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Extrafloral nectary composition within and across selected *Passiflora* species: Do patterns support the hypothesis that differential herbivore tactics promote alternative EFN traits?

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Extrafloral nectary composition within and across selected *Passiflora* species: Do patterns support the hypothesis that differential herbivore tactics promote alternative EFN traits?

by

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Dedication

This thesis is dedicated to my family who have always supported and encouraged me to follow my heart.

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I would like to acknowledge my advisor, Larry, for allowing me to join his lab and for all the guidance he showed during my graduate career. He was always generous with sharing his knowledge of the system and encouraged pursuing independent questions about the system. I would like to thank many for their advice and guidance along the way, especially Mona, Beryl, Ulrich, and Randy who helped guide this project through the years.

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Abstract

Extrafloral nectary composition within and across selected *Passiflora* species: Do patterns support the hypothesis that differential herbivore tactics promote alternative EFN traits?

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The University of Texas at Austin, 2020

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This study determined extrafloral nectar (EFN) amino acid composition of 31 species of the salicoid genus *Passiflora* known for diverse EFN morphology to characterize quantitative and qualitative variation in EFN amino acid profiles within and between species. The work was motivated by the question of whether such diversity is driven by distinct tactics used by specialists herbivores i.e. whether EFN traits attract mutualistic defenders appropriate to tactics used by specialists herbivores. The coevolutionary relationship between *Passiflora* and *Heliconius* butterflies presents an ideal system to understand how herbivore pressure can drive indirect defenses such as EFNs. EFN nectar composition might differentially favor mutualistic defenders ranging from tiny egg parasitoids to large ants and wasps. While most *Passiflora* species analyzed are host to several heliconiine species, our study focused on the oviposition behavior of *Heliconius* that impacts vulnerable new shoots. Past observations indicate that *Heliconius*

species that deposit single eggs on host new shoots experience high mortality from egg-parasitoids. By contrast, *Heliconius* that lay groups of eggs on new shoots, are thought to satiate local egg parasitoids. Thus, *Passiflora* species vulnerable to group attack may rely on attracting predators like social insects with potential of a functional response to richer resources. This study sought evidence that signals EFN traits reflect these extreme modes of *Heliconius* oviposition tactics. Though *Passiflora* subgenus classifications were a significant contributor to EFN amino acid composition (r^2=0.299) pointing to a taxonomic origin of specific amino acid compositions, species classifications were a larger contributor (r^2=0.526), highlighting that composition could be driven by species specific herbivore pressure. The final part of the study examined known ant defenders that patrol two species, *P. auriculata* and *P. vitifolia,* hosts to heliconiine/*Heliconius* species with mass egg laying strategies. Neither *Passiflora* species showed significant difference in EFN amino acid composition between field populations and were used to model synthetic nectars for lab-testing preferences of the ants *Crematogaster laeviscula* and *Pseudomyrmex gracilis*. Synthetic nectar trials revealed ant preferences reflective of relationships witnessed in the field and preference for higher diversity of EFN amino acid composition.

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INTRODUCTION

Extrafloral nectaries (EFNs) are nectar-bearing glands not associated with pollination. They have been described on almost all above ground plant tissue including leaves, petioles, stipules, bud bracts, stems, cotyledons, and fruit (Elias 1983). Structurally, they can be single-celled nectar secreting hairs, complex raised cups, bowl-like depressions; and range from being vascularized to completely lacking vascularization (Elias 1983). Classification of structures as either EFNs or other porous features is dependent on composition of excretion and related vascular tissues (Heil 2015) along with ecological function as nectaries not associated with pollination (Weber and Keeler 2013) and as an indirect plant defense (Bentley 1977) by attracting mutualistic defenders, specifically ant species, that deter oviposition by butterflies or actively remove eggs, caterpillars, and other herbivores. Delphino,1886, first formulated the hypothesis that EFNs function as a defense mechanism for the plants they are found on (Mancuso 2010). Today, EFNs are described on 3,941 species in 108 families (Weber and Keeler 2013). Within these clades, a two-fold increase in diversification rates in families containing instances of species with EFNs compared to families without suggests that EFNs may facilitate species radiations due to insect-plant mutualisms (Weber and Agrawal 2014).

