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Intra- and Interspecific Secondary Metabolite Variation Between Fruit and Leaf Tissues in the Hyperdiverse Psychotria Genus

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INTRA- AND INTERSPECIFIC SECONDARY METABOLITE VARIATION BETWEEN FRUIT AND LEAF TISSUES IN THE HYPERDIVERSE *PSYCHOTRIA* GENUS

by

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Abstract

Secondary metabolites are chemical compounds that are considered to mediate a variety of plant interactions with their environment and are not involved in basic metabolism. Recently, there has been an interest in understanding the function and allocation of these metabolites in fruit tissues. In contrast to leaves, the chemistry in fruit tissue mediates exclusive interactions with seed dispersers that directly affect plant fitness and are under different evolutionary selective pressures. Only a few studies outline the patterns of chemistry between fruit and leaf tissues. This study aims to understand how secondary metabolites in two species of the hyperdiverse congeneric genus (*Psychotria*) differ between fruit and leaf tissues within each species, how plant tissue chemistry differs across species, and what implications this has for ecological interactions, seed dispersal, and the understanding of evolutionary processes. Plant samples from seed, leaf, and pulp tissue were collected from two species of the hyperdiverse *Psychotria* genus, *P. marginata* and *P. limonensis*. Plant samples were collected in a Neotropical forest on Barro Colorado Island in Panama. The secondary metabolites from these plant tissues were extracted using a [99.9: 0.1] ethanol to formic acid solution. The plant extracts will be analyzed using liquid chromatography coupled with mass spectrometry methods. The data will then further be analyzed using novel modeling methods to elucidate and compare the chemical structural diversity of each tissue and species.

Within species, I predict the chemical makeup of leaf tissues is different than that of pulp and seed tissues. This would further support the hypothesis that secondary metabolites in fruit tissues have an adaptive function. Across species, I predict that the differences in secondary metabolite diversity in leaf tissues will be greater than the differences within species. These results would suggest an evolutionary mechanism in which defensive leaf chemistry is selected

upon. This selection of leaf chemistry contributes to the diversification of the genus to fill novel niches and allows for the hyperdiverse genus to coexist within a small area. Though the chemistry between the leaf tissues is predicted to be divergent when compared across species, the question remains if this trend will be exhibited in fruits. I predict that fruit tissue chemistry will be similar between species in order to conserve the important function these metabolites play in mediating interactions with the same seed disperser within the genus. This study will lead to a better understanding of the evolutionary selective processes imposed on different plant tissues and the significance that these metabolites play in the diversification of plant genera.

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Introduction

Plants have always been attacked by herbivores and over time have evolved a variety of unique defense strategies to protect themselves. Different strategies or "syndromes" have been identified in leaf tissue and are defined as a combination of functional traits that contribute together to the plant's overall fitness (Kursar and Coley 2003, Agrawal and Fishbein 2006). Among these functional traits, secondary metabolites have been shown to play one of the largest roles in leaf defense. Secondary metabolites are organic compounds produced by plants that are not directly involved in growth, development, or reproduction. Different classes of metabolites are associated with certain functional roles and are found in all plant tissues. These metabolites increase fitness by mediating negative interactions with herbivores (Agrawal and Weber 2015) and have revolutionized the understanding of plant-herbivore and plant-pathogen interactions with regards to leaf tissue (Scoohoven et al. 2005). Though studies have been conducted considering leaf tissue and the function of metabolites they contain, only recently have fruit secondary metabolites received similar attention.

