**Attract or Defend?   
Secondary Compounds among tissues in two species of Lupine (Fabaceae)**

**Overview**

This 50- to 75-minute activity leads sophomore-level students through thinking about the possible roles of secondary compounds in plants and analyzing real data to address their predictions. Students consider the unique selective pressures facing plants (i.e., they often can’t move but must interact with the world around them) and relate this to the presence of secondary compounds. Students make hypotheses and predictions about how the presence of these chemical compounds might relate to interactions between the plant and its environment. Then, they explore and analyze relevant data using bar figures and ANOVAs in Microsoft Excel 

One fairly obvious result is that bad-tasting compounds are found in higher concentrations in tissues most vulnerable to herbivores and vital to survival/reproduction (flowers, stems, leaves). A less-obvious finding is that pollen has higher caffeine concentrations than other tissues. An extension activity leads students to investigate this potentially surprising result. 

**Key Concepts**

* Plant trade-offs between attracting pollinators and deterring herbivores
* Secondary compounds in plant: purposes and variation among tissue types
* Organizing data in bar figures
* Analyzing data through visualization and statistical inference (ANOVA)

**Student Learning Targets**

* Students will be able to identify possible roles of secondary compounds in plant tissues
* Students will be able to explain how and why certain compounds might vary among tissue types within an individual.
* Students will be able to describe the type of data analysis question that requires an ANOVA.
* Students will be able to answer a research question given the results of an ANOVA (primarily the p-value) and a bar figure with error bars.

**Prior Knowledge**

Students should:

* Have basic computer skills (typing, copy-paste, usage of hyperlinks)
* Be able to construct reasonable and relevant hypotheses and predictions based on given background information
* Have basic familiarity with interpreting and manipulating spreadsheets
* Have familiarity with interpreting bar plots and statistical inference
* Prior introduction to plant secondary compounds and the idea of trade-offs in resource use would be useful but are not required
* Optional: Have basic research skills that would enable some independent investigation of provided resources

**Background**

Unlike most animals, the vast majority of plants are stationary and thus have unique ways of defending themselves against enemies and attracting attention from allies. So-called secondary compounds have important roles in these interactions. Although the plant as a whole is subject to the general action of natural selection, different plant parts may have different vulnerabilities, roles, and interactions with the local environment. Thus, we might expect different compounds to be more or less present in certain tissues.

Alkaloids are bitter-tasting secondary compounds found in many plants. Many alkaloids have pronounced effects on physiological processes within animals. In high concentrations, they are often toxic and interfere with physiological processes in animals. In low concentrations, their effects may be pleasurable and/or habit-forming (e.g., with caffeine, morphine, etc).

*Lupinus* is a genus of flowering plants in the pea family (Fabaceae). Lupines are nectarless and pollinated by medium and large bees that collect and consume pollen as their reward. Plants must present copious pollen to attract these pollinating insects. These plants are also subject to herbivory by lycaenid butterfly larvae and small mammals.

**Teaching Tips**

* This activity is designed for sophomore-level students with some familiarity with MS excel and *the idea of* statistical tests. With additional help, this activity could be used with introductory-level students. With added rigor, this could be assigned for higher levels.
* The pre-activity questions could be assigned before the relevant class period.
  + Question 2 implies that the students have some prior knowledge of secondary compounds. This is not essential. If the topic hasn’t been covered in class, the educator may want to edit the handout to refer students to relevant textbook pages for that question.
* A recommended approach for the data analysis and interpretation section, is to have students collaborate with a single partner to complete Parts 1 - 4 for just one of the two species included in the data set.
  + The teacher should assign these to have equal representation across the class.
  + Then, for Part 5, student pairs could integrate into groups of four or could reform pairs with a student studying the other species.
  + The students are asked to use an ANOVA and have directions to walk then through this, but you may wish to go over this before, after, or during the activity
  + Ideally, this portion could be completed synchronously in a computer lab, but asynchronous or at-home collaboration could work given sufficient scaffolding and accountability.
* The post-activity questions could be excluded, modified, or assigned for post-class work.
  + The educator could provide the resources suggested in the answer key (below) or could allow students to conduct their own searches.