Several studies have illustrated the protective function of EFNs with increased plant fitness through an increase in long-term vegetative growth (Heil 2001) and reduction of damage to foliage (Janzen 1966, Bently 1976, Koptur 1979, de la Fuente & Maguis 1999, del-Claro et al. 1996, Ness et al. 2006), and seed set (Leal et al 2006) in

various plant lineages. However, the protective benefits in facultative ant-plant relationships will vary depending on associated ant species (Freitas et al., 2000, Djiéto-Lordon, et al. 2004, Rudgers and Gardener 2004), ant community composition (Melati 2018, Xu 2010, Blüthgen et al. 2004), and interactions with non-ant defenders, like spiders (Nahas 2012) or EFN nectar thieves (Heil 2004). While morphological structures of EFNs influence associated defender species and strength of defense due to nectary shape and size (Baker-Méio 2012), nectar volume and concentration (Alvas-Silva and Del-Claro 2013); the components (sugars, amino acids, lipids, and additional organic molecules), and the ratios of these components to each other (Lanza 1993, Koptur 1994, Blüthgen et al. 2004, González-Teuber and Heil 2009) should also influence the type of mutualistic defender attracted to the nectary, as well as the duration or strength of protection provided by the defender. Maintenance of EFN nectar chemical composition in plant lineages where coevolved host plant-herbivore relationships occur could be an indicator of selection for specific mutualistic defenders.

Passifloraceae, and genus *Passiflora,* is a model clade to study the relationship between EFN nectar chemical composition and herbivore pressure due to the morphological diversity of EFNs within the genus (number, shape, size, color, and position) and diversity of its coevolved butterfly herbivore, *Heliconius* whose species utilize different oviposition strategies that will vary in the herbivore pressure in each species specific hostplant-herbivore relationship (Benson et al. 1975, De Castro et al. 2018) (**Figure 1.1**). Previous work has shown variation in visitor type and frequency on *Passiflora* species exhibiting dissimilar EFNs. Apple and Feener (2001) surveyed ant abundance on *P. oerstedii* , *P. biflora* and *P. auriculata* in paired association on successional strips at La Selva Biological Station in Costa Rica. Termite bait found by patrolling ants was infrequently recovered on *P. oerstedii*, which possess small numerous

paired petiole nectaries (**Figure 1.1C)** and had less observed visitors overall. *P. auriculata* which possess petiole nectaries experienced the highest visitation rates and bait removal (**Figure 1.1J-L).** Smiley (1978) characterized ant attraction for *P. auriculata, P. biflora,* and *P. oerstedii* as high, medium, and low based on ant visitor frequency observations also performed at La Selva. EFN chemical composition is largely composed of simple sugars (sucrose, glucose, and fructose) and the ratio of these sugars to one another can result in differential attraction from mutualistic defenders (González-Teuber and Heil 2009), however, concentration and composition of amino acids in extrafloral nectar are potentially more important in the function of attraction to specific defenders (Blüthgen 2004, González-Teuber and Heil 2009, Escalante-Pérez 2012b). Studies within *Passiflora* have focused on interspecific differences in nectary morphology, sugar concentration and ratio, and total amino acid content (Durkee 1982, Cardoso-Gustavson 2013), leaving EFN amino acid composition variation largely undetermined in this genus.

In this study, we examined variation in EFN amino acid composition in *Passiflora* species, related this variation to *Heliconius* oviposition strategies and resulting herbivore pressure, and related how herbivore pressure may be a selective pressure on EFN amino acid composition leading to the attraction of specific mutualistic defenders. We measured the intra- and interspecific variation related to EFN amino acid composition within the genus *Passiflora* to understand if variation occurs at the levels of individual, population, or species within this genus. This variation can then be looked at in context of variation in ecological pressure to improve our understanding of how extrafloral trait variation is associated with mutualistic defenders and a response to herbivore oviposition strategy. Preference experiments with known mutualistic defender ant species with artificial nectar

resembling compositions found in *Passiflora* species of interest were carried out to further test drivers behind EFN amino acid composition.