It has been argued that leaf and fruit chemistry are conserved within a species and the function of fruit metabolites is a consequence of the previously evolved leaf chemistry (Ericksson & Ehrlé 1998); but the main emerging hypothesis is that the function of metabolites in reproductive tissue are adaptively significant (Cipollini and Levey 1997). This hypothesis postulates that there are qualitative and quantitative differences in the secondary metabolites found in the pulp and seeds of fruit, which function in order to maximize seed dispersal and mediate exclusive interactions that fruits have with mutualistic and antagonistic frugivores, and abiotic factors. Known functions of secondary metabolites in fruits are diverse and include regulating gut retention time in seed dispersers, inhibition of seed germination in intact fruit, defense against pathogens and non-dispersing frugivores, and attraction of seed dispersers (Cipollini and Levey 1997). Recent studies provide support for this hypothesis and indicate that fruits within one species included a higher concentration and more individual compounds of secondary metabolites (Whitehead et al. 2013). They also observed that there was a negative relationship between the concentration of these metabolites and fruit damage (Whitehead et al. 2013). Biotic factors have also been shown to regulate certain defensive metabolites in fruit tissues. The observation of an evolutionary trade-off between toxicity and attractiveness a fruit has toward a seed disperser has been shown in recent literature. Tewksbury et al. (2008) found that damage caused by pathogens was positively correlated with toxic metabolites. They concluded that the plant will trade the potential attractiveness toward seed dispersers for the protection of the fruit in response to increased pathogen or predator attack. Along with biotic factors, plants have been shown to regulate fruit metabolites differently than leaf metabolites in the face of abiotic factors. One study limited essential nutrients required by plants and quantified both leaf and fruit chemistry after a period of time. Leaf chemistry was shown to be greatly affected while fruit chemistry remained the same at the cost of fewer and smaller fruits (Cipollini 2004). These studies show the different types of interactions these metabolites may mediate, the regulation of these metabolites in response to biotic and abiotic factors, and the importance of these metabolites that are directly related to plant fitness and unique to fruit tissues.

Few studies have compared the natural variation in fruit and leaf metabolites across closely related species. This line of inquiry can be beneficial in a number of different ways and is important in understanding seed dispersal, evolutionary mechanisms and plant defense structure. It is known that there is intraspecific variation in seed dispersal within a population (Schupp et al. 2019). By quantifying differences in the chemical makeup of fruit tissues we can gain insight into whether intraspecific seed dispersal variation is possibly driven by these differences. Furthermore, understanding the difference in metabolite diversity in plant tissues within and among closely related species allows us to focus on evolutionary factors shaping plant chemistry. This is important because selection of chemical defense mechanisms in fruit tissues may regulate seed dispersal in different ways which will affect diversity and abundance in the community, coexistence, and dynamics between populations (Beckman and Rogers 2013).This knowledge can also help us to understand how plant defense in fruit and leaf tissues are related, how they are selected for, and how plant defense develops, focusing on a whole-plant context.

This study quantifies and compares secondary metabolites across plant tissues within two species of the genus *Psychotria*. *Psychotria* is considered a hyperdiverse "swarm species" (Gentry 1982), which contributes a disproportionately high amount of alpha diversity in tropical communities. These types of genera/clades are unique because they challenge the view of community assembly that greater niche overlap of closely related species should increase competitive exclusion. This view is challenged because there are more than 20 species co-occuring on BCI (Barro Colorado Island, Panama) in the Neotropics. Experiments were carried out to understand how these species differ ecologically to allow them to coexist in this fashion. The first tested to see if *Psychotria* utilized hydraulic traits and responses to light and water in order to fill different niches, but functional trait analysis revealed little or no evidence of niche partitioning within sites (Sedio et al. 2012). Another study focused on secondary metabolites in leaf tissue provided support for the hypothesis that niche segregation in this genus is based on differences in chemical leaf defense against insects and pathogens (Sedio et al. 2017). This study used molecular networks that quantify the chemical similarity of its compounds and showed there was a greater difference of secondary metabolites in leaves between species of *Psychotria* than within *Psychotria* species. This analysis supports the hypothesis that this niche segregation is due to exploiting distinct chemical niches.

These studies leave out the consideration of important fruit metabolites. This study will use similar molecular networking models to assess secondary metabolite structure of pulp, leaf, expanding leaf, and seed tissue in two species of *Psychotria, P. marginata* and *P. limonensis*. The plant samples were collected on Barro Colorado Island which is the same community that the study of leaf defense and its role in niche segregation was investigated. The species metabolic structure will be compared within-species and between-species. Two main questions are asked that can shed light on understanding the role and implications of leaf and fruit metabolites in a community. These questions attempt to support previously established hypotheses as well as provide knowledge into the way fruit metabolites fit into the evolutionary understanding of this genus, and the implications this has for understanding seed dispersal and community dynamics.

