper and contribute to finalizing the paper.
* Collaborate with your group and develop both a plan and schedule that makes sense. Agree on deadlines for stages of the work, including completing drafts of sections and finalizing the paper. Allow adequate time for final revisions by all group members. (An hour or two before the paper is due is NOT a good plan.)
* Length is not important; being thorough, clear, and consistent is. (You are asking the wrong question if you ask “How long should my report be?” The question to ask is: “What should be included in my report”?)
* You do not need to seek out additional citations for this assignment, however, if you are having trouble interpreting your results, you may seek primary literature sources and cite them in the intro and/or the discussion. You may not cite websites or other non-primary literature such as review articles or materials written for non-scientists. Do not plagiarize. Citations (in-text and at the end of your paper) should be in APA format. **If you use outside sources in preparing your report, YOU MUST CITE THEM.**
* For the introduction:
  + You should briefly discuss the study by De Kort et al., 2020 and pay particular attention to the maternal effects in question.
  + There should be a clear statement on how/why a two-way ANOVA will help you understand the maternal effects in De Kort et al., 2020.
  + Include your hypotheses in the introduction.
* In the methods section:
  + Describe how the data you plan to test was collected by De Kort et al. 2020 and where we obtained the data. You do not need to describe the entire study; focus on the parts of the study that are relevant to us. Hint: look at what is annotated, and what is not, in the annotated PDF.
  + Describe our statistical methods (do not write your methods in a cook-book fashion; avoid step by step directions).
* In the results section:
  + Include the tables that you made above.
  + Include the figure that you made above.
  + **The results section contains text explaining your analysis and the result of your hypothesis tests**.
  + Do not explain WHY you got a particular result, but do report WHAT your results are.
    - This is acceptable: “The Shapiro-Wilk test indicated that it was reasonable to assume normality (p=0.54, α= 0.25)”
    - This is **not** acceptable: “The Shapiro-Wilk test indicated the data was normal (p=0.54, α=0.25), which was surprising given that the data was collected in France.”
  + Use correct statistical terminology when describing your results.
* In the discussion:
  + Broadly summarize the result of your hypothesis tests and explain why you think you obtained these particular results. (That is, what could have caused these results?)
  + Put our study into a broader context (the “big picture”). Can we trust the results of De Kort et al., 2020? What should De Kort et al., 2020 do with our results?
* **Submission Details**
  + Work with your group on this assignment.
  + You do not need to submit answers to questions in these instructions (but do answer them carefully, to help you prepare to write the paper.)
  + Submit your report to the assignment link in Canvas.
  + **The lab report is due in two weeks**, but you are welcome to submit earlier. See canvas for the exact due data and time for your section.

**References**

Main Publication:

De Kort H, Panis B, Helsen K, Douzet R, Janssens SB, and Honnay O. 2020. Pre-adaptation to climate change through topography-driven phenotypic plasticity. *Journal of Ecology*, 108:4, 1465-1474. <https://doi.org/10.1111/1365-2745.13365>

Published Data:

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F.Vesca Image:

“Fragaria Vesca.” *Wikipedia*, Wikimedia Foundation, 4 June 2021, en.wikipedia.org/wiki/Fragaria\_vesca.