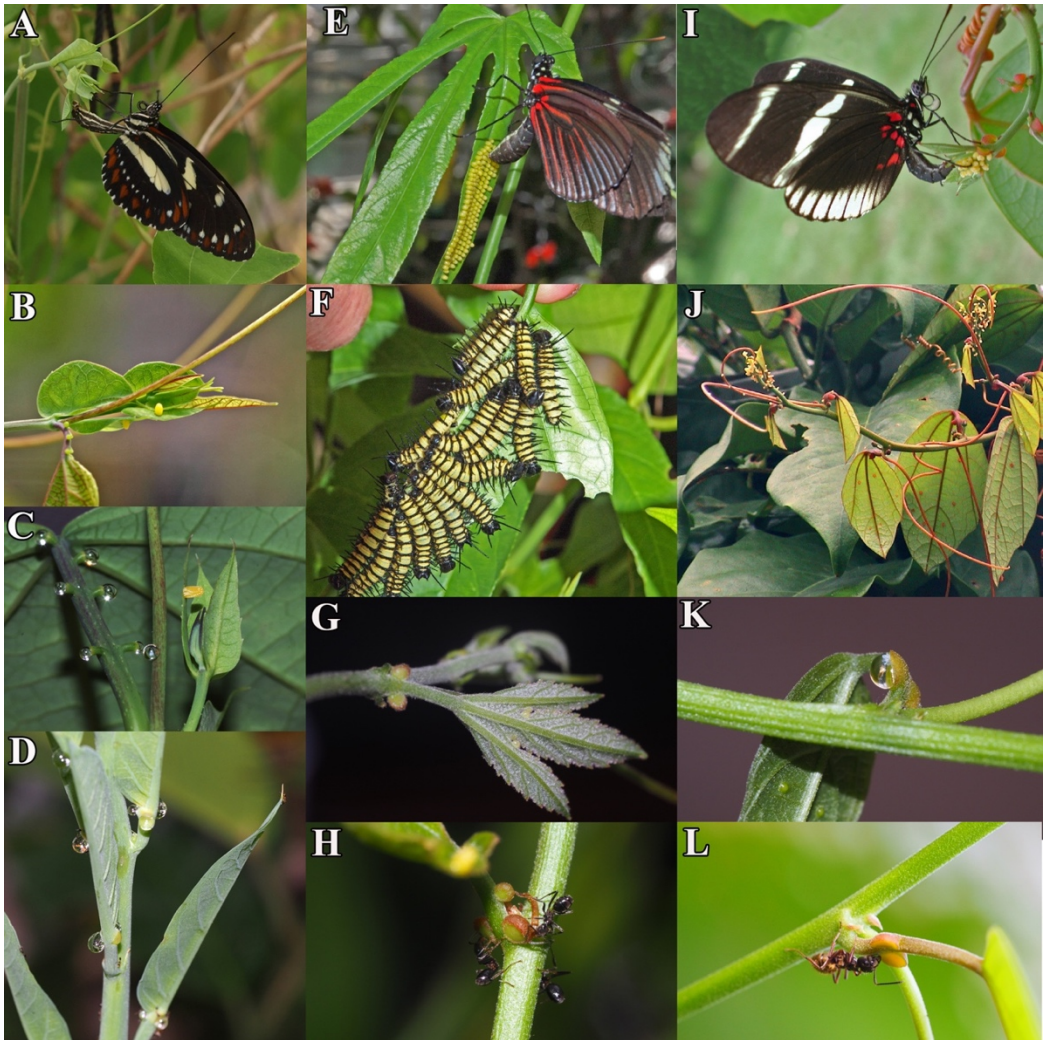

Figure 1.1: Solitary egg laying *Heliconius atthis* (**A**) on *Passiflora oerstedii* (**B**) with multiple minute extrafloral nectaries (**C**) compared to *H. doris* (**E**) which lays rafts of eggs resulting in masses of caterpillars (**F**) or *H. sara* (**I**) laying large clusters of eggs on the shoot meristems of *P. auriculata*. Numerous extrafloral nectaries of new shoot growth in *P. pittieri* **(D)** and the large cup like petiole nectaries of *P. vitifolia* and *P. auriculata* deliver large quantities of nectar and attract numerous visitors (**H, L**). (photo credit: L. Gilbert A-C, E-F, I-J; E. Rees D, G-H, K-L).