**Answer Key--*Note, this key does not contain all student avtivity instructions!***

## **Pre-Activity Questions**

1. All organisms face similar requirements for success in an evolutionary context. Namely, organisms need to survive (at least for a little while) and reproduce in order to contribute to the next generation. Consider the *specific, unique challenges that a plant* might face in terms of survival and reproduction.   
What factors have likely influenced the evolution of most plants? List four or five possible factors and star the two you think are most important and universal among plants.

* \*Defense of “body” tissues against herbivores
* \*Attraction of pollen-spreading animals
* Defense of reproductive propagules against the dry air (for spores and swimming sperm in the non-see plants) or against consumers (for seed-plants)
* Attraction of seed-dispersing animals

2. Reflect on what you have learned so far about plant secondary compounds (or, do some independent research).   
Generally, what are secondary compounds, and why might plants produce them? In the context of plants, what are alkaloids and how do most herbivores respond to them?

Secondary compounds (or “secondary metabolites”) are organic compounds, produced by bacteria, fungi, and plants that may be useful but aren’t required for basic (primary) life functions and processes like growth and reproduction. These might include volatile compounds (i.e., odors) that attract pollinators or defensive compounds that deter herbivores through bitter taste or other means.

Alkaloids are bitter-tasting secondary compounds found in many plants. In high concentrations, they are often toxic and interfere with physiological processes in animals. Many plants use alkaloids for defense. In low concentrations, their effects may be habit forming and result in repeated visits by the animal (e.g., with caffeine, morphine, etc).

2b. Consider a group of “pollen-rewarding” plant species, like the genus *Lupinus*. These plants are pollinated by bees and reward visiting bees with pollen that is consumed. The presence of edible pollen ensures continued visits to the plant’s many flowers. In terms of secondary compounds, what might you expect about the pollen in one of these species relative to its other tissues?

* These plants might produce lower levels of defensive compounds in pollen.
* These plants might produce higher levels of attractive compounds in their pollen

3a. Based on what you know so far, do you think that levels of certain secondary compounds are likely to differ among tissue types within a single plant? State a hypothesis related to your educated guess.

* Yes, levels of secondary compounds are likely to be different among different tissue types.

3b. Based on your hypothesis above, state a prediction related to the question posed in 3a. A prediction states a specific expected outcome, so your prediction should mention what the secondary compound is or does and what specific differences are expected among tissue types (stems, leaves, flowers, pollen). Example: *The hormone oxytocin will be found at higher concentrations in brain tissue.*

* Levels of secondary compounds related to herbivore defense are likely to be greater in the leaf and stem tissue than in the flowers or pollen.
* Levels of secondary compounds related to pollinator attraction are likely to be greater in the floral tissue for plants with nectar-seeking pollinators and greater in pollen for species with pollen-seeking pollinators

3c. Provide a justification for your prediction in 3b. Why do you think your prediction is reasonable? Example: *Oxytocin is produced in the brain, so that is probably where most of it is used.*Note: your prediction may or may not be accurate! We need an experiment to test it. In the case of oxytocin, this prediction is not quite right.

* Secondary compounds are expensive to produce, so the plant will probably concentrate them in the tissues where they are most useful/most likely to have a benefit
* Tissues have different functions, so a compound that is useful in one tissue might be detrimental to another. For instance, a plant wouldn’t want to deter pollinators from drinking its nectar and wouldn’t want to lure pollinators to it’s non-reproductive tissues like leaves.

## **Data Exploration and Analysis**

1. Open the Data File “Secondary compounds in plant tissues.xlsx” in Microsoft Excel, and consider the first worksheet. **Take a few minutes to familiarize yourself with the data.**

Note: Make sure that students have the Data Analysis add-on installed in their version of Excel. If students are completing this activity asynchronously, you may wish to provide additional instructions for this.

a. What variables are present? Are they continuous (if so, what are the units?) or discrete (if so, what are the categories)?

Species - discrete (n = 2), Individual - discrete (n = 28 / 8), Site - discrete (n = 4 / 1)

Tissue type - discrete (n =4)

Caffeine concentration - continuous, ug/g

Micrograms per gram - continuous, ug/g

b. Brainstorm comparisons that you could make using these data. List two or three of these possible comparisons below.