The first question is how fruit metabolites (seed and pulp) will differ between leaf and fruit tissues within each species? It is predicted that fruit metabolites will be significantly different from leaf metabolites within each species. This can be explained by the adaptive significance hypothesis. Fruits will have different overall metabolite profiles because these metabolites are adaptively significant and will mediate different interactions than leaves. These interactions will attempt to maximize seed dispersal while also mediating other negative interactions that are imposed on the fruit. A null hypothesis is that these plant parts will not be significantly different in their metabolite profiles, indicating that fruit metabolites are most likely a pleotropic consequence of leaf tissue and have not significantly adapted to mediate specific interactions with seed dispersers. It will also be of interest to understand the degree to which fruit tissues differ from leaf tissue in each species and if there are differences in overlap of their metabolite makeup.

The second question is how metabolites will differ across these two species. It is predicted that metabolites in fruits will be more similar across species and will have less intraspecific variation in each species compared to leaves. Each species of *Psychotria* shares the same seed dispersers (Poulin 1998) and fruit metabolites must mediate interactions with these dispersers that are similar across species. It will also be important to understand how intraspecific metabolite structure varies in the fruits of each species. The degree to which there is intraspecific variation in fruit chemistry may result in variation in dispersal within species, with important consequences for plant populations and communities (Snell et al. 2019). An alternative hypothesis is that fruits metabolite profiles will be significantly different across species. This will be due to the fact that metabolites in fruits also provide an opportunity for niche segregation similar to leaves. The interactions with seed dispersers and non-dispersing antagonist frugivores may be mediated in different ways and plants may face different abiotic and biotic factors, that will drive formation of different chemical structures in fruits. This may also support a hypothesis that leaf and fruit tissues are in some way conserved and as leaves formed distinct chemical niches, fruits do as well. Leaf tissues are suspected to follow the same trend as in Sedio et al. (2017) and will be significantly different across species in order to provide niches for these species to coexist. The intraspecific variation is also predicted to be higher in leaves compared to fruits, which will support the fact that leaf chemistry provides the variation for natural selection to act, resulting in the formation of distinct leaf chemical niches.

Methods

Study Site:

Barro Colorado Island (9 9'N, 79 51'W) is a lowland, moist tropical forest with average annual rainfall of 2,600 mm (Leigh et al.,1996, Paton, 2007) The island follows a 4-month dry season that begins around mid-December and ends in April.

Psychotria:

Psychotria is globally one of the largest plant genera and is comprised almost entirely of shrubs and smaller plants in the understory, with approximately 1650 species distributed throughout the tropics and subtropics (Taylor 1996). BCI contains a high diversity of *Psychotria* species and more than 20 species of *Psychotria* are found on BCI in two well defined sub-genera. Mean density of this genus on BCI is .66 stems m-2 (Sedio et. al 2012) to put the abundance of this genus into perspective. *Psychotria* fruits on BCI are dispersed primarily by two types of birds, specifically three species of manakins and three species of migratory thrushes (Poulin et. al 1999), making the main seed dispersal method the same in this genus. *Psychotria* have slow-expanding leaves and high chemical defenses in leaves; properties of the species that will be important when considering leaf and fruit chemistry (Kursar and Coley 2003). *Psychotria* are also known to contain a diverse amount of bioactive compounds that may play a role as therapeutic agents (Porto et al 2009).