PASSIFLORA BACKGROUND

Passiflora is a genus of about 550 species within the family Passifloraceae, order Malpighiales. The genus is usually recognizable by its often showy flowers that attract a variety of pollinators depending on the species and geographic location. The sterile plant material can vary from woody lianas to herbaceous smaller shrubs. Killip laid the foundation for *Passiflora* systematics in the early 20th century, originally dividing the species into 22 subgenera based on floral morphology (Killip 1938). Most recent taxonomic revisions agree on five subgenera based on morphological traits (Feuillet and MacDougal 2003, Krosnick et. al. 2009) four of which are monophyletic: *P.* subg. *Passiflora, P.* subg. *Astrophea* (DC.) Mast., *P.* subg. *Decaloba* (DC.) Rchb. (Feuillet and MacDougal 2003) and *P*. subg. *Tetrapathea* (DC.) P. S. Green. *P*. subg. *Deidamioides* (Harms) Killip has been characterized as polyphyletic in a phylogenetic analysis utilizing two nuclear and two plastid regions (Krosnick et al. 2013) with section *Tryphostemmatoides* sister to *P.* subg. *Astrophea* and representative of the most basal branch of *Passiflora.* Species within the *Deidamioides*' other sections (*Tetrastylis, Polyanthea,* and *Deidamioides*) form a clade sister to *P.* subg. *Passiflora*. Subgenus *Astrophea* includes ca. 60 species (Krosnick 2013) and is currently organized into two supersections (Ulmer & MacDougal 2004); however pollen analysis (Mezzonato-Pires et al 2015) does not align with current taxonomic classiflcations for this subgenus based on other morphological characteristics. Subgenus *Decaloba* contains ca. 230 species divided into 8 supersections (Feuillet & MacDougal 2003); of these 8, supersection *Auriculata* and supersection *Multiflora* were resolved as paraphyletic to each other by Krosnick et al. 2013. Subgenus *Passiflora* contains ca. 250 species (Krosnick 2013) divided into 6 supersections (Feuillet & MacDougal 2003). Within genus *Passiflora,* extrafloral nectaries are widely used in identification and classification of species as well as groups

due to location, number, and shape of nectaries. Petiole nectaries are common within the genus, are usually paired (**Figure 1.1C,G,K**), but species can be unique in numbers of pairs as well as petiole nectary structure (ie. flat and scar-like, raised large cups, raised small stalks) and placement along the stalk. For example, species in the subgenus *Astrophea* consists of ca. 60 species (Krosnick 2013) of woody lianas and trees with two flat extrafloral nectary glands on the apex of the petiole (**Figure 1.1D**) or the base of the leaf blade(Ulmer & MacDougal 2004). Laminar nectaries can also aid in identification between groups within subg. *Decaloba* whether they are confined within the 3 major leaf veins (*P. biflora*) or scattered on the leaf surface (*P. auriculata*) (Ulmer & MacDougal 2004). Similar to utilizing the morphology of EFNs to identify species, the lack of petiole nectaries or the complete absence of EFNs defines species within supersection *Decaloba (*subg. *Decaloba*) (Ulmer & MacDougal 2004). While these examples could lead one to assume that EFNs within *Passiflora,* follow a clear cut pattern across the phylogeny, there are more outliers than norms. *P. vitifolia* and *P. oerstedii* are both in subgenus *Passiflora,* however, petiole nectaries of *P. vitifolia* (**Figure 1.1G**) more closely resemble the petiole nectaries of *P. auriculata* (**Figure 1.1K**) in subgenus *Decaloba.* If herbivore pressure and selection for mutualistic defenders and not shared ancestry is driving variation in EFN morphology then similar patterns of variation will be observed in EFN chemical composition.

METHODS

Study area-greenhouse and field locations

This study occurred at the University of Texas at Austin, utilizing greenhouse facilities and Passifloraceae species established and maintained by the lab of Dr. Lawrence Gilbert. Sampling occurred under greenhouse and field conditions.

Greenhouses on the roof of the Patterson building on UT campus and at Brackenridge Field Laboratory maintain *Passiflora* and *Heliconius* populations under controlled environmental conditions. These greenhouses have been maintained since 1971 and contain over 300 individual plants representing 100 *Passiflora* species originating from neotropical locations. We also conducted field sampling in Texas and Costa Rica. Field sampling for Costa Rica occurred during the summer of 2016 and 2017. Texas field sampling occurred from spring to fall in 2017 and 2018 with locations established through the Texas Ecolab program. Sampling location and specimen's original collection location are listed in **Table 1.1**.