## **2. Visualize**

## **[Note: For figures, see the Excel spreadsheet KEY]**

1. Familiarize yourself with the data by making and briefly considering the following figures:
   1. Histogram for Average Alkaloid Concentration. [...instructions deleted…]
      1. Do the data have a bell-shape: a peak and lower values on either side? Are there any points that seem like outliers?   
         Note: we can’t just delete outlying values from our data set! But, it can be useful to make note when they exist.

Mostly normal, but longer tail of large values and a few larger outlying values. Note: “Outlier” is a statistical term, and we aren’t doing the analyses that would allow us to exclude data.

* 1. Boxplot relating Average Alkaloid concentration (y) and Caffeine Concentration (x) among tissue types [...instructions deleted…]
     1. Do you notice any differences (in magnitude or variation) among tissue types or between defensive alkaloids and caffeine? If so, what do you see?

Flowers seem most variable, pollen and stem least variable. Pollen concentration looks lower, but hard to be certain given the variation.

Educator Note: It may also be interesting to come back to a relationship between Average Alkaloid concentration (y) and Caffeine Concentration (x), grouped by tissue type (esp. if students pursue the caffeine extension). That figure shows that, while Alkaloid concentrations vary considerably within flower, stem, and leaf tissues, caffeine concentrations vary less within these groups. It is also clear that pollen caffeine concentrations are greater and alkaloid concentrations are smaller in the pollen than in other tissue types.

1. Reconsider your question, hypothesis, and prediction from Pre-activity question #3. What independent variable did you identify (is it discrete or continuous)?   
   What dependent variable did you identify (is it discrete or continuous)?  
   What figure type is appropriate to address your hypothesis?

Answers will vary. Make sure students are using an appropriate figure type (scatter/line for continuous x-axis/IV; bars for discrete x-axis/IV)

**We will first visualize, then analyze, data related to the following questions.**You should have the background to make an informed prediction about defensive-alkaloid concentrations in plant tissues, but you might be unsure about the expectation for caffeine. That’s OK.

For each hypothesis below, state a specific prediction:

- Alkaloid concentrations differ significantly among tissue types (flower, leaf, pollen, stem).

Prediction: Concentration in pollen will be lower than in other tissues

- Caffeine concentrations differ significantly among tissue types (flower, leaf, pollen, stem)..

Prediction: Pollen concentration will be higher than in other tissues.

Educator Note: students may not know what to think here as they haven’t been presented with information about caffeine yet.

1. Make a Column figure showing Average Alkaloid Concentration in each tissue type for your species.

[...instructions deleted…]

1. What does your figure suggest about the relative alkaloid concentrations among tissue types in your species? Is this in line with your hypothesis?

Alkaloids lower in pollen than in other tissues.

Relationship to hypothesis will vary

1. Repeat the steps from c. To add a new bar graph for caffeine concentration among tissue types in your species.
2. What does your figure suggest about the relative caffeine concentrations among tissue types in your species? Is this in line with your hypothesis?

Caffeine higher in pollen than in other tissues

## **3. Analyze**

Use the “Data for ANOVA” worksheet in the MS Excel file to complete an ANOVA that compares average alkaloid levels among samples from different tissues. [...instructions deleted…]

**Table 1: Average alkaloid and Caffeine concentrations in each tissue type within the species: *L. argenteus***

|  |  |  |
| --- | --- | --- |
| Tissue type | Average Alkaloid Concentration (ug/g), N | Average Caffeine Concentration (ug/g), N |
| flower | 3116.24 (28) | 2239.26 (27) |
| leaves | 2673.09 (28) | 2263.58 (28) |
| pollen | 1374.61 (27) | 2363.08 (27) |
| stem | 1796.49 (28) | 2196.72 (28) |
| p-value for comparison among tissues | P < 0.0001 | P = 0.002 |

**Table 1: Average alkaloid and Caffeine concentrations in each tissue type within the species: *L. sulphureus***

|  |  |  |
| --- | --- | --- |
| Tissue type | Average Alkaloid Concentration (ug/g), N | Average Caffeine Concentration (ug/g), N |
| flower | 6296.60 (7) | 2199.94 (7) |
| leaves | 5023.38 (7) | 2142.74 (7) |
| pollen | 1293.43 (7) | 2419.55 (7) |
| stem | 2669.47 (7) | 2191.75 (7) |
| p-value for comparison among tissues | P = 0.0004 | P = 0.028 |

## **4. Summarize your findings**

Now that you have visualized and analyzed data related to our question, we can make well-supported statements to address our hypothesis and prediction.