Plant Collection:

Leaf, expanding leaf, pulp and seed tissues were collected across Barro Colorado Island (BCI). I identified plants to species and collected plant tissues from 15 individuals of *P. marginata* and *P. limonensis*. The tissues were collected from plants located in the understory with exclusion of plants that are located in light gaps within the forest, in order to control for the presence of light. The tissues were collected during the wet season from May to August of 2019. Once collected, the tissues were immediately placed on ice and taken back to the lab to be processed for extraction. The samples were placed on ice in order to stop metabolic reactions from continuing and further changing the chemical makeup of the tissues. Leaf tissues were weighed in scintillation vials and placed in a -80 C freezer. Fruit tissues were separated into pulp and seed tissues in a petri dish, weighed in scintillation vials and placed in the -80 C freezer. After dissection leftover juice was rinsed with ethanol and placed into the pulp vial. After a minimum of 24 hours, the samples were placed in a freeze dryer for approximately 3 days to obtain a dry sample. During this time water from the plant tissue and the ethanol used to rinse the juice was evaporated from the plant tissue samples. Dry weights were taken from each of the samples and the samples were ground and placed back into the -80 C freezer to await chemical analysis. In December of 2019 the samples were placed on ice and shipped to USU with all of the appropriate permits required by the United States of America and the Republic of Panama.

Chemical Extraction:

Chemical extraction of plant secondary metabolites was carried out at USU. A solvent of 99.9 % ethanol and 0.1% percent formic acid was used in the extraction process. This solvent is efficient because it is able to extract secondary metabolites that are diverse and range greatly in polarity. 80 mg of dry plant tissue was then placed in an eppendorf tube. 1.4ml of the solvent was added to each eppendorf tube followed by 5 minutes of vortexing and 5 minutes of centrifuging. The supernatant was gathered using a glass syringe and was placed in pre-weighed scintillation vials. The process was repeated a total of 5 times to allow for optimal extraction of the metabolites.

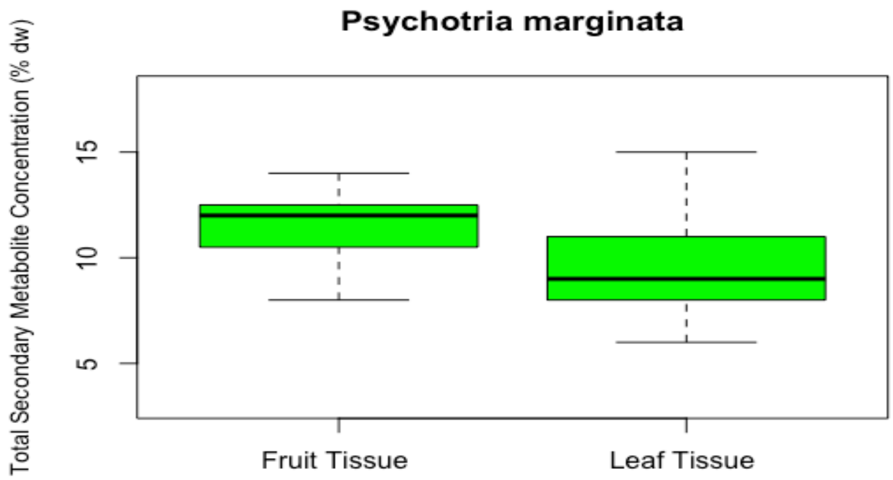
After the last extraction the scintillation and eppendorf tubes were dried using a speed-vac for approximately 4 hours. The dry weights of each extract were subtracted from the original dry weight of the tissue in order to calculate the quantity of total secondary metabolites extracted from each sample. This measurement, the total photochemical concentration, can be used as a means of comparing the samples in order to better understand the questions presented. The extracts will then be sent to USU Proteomics Core Lab in order to undergo liquid chromatography coupled with tandem mass spectrometry methods.