Species

In this study, 31 species from four subgenera (*P.* subg. *Deidamioides, P.* subg. *Astrophea, P.* subg. *Passiflora, P.* subg. *Decaloba)* of the 5 commonly recognized subgenera *Passiflora* were analyzed for amino acid composition in extrafloral nectar samples (**Table 1.1**). *Passiflora* subg. *Tetrapathea* is not represented in this study, this subgenus that includes only 3 species found only in northeast Australia, Papua New Guinea, and New Zealand and was only recently included within the genus *Passiflora* (Krosnick 2009). Within the genus *Passiflora,* finer scale classification results in 17 recognized supersections, though only a few have been are supported as monophyletic through genomic analysis (Krosnick 2013). Our data set includes representatives from 11 of these commonly recognized supersections within four of the subgenera of *Passiflora*. Three species (**Table 1.1**) from supersection *Astrophea* are represented in this study with repeat sampling of *Passiflora pittieri* under greenhouse and field conditions. Paraphyletic subgenus *Deidamioides* is represented by two species in this study, *P. contracta* from the

section Tetrastylis within *Deidamioides*, resolved as sister to subg. *Passiflora* and *P. arbaelezii* from section Tryphostemmatoides, resolved as sister to *Astrophea* (Krosnick 2013). Subgenera *Decaloba* and *Passiflora* (**Table 1.1**) have the most representatives in this study but this may not fully capture potential EFN amino acid variation in these subgenera due to the size and further division of clades within these groups. Within *Decaloba,* species from monophyletic supersections *Cieca, Decaloba, and Pterosperma* are represented in this study; supersection *Auriuclata* is well represented with 4/10 species sampled. Within subgenus *Passiflora,* 4 of the 6 supersections are represented in this study but still only a small number of the total species (18/250) of this subgenus are represented in this study. Species from genus *Dilkea* and genus *Adenia*, both from family Passifloraceae, were sampled as an outgroup comparison. Both of these genera are small in size compared to *Passiflora* but contained species with EFNs and experience similar herbivore pressures from butterflies with the Heliconiini tribe.

Table 1.1: Species sampled (n (acc./sp.)=individual plant for species), n(sample/sp.)=nectar samples per species) for EFN and floral amino acid analysis with type of nectary noted (parentheses note number of samples for each nectary type if multiple nectary type samples occur for that listing) , plant location (GH=greenhouse, FIELD=field collection) when nectar was sampled, and original collection location for each accession number (if sample was field collected, then location of that collection is listed). Taxonomic classifications are based on currently recognized organization based on morphological and genomic information (Feuillet and MacDougal 2003, Krosnick 2009, Krosnick 2013).