1. Does alkaloid concentration differ significantly among tissues?
   1. If so, what specific differences exist, reference your figure/statistical results.

Yes. The difference among tissues is significant in *L. argenteus* (p < 0.0001). Pollen appears to have lower Alkaloid concentrations (Figure 1)

Yes. The difference among tissues is significant in *L. sulphureus* (p = 0.0004). Pollen appears to have lower Alkaloid concentrations (Figure 1)

1. Does caffeine concentration differ significantly among tissues?
   1. If so, what specific differences exist.

Yes. The difference among tissues is significant in *L. argenteus* (p < 0.0001). Pollen appears to have lower Caffeine concentrations (Figure 2)

Yes. The difference among tissues is significant in *L. sulphureus* (p = 0.028). Pollen appears to have lower Caffeine concentrations (Figure 2)

1. What possible explanations exist for the pattern you observed for alkaloid concentrations among tissue types? In your answer, consider the role of alkaloids in plants as well as how the tissues differ in their interactions with other organisms.

Alkaloids are defensive in tissues preferred by herbivores (flowers and leaves, especially). Plants produce large quantities to protect their essential body parts from damage.

Tissues that need to attract other organisms, i.e., pollen, lack these bad-tasting compounds as to not deter their pollinators

1. What possible explanations exist for the pattern you observed for caffeine concentrations among tissue types? In your answer, consider the role of caffeine in plants as well as how the tissues differ in their interactions with other organisms.

Caffeine may be attractive to pollinators, so plants that produce it in their pollen receive more visits and thus have higher fitness.

## **5. Collaborate and Compare**

Find another group working on the other species. Share and compare your results

1. Are the major findings the same between species? If not, how do they differ?

The overall pattern is the same.

To visualize the answer to this question, let’s make two new column figures formatted to compare alkaloid and caffeine concentrations across tissues and between species.

1. [...instructions deleted…]
2. [...instructions deleted…]
3. What between-species differences do you notice here?

Flower, stem, and leaf alkaloid averages are higher in *L. sulphureus*

1. Generate one testable hypothesis that would account for the difference you noted between species.

H1: *L. sulphureus* evolved in/has adapted to higher levels of herbivory

H2: *L. sulphureus* populations were located in higher-resource environments that allowed for greater production levels of secondary compounds

H#: certainly others are possible

## **6. Post-Activity questions**

As an extension to this activity, consider one or more of the following.

Note: The Instructor may wish to prescribe an activity for all students / groups, and should edit the handout accordingly.

1. Conduct some outside research to help explain your findings related to caffeine levels in pollen. Why is caffeine production higher in the pollen when other alkaloids are lower?

Students are likely to easily come across the news articles below, but you may wish to provide them instead of having students search.

Bees are more likely to remember flowers that have moderately high concentrations of caffeine in the nectar. These levels activate neurons for memory but do not surpass a bitter-taste avoidance threshold. (Wright, et al. 2013). The press release about the research summarizes key points and mentions connections to honey bee conservation (Newcastle, 2013)

A modeling study shows that this fidelity offers a further benefit to the plant (beyond pollination). Plants can produce less nectar and still attract/be favored by the bees. Producing a small amount of caffeine is probably less resource-intensive than producing more nectar. So, it saves energy/resources for the plants. However, this has a negative effect on the pollinators as reduced nectar collection leads to reduced honey production (Couvillon, “Caffeinated”). The news is descriptive and engaging and has links to other articles (Sokol, “Plants”).

A [1999 article about](https://www.sciencedirect.com/science/article/abs/pii/S0031942299001193) caffeine concentrations in nectar and pollen. Jargon alert.