LC/MS-MS and Molecular Networking:

The extracts will be analyzed using high-performance liquid chromatography and tandem mass spectrometry. This study utilizes novel methods that are able to assemble mass spectra into molecular networks and can quantify the structural similarity of all metabolites in a compound. The data received from LC/MS-MS will be compared to the Global Natural Products Social Molecular Networking database of natural products (GNSP; http://gnps.ucsd.edu). This database is a source of LC/MS-MS natural plant products data, and certain spectra have already been associated with specific metabolites (Wang et al. 2016). Examples of the different classes of metabolites that this database contains includes flavonoids, alkaloids, and terpenoids. These classes have associated roles in plant defense and can give some insight into the plant defense mechanisms of this genus (War et al. 2012). Though some compounds may match the database, many will be unknown. The structure of unknown compounds is able to be understood because of the mechanism of tandem mass-spectrometry. Molecules with similar structures or substructures will have similar fragmentation patterns, and these fragmentation patterns can be compared to one another in order to quantify their structural similarities. In this way we can quantify structural similarity for every pair of compounds and create a molecular network. More information on the precise techniques of creating a molecular network can be found in (Wang et al. 2016).

Results

Total Phytochemical Concentration:



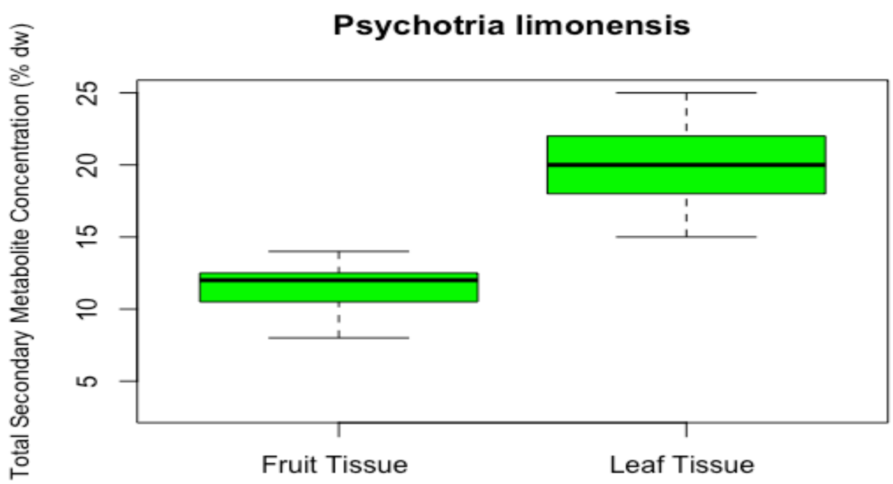


Figure 1: Average secondary metabolite concentrations (percentage of dry weight [dw]of the extract) in leaves and fruit tissues. These box and whisker plots show the median, twenty-fifth and seventy-fifth percentials and range of total secondary metabolites. N=15 in each of the species (P. marginata & P. limonensis).

Figure 1 is a hypothetical example of the data I expect for the percent mass of secondary metabolites between leaf and fruit parts, or in other words the total phytochemical concentration. These graphs display the data of leaf vs. fruit tissues, but each tissue (fruits, seeds, expanding leaves, and leaves) will all be taken into account in the actual results. It is expected that there will be a significant difference in the total secondary metabolite concentrations between plant parts within a species, and that leaves will differ significantly between the species while fruits will not. Both of these expected results are displayed in the graphs above. Statistical analysis will be conducted using Tukey's HSD post hoc comparisons for plant tissues within and across species in order to determine which tissues differ statistically. These measurements and descriptive statistics can give insight into answering the questions presented in this study. This measurement only gives us a surface understanding of metabolites. These measurements will be combined with qualitative measurements using molecular networking techniques in order to give a bigger picture into the makeup of plant secondary metabolites in these two species of *Psychotria.*

Discussion

This experiment will help bring together scientific fields of plant-herbivore studies with those of fruit consumer and seed dispersal studies to better understand plant defense in a whole-plant context. The allocation of different metabolites may help us to begin to understand how leaf defense is related to fruit defense in certain species and if these strategies vary between closely related species and understory plants in general. Though the functions of many secondary metabolites have been confirmed, further studies are needed to quantify the functions of many lesser studied metabolites in order to understand the differences in leaf defense and fruit defense. It is important to note that metabolites may exhibit multifunctionality by taking on multiple roles in the interactions they mediate, in order to provide maximum defense of plant tissues.