Genus	Subgenera	Supersection	Section	Series	species (33)	n (acc./sp.)	n (sample/sp.)	Sample type				EFN sample Collection Location	Accession Collection Location
								laminar	petiole	other EFN	floral		
Adenia	*NA*	NA	NA	NA	*mannii*	1	1		X			GH	7008 – Kumba, Cameroon
Dilkea	*NA*	NA	NA	NA	*Dilkea sp*	1	3	X (leaf tip)				GH	9246 – Ecuador
Passiflora	*Astrophea*	*Astrophea*	*Astrophea*	NA	*sphaerocarpa*	1	1		X			GH	9263 – Colombia
Passiflora	*Astrophea*	*Astrophea*	*Capreolata*	NA	*citrifolia*	1	1		X			GH	9282 – unknown original location
Passiflora	*Astrophea*	*Astrophea*	*Capreolata*	NA	*pittieri*	2	4		X			GH	925 – Corcovado, Costa Rica
							1		X				8051 – Corcovado, Costa Rica
						1	2		X			FIELD	Osa Penisula, Costa Rica
Passiflora	*Deidamioide*	NA	*Tetrastylis*	NA	*contracta*	1	1		X			GH	8071 – Brazil
Passiflora	*Deidamioide*	NA	*Tryphostemmatoides*	NA	*arbaelezii*	1	1		X			GH	8027 – Puerto Viejo, Costa Rica
Passiflora	*Passiflora*	*Coccinea*	NA	NA	*quadriglandulosa*	1	1		X			GH	9483 – Colombia
Passiflora	*Passiflora*	*Coccinea*	NA	NA	*vitifolia*	1	2		X	X (floral bract)		GH	9041 – Costa Rica
						19	6	X (1)	X (5)			FIELD	Gandoca, Costa Rica
							13	X (1)	X (12)				Osa Penisula, Costa Rica
Passiflora	*Passiflora*	*Laurifolia*	NA	*Laurifoliae*	*ambigua*	1	1		X			GH	9429 – unknown origin
						1	1		X			FIELD	La Palma, Costa Rica
Passiflora	*Passiflora*	*Laurifolia*	NA	*Laurifoliae*	*laurifolia*	1	1		X			GH	9109 – French Guiana
Passiflora	*Passiflora*	*Laurifolia*	NA	*Laurifoliae*	*nitida*	1	1		X			GH	8060 – Manaus, Brazil
Passiflora	*Passiflora*	*Laurifolia*	NA	*Quadrangulares*	*alata*	1	1		X			GH	9466 – unknown original location
Passiflora	*Passiflora*	*Laurifolia*	NA	*Quadrangulares*	*quadrangularis*	1	1		X			GH	9056 - Brazil
						1	1		X			FIELD	Gandoca, Costa Rica
Passiflora	*Passiflora*	*Laurifolia*	NA	*Tiliaefolia*	*seemannii*	1	1		X			GH	9455 – French Guiana
						2	3		X			FIELD	Gandoca, Costa Rica
Passiflora	*Passiflora*	*Passiflora*	NA	*Passiflora*	*cincinnata*	1	1		X			GH	8029 – Argentina
Passiflora	*Passiflora*	*Passiflora*	NA	*Passiflora*	*edulis*	1	1		X			GH	8035 – Huila, Columbia
Passiflora	*Passiflora*	*Passiflora*	NA	*Passiflora*	*serratifolia*	1	1		X			GH	8058 – Belize
Passiflora	*Passiflora*	*Stipulata*	*Granadillastrum*	NA	*actinia*	1	1		X			GH	9465 – Brazil
Passiflora	*Passiflora*	*Stipulata*	*Granadillastrum*	NA	*eichleriana*	1	1		X			GH	9190 – South America
Passiflora	*Passiflora*	*Stipulata*	*Granadillastrum*	NA	*garckei*	1	1		X			GH	9080 – French Guiana
Passiflora	*Passiflora*	*Stipulata*	*Granadillastrum*	NA	*menispermifolia*	2	1		X			GH	8039 – Corcovado, Costa Rica
							1		X				9045 – Corcovado, Costa Rica
						4	6		X			FIELD	Gandoca, Costa Rica
							1		X				Osa Penisula, Costa Rica
Passiflora	*Passiflora*	*Stipulata*	*Granadillastrum*	NA	*mucronata*	1	1		X			GH	9253 – Brazil
Passiflora	*Passiflora*	*Stipulata*	*Granadillastrum*	NA	*oerstedii*	1	8		X			GH	7005 – Costa Rica
Passiflora	*Passiflora*	*Stipulata*	*Granadillastrum*	NA	*sprucei*	2	2		X		X	GH	9410 – Ecuador
							1		X				9487 – Ecuador

Table 1.1 continued: Species sampled (n (acc./sp.)=individual plant for species), n(sample/sp.)=nectar samples per species) for EFN and floral amino acid analysis with type of nectary noted (parentheses note number of samples for each nectary type if multiple nectary type samples occur for that listing) , plant location (GH=greenhouse, FIELD=field collection) when nectar was sampled, and original collection location for each accession number (if sample was field collected, then location of that collection is listed). Taxonomic classifications are based on currently recognized organization based on morphological and genomic information (Feuillet and MacDougal 2003, Krosnick 2009, Krosnick 2013).