1. Research alkaloids both generally and in the context of plants. Briefly describe the function of alkaloid compounds and how your findings make sense in light of this information.
2. Return to your figures from 5b. and compare alkaloid levels *within* tissues *between* species. Identify one or more tissues for which there is a large disparity between species (i.e., where one species concentration appears significantly greater than the other). Based on your knowledge of secondary compounds, generate a hypothesis to explain this inter-specific difference.

*L. sulphureus* has higher levels in flowers, leaves, and stems (the latter to a lesser degree). Perhaps it is subject to greater levels of herbivory. Perhaps it is subject to colder temperatures and high solute levels reduce freezing risk...

**References**

Couvillon, Margaret J.; Al Toufailia, Hasan; Butterfield, Thomas M.; Schrell, Felix; Ratnieks, Francis L.W.; Schürch, Roger. “Caffeinated Forage Tricks Honeybees into Increasing Foraging and Recruitment Behaviors” *Current Biology* 25, no. 21 (2015):Pages 2815-2818

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Heiling, Jacob M.; Cook, Daniel; Lee, Stephen T.; Irwin, Rebecca E. “Pollen and vegetative secondary chemistry of three pollen‐rewarding lupines.” *American Journal of Botany*, no. 106 (2019): 643-655. <https://doi.org/10.1002/ajb2.1283>

Kretschmar, Josef A.; Baumann, Thomas W. “Caffeine in Citrus flowers.” *Phytochemistry* 52 no. 1 (1999): 19-23. <https://doi.org/10.1016/S0031-9422(99)00119-3>

Newcastle University. "Bees get a buzz from flower nectar containing caffeine." ScienceDaily, March 7, 2013. www.sciencedaily.com/releases/2013/03/130307145257.htm (accessed July 1, 2020).

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Wright, G. A.; Baker, D.D.; Palmer, M. J.; Stabler, D.; Mustard, J.A.; Power,E.F.;Borland, A.M.; Stevenson, P.C. “Caffeine in Floral Nectar Enhances a Pollinator's Memory of Reward.” *Science* 339, no. 6124 (2013):1202-1204. DOI: [10.1126/science.1228806](http://dx.doi.org/10.1126/science.1228806)

**Credits**

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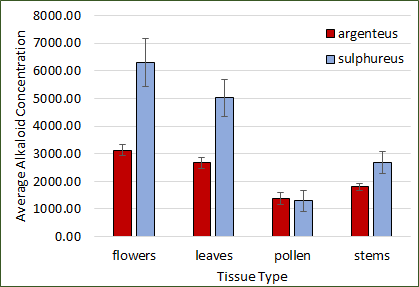
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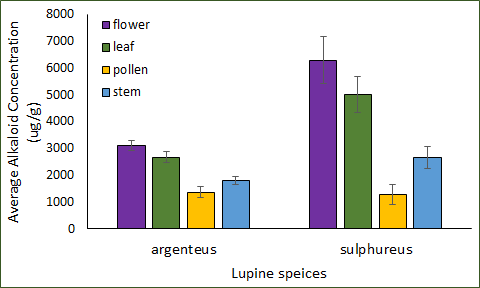
Scientific review by NAME, AFFILIATION

Figure 1: “Mary Vaux Walcott - Blue Lupine (Lupinus argenteus) - 1970.355.99 -” by Smithsonian American Art Museum, used under Public Domain <https://americanart.si.edu/artwork/blue-lupine-lupinus-argenteus-25889>

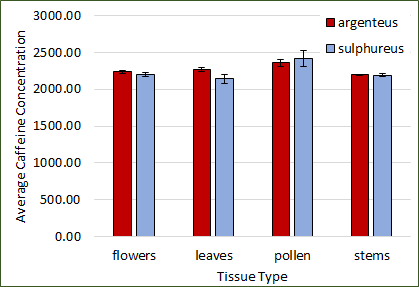
# **Appendix - Excel Figures for references (for educator; See also KEY Excel file)**

**Average of Alkaloids - Species nested within tissue type, Error bars ±1SE**



**Average of Alkaloids - Tissue nested within species, Error bars ±1SE**

**Caffeine - Species nested within tissue type, Error bars ±1SE**



**Caffeine - Tissue nested within species, Error bars ±1SE**