From the data we can deduce evolutionary processes that may be different or the same for plant and leaf tissues. We can begin to better understand how fruits are involved in these processes, which will lead to a better understanding of how hyperdiverse species may coexist in a given area. More studies of this type in a genus are necessary to see if these evolutionary processes are apparent for certain genera or if evolutionary processes are different based on the properties of metabolites and the differing interactions they mediate.

This study will also shed light on seed dispersal. The variation between plant species is predicted to be similar but can also be different. This difference would indicate that similar plants have adapted secondary metabolites to mediate certain interactions in different ways that allow for efficient seed dispersal, in the face of different abiotic and biotic factors. Quantification of secondary metabolites of the fruit tissues across species within a genus that utilizes the same seed disperser can also give us important information that leads to further studies understanding if these secondary metabolites play a role in seed dispersal. A question of importance also lies in whether intraspecific variation in seed dispersal is driven by intraspecific variation of secondary metabolites in plants.

Little emphasis in the past has been placed on the examination of fleshy fruits for biologically active compounds that may be of value in agriculture and medicine. Conventionally, pharmaceutical companies have contracted with botanists who make random collections of plants for bioassays (Coley et al. 2003). One study demonstrated that leaf tissues in certain classes of plants contain a better potential for finding more bioactive compounds that can be useful in medicine (Coley et al. 2003). Though studies in potential bioprospecting mainly focus on leaf chemistry; fruit chemistry may be of more importance. Most fleshy fruits are evolutionary designed to be consumable by vertebrates and are a prime location to search for compounds that may be of value to human health. For example, leaves may contain anti-fungal compounds and compounds that deter vertebrate herbivores while fruit tissues will most likely only deter the fungal pathogen while being consumable by vertebrate that disperse their seeds. A better understanding of the interactions metabolites mediate and how these metabolites change across species within genera can be useful in formulating specific ecological guidelines that will be most beneficial in looking for these compounds in plants. More research is needed in understanding what metabolites are in plant tissues and what roles they play in plant defense.

While so much research has focused on that of leaf tissues, I emphasize the importance of understanding fruit tissues and their roles in the community in combination with leaf tissue. This will lead to a better understanding of population dynamics and the understanding of community ecology.

Word Count: 3302

Reflective Writing

During my capstone project I was given the opportunity to travel to Panama and conduct research on BCI (Barro Colorado Island) in association with the Smithsonian Tropical Research Institute. This Island is located on the Panama Canal and is well known for its ecological tropical research and incomparable biodiversity. I would like to take a moment and reflect on some of the things I have learned about science and research, choosing a profession, and culture along with how these things have helped me grow and view science in a different way.

Before this trip, I did not think much about science or research, or how the world of academia worked. I would go to class; I would memorize what the teacher told me to memorize. I would do well in class and move on to the next, retaining some of the information I was learning and not thinking too much about the material in a critical manner. Never asking meaningful questions, never thinking about where this information came from. Just memorizing and hoping I would retain some of the information I was being taught. In Panama, I came to the realization that all of these things I have studied in science have been the result of people asking questions about how life and the world functions. Asking the important question of why? All of the things I have read in textbooks, someone has discovered by asking questions and putting their curiosity to the test. Most of these questions were asked by people that were once undergraduates thinking about going into academia like myself. I remember at the beginning of my stay in Panama, I had a talk with a staff scientist who worked at STRI and was known for his revolutionary research on insects. He described to me how when he was an undergraduate student someone gave him a chance to ask questions and experiment with something he thought was interesting. This led him to discovery and a lifetime of research in something he truly enjoyed. I feel that the honors program was able to give me a similar opportunity. His story and my experience in Panama have encouraged me to find out what I am motivated to do, and how I can contribute to this growing knowledge of the world in some small way. It has also given me a perspective that academia and research is something I can possibly go into in the future. All of these accredited staff scientists were once undergraduates not too different from myself, and this fact has given me a different, more realistic view of a profession in academia and what is possible in my future career. This newfound way of thinking about science has furthermore led me to look at biology in a broader sense. In my mind all of these subjects were separate and I never understood or thought about their relationship to each other. Physics, Biology, Math, and Chemistry all paint a different picture of life and fit with each other as if each subject and the research conducted therein is part of a puzzle. Each level of organization works together to create an understanding of the world in which we live. I have always known these things and have been taught them in class, but actually experiencing them is something that brought all these concepts together and makes them more than just a teacher lecturing in front of a class of 300. This makes my view of life beautiful and gives me a sense of purpose when I sit down to study or wonder about science. These ways of thinking about science, research, and the processes to start researching have been a direct result from my experience in Panama.