						n		Sample type				EFN sample	Accession
Genus	**Subgenera**	**Supersection**	**Section**	**Series**	**species (33)**	**(acc./sp.)**	**n (sample/sp.)**	**laminar**	**petiole**	**other EFN**	**floral**	**Collection Location**	**Collection Location**
Passiflora	*Decaloba*	*Pterosperma*	*Pterosperma*	NA	*microstipula*	1	1		X			GH	9271 – Vera Cruz, Mexico
Passiflora	*Decaloba*	*Auriculata*	NA	NA	*auriculata*	4	12	X (2)	X (9)		X (1)	GH	8028 – Corcovado, Costa Rica
							1		X				9208 – Gamboa, Panama
							4		X (3)		X (1)		9406 – French Guiana
							2		X				9407 – Surinam
						22	14	X (5)	X (9)			FIELD	Osa Penisula, Costa Rica
							17	X (2)	X (15)				Gandoca, Costa Rica
							1		X				San Jose, Costa Rica
Passiflora	*Decaloba*	*Auriculata*	NA	NA	*jatunsachensis*	1	8	X (1)	X (4)		X (3)	GH	9402 – Ecuador
Passiflora	*Decaloba*	*Auriculata*	NA	NA	*rufa*	1	7	X (1)	X (4)		X (2)	GH	9086 – French Guiana
Passiflora	*Decaloba*	*Cieca*	NA	NA	*suberosa*	1	1		X			GH	9494 – Harlingen, TX
Passiflora	*Decaloba*	*Cieca*	NA	NA	*tenuiloba*	4	2		X			GH	3100 – Texas
							1		X				3101 – Hays county, Texas
							1		X				3102 – Hays county, Texas
							1		X				3013 – Comal county, Texas
						2	2		X			FIELD	3126 – Real county, Texas
							1		X				Comal county, Texas
Passiflora	*Decaloba*	*Decaloba*	*Decaloba*	NA	*affinis*	9	1	X				GH	3080 – grown from seed of 9493
							1	X					3081 – grown from seed of 9493
							2	X					3082 – grown from seed of 9493
							5	X					3083 – grown from seed of 3091
							1	X					3084 – Dripping Springs, Texas
							3	X					3085 – Bexar county, Texas
							1	X					No acc. – Bexar county, Texas
							3	X					3086 – Dripping Springs, Texas
							1	X					3091 – Dripping Springs, Texas
						4	3	X (2)			X	FIELD	9493 – Austin, Texas
							1	X					3085 – Bexar county, Texas
							1	X					3086 – Dripping Springs, Texas
							2	X					Dripping Springs, Texas
							1	X					Bexar county, Texas
							3	X					Travis county, Texas
Passiflora	*Decaloba*	*Decaloba*	*Decaloba*	NA	*misera*	1	1	X				GH	9335 – Matto Grosso de Sul, Brazil
TOTAL						106	181						

Sample collection

Extrafloral nectar samples were collected via a Hamilton syringe or microcapillary tube and stored in -4ºC. Samples were collected from extrafloral nectaries free of debris and fungal growth and from the youngest tissue possible. Depending on the *Passiflora* species sampled, nectary type, and nectary size; samples are composed of nectar from a single nectary to several nectaries on the same leaf surface or adjacent petioles sampled together. Samples were collected by actively collecting nectar through a Hamilton syringe or passively collecting nectar by placing a microcapillary tube on the nectar. Method discrepancy is due to viscosity differences between species due to nectar morphology and environmental differences related to time of day and season nectar was collected but should not affect analysis of amino acid composition. Sample amounts range from 0.5uL to 10uL. Greenhouse samples were collected between 10-17h with no additional plant specimen prep or shoot isolation to acquire adequate nectar sample volume due to high humidity and low insect populations in greenhouses. To collect adequate sample volume for field samples, plant shoots were bagged for 4 hours or more (up to 12 hr overnight) isolating the nectary from visitors and reducing evaporation. Method discrepancy will influence total amino acid concentration but not amino acid composition, for this reason comparisons between field and greenhouse samples only evaluate amino acid composition (% of total) and not concentration (nmol/uL). Only one type of nectary was ever collected for an individual sample to allow analysis of variation between nectary types for an individual. Samples were expelled from collection instrument directly into 0.6 mL Eppendorf tubes at stored in -4ºC until analysis of free amino acids via HPLC was carried through a contract with Texas A&M University Protein Chemistry Laboratory.

Sample HPLC analysis

Samples were spun and dried via speedvac in original collection tube before shipment to Texas A&M to reduce movement of sample to sides of tubes during transit. Samples were packed in test-tube freezer boxes and shipped via 2day FedEx.

Texas A&M amino acid analysis protocol is an updated adaptation of the Hewlett Packard AminoQuant method. The system consists of a Agilent 1260 liquid chromatograph with a variable wavelength UV detector for low-moderate sensitivity (Agilent G1365D) analyses and an in-line Agilent G1321B fluorescence detector for high-sensitivity analyses (fluorescent detection). All system control and data analysis is performed by Agilent Chemstation software. Free amino acids are derivatized pre-column with *o*-phthalaldehyde (OPA) and 9-fluoromethyl-chloroformate (FMOC) prior to separation and quantitation by reverse phase HPLC (Texas A&M "Amino Acid Assay Description") .

Samples analyzed in 2016 are true duplicates due to sample division into two aliquots before analysis. The amino acid glutamine varied between duplicates with this method due to the pH of the samples, 0.4 N Borate Buffer is added to samples before injection to bring the pH to 10 for optimum derivatization but the addition of the buffer didn't fix this issue. All subsequent analysis batches, 2017-2019, had the internal standard and buffer added directly to original vial and were then divided into two injections to avoid issues presented in the 2016 sample batch. Internal standards are added to all samples to control errors due to sample loss, injection variations and variability in preparing dilutions.

Data Subsets

Raw data from each batch of samples analyzed by Texas A&M was individually reviewed for large discrepancy between replicates leading to additional replicate sampling by Texas A&M to determine true outlier leading to removal of that replicate. These datasets were then combined to the complete set of samples analyzed. No full samples were removed from analysis since determining if discrepancies between samples of the same *Passiflora* species is due to error or truly captures nectary variation was impossible due to small sample size for any one species. Subsets of data are examined to determine variation on an individual level, population level, and species level; sample size of these subsets will determine statistical tests applied.

Interspecific variation across genus

Total concentration of all amino acids is briefly compared from nectar samples collected under greenhouse conditions. Concentration of nectar is known to fluctuate more than composition of nectar (Gardener and Gillman 2001) and can be greatly influenced by environmental conditions like temperature or humidity as well soil conditions, nutrient levels, or hormone inducibility influencing nectar product processes (Escalante-Pérez 2012a). Due to these factors, total amino acid concentration is only briefly analyzed in specific cases. Instead individual amino acid proportions of the total concentration and structure of composition is compared between species.

Shannon-Weaver diversity index was used as a basis for determining structure and evenness for *Passiflora* species amino acid composition. For this method, nectar samples were treated as ‘sites’ and the 19 measured amino acids were considered as ‘species’. Since all samples were analyzed for the same amino acids and only one or two

samples actually had no trace of any individual amino acid, richness is constant. If all 19 'species' are in equal proportion than that sample would have the max evenness and diversity possible and the proportion of all amino acids would be 5.263% (H'=2.944), a median diversity with 10 amino acids at 9.1% (H'=2.310) with the other 9 at trace amounts. Additional high and low quadrants for diversity were set at 15 amino acids at 6.8% (H'=2.79) with 4 at trace amounts and 5 amino acids at 17.2% (H'=1.94) with 14 at trace amounts. Individual *Passiflora* species are contrasted to this median and boundaries to quantify EFN amino acid composition diversity for a given species as high or low diversity.

EFN amino acid composition for 31 *Passiflora* species and outgroups *Dilkea* and *Adenia* under greenhouse conditions was visualized as an unconstrained ordination using non-metric multidimensional scaling (NMDS) ordination plot (Clarke 1993) with Bray-Curtis dissimilarities with 95% confidence intervals highlighting subgenera and species clusters when sample size allows. Comparisons between species and subgenera quantified with PERMANOVA analysis. Individual amino acid contributions to variation between species was analyzed with Kruskal Wallis rank summed tests with Bonferroni corrections. EFN amino acid composition can be attributed to taxonomic relationships or driven by and maintained through herbivore pressure and selection for specific mutualistic defenders; disentangling these connections was achieved by within subgenera comparisons for *Decaloba* and *Passiflora* and post-PERMANOVA pairwise comparisons between species where n>2. Pairwise comparisons should be significantly different between species of different subgenera and not within the same subgenus if EFN amino acid composition is derived from taxonomic relationships.

Floral and extrafloral nectar comparison

Floral samples were collected opportunistically at the same time as EFN samples were collected. Extrafloral nectaries are similar to floral nectaries in both structure and biosynthetic pathways, but vary in function and chemical composition. Floral nectar is associated with pollination and generally differs in sugar present, type of amino acids present, and overall concentration and composition of amino acids (Baker et al. 1978, Baker & Baker 1983, 1986). Floral nectar tends to be high in a single amino acids, proline, attractive to bees and essential for the energy requirements of flight (Teulier 2016). Comparisons between floral and extrafloral nectar were carried out in this study to confirm previously findings that floral and extrafloral differs within a species (Baker 1978) as well as determine the breadth of difference between the two nectary types within *Passiflora*. Nine floral – extrafloral nectar comparisons were performed for 6 *Passiflora* species (**Table 1.2**). These samples were collected at the same time point on 5 incidences, other floral and extrafloral nectar samples used in these comparisons were collected at different timepoints due to either plant or environmental conditions limiting an adequate sample amount of one or the other nectar type (**Table 1.2**). When sampling of both nectary types couldn't occur at the same time point, we still used the same individual to sampled