Along with these ideas about science, my stay in Panama gave me a greater understanding of how to go about choosing a profession, whether that be in academia or medicine. One thing that I have noticed with PhD students on BCI, who have basically dedicated their lives to the study of their choice, is that most of them truly love what they are researching. When you talk with them you can see it in their eyes and the way they talk about what they are trying to discover. Whether they are explaining how lightning strikes influence forest populations, or about evacuation processes of ants from their nests and the factors that play a role in this. Seeing this motivation and inspiration is something I desire in my work. It has taught me that in order to be happy and motivated I need to truly love what I do. I believe that this has helped me to consider a career in medicine. It has inspired me to have experiences and activities that revolve around this profession and has helped me understand that this is something I will truly enjoy doing in the future.

The final and most important thing I have taken away from my experience in Panama has been in a social and cultural sense. I have learned much about culture in the science community, and the important role of diversity in research and life in general. I have met people from all over the world and from all aspects of life. On this project I personally worked with two individuals from India and Colombia. Though we all came from completely different backgrounds, upbringings, languages, and societies, we all had something in common. The project and research we were working on. We could all discuss a scientific question and use our different backgrounds to help with ideas that benefited the project. I have come to understand that having these different backgrounds and different ways of thinking is beneficial to the success of research and trying to solve any problem. It was fascinating to see science as something that can unite and bring people together.

My advice to future students would be to get involved and learn from what you are doing and those around you. Getting involved was where the start of this project began. Once involved in something, one activity leads to another until you develop relationships and are able to have experiences you can learn from in an integrative manner. Another piece of advice to future students would be to keep in mind that not everything goes as planned in the scientific world. When I arrived in Panama, I realized my initial project was not going to be possible due to an unusually prolonged dry season. I was able to get advice and adapt to the situation in order to develop a project that would work for my new circumstance. It is important to understand that putting aside stress and fear, and the ability to adapt with confidence when something does not go as planned, are important skills to have for a future professional career.

These are a few of my thoughts and what I have taken away as I reflect on my capstone experience. I am glad I was given this incredible opportunity to come to Panama. At first, I did not know what to expect, I thought it would just be another class/project. It turned out to be an amazing experience that has taught me a lot about life and biology. One that I will always remember.

Word Count: 1211

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Professional Author Bio

Cole Carlson was raised in Redlands California and attended BYU for a year after High School. After attending BYU he served an LDS mission in Sweden, and upon returning made the decision to transfer to USU. Here his desire for the study of biology and medicine has grown and he has been pursuing these interests ever since. He will be graduating in Human Biology, with a minor in Psychology and Chemistry with a 3.86 GPA. During his time at USU he was able to participate in the Honors Program, Community Engaged Scholars program, and has worked as an Undergraduate Teaching Fellow and TA in Ecology and Statistics courses. As a sophomore Cole began working in the Beckman lab, where research primarily focuses on ecology principles revolving around plants and seed dispersal. He was able to integrate these concepts and apply them to the study of natural medicinal compounds derived from plants. He has presented at three Biology Symposiums during his time at USU and has received two Biology Scholarships, an URCO grant, and honors research funding that has helped him fund his research and capstone experience. Along with his involvement in research he has also received an Honors Service Scholarship that has allowed him to devote more time to learning about and helping the community of Cache Valley. This scholarship has helped to support him financially and allowed him to focus more on participation in the Community Engaged Scholars Program. After graduating Cole plans to continue his research in the Beckman lab part-time while he applies to medical school and will work in a medical setting throughout the upcoming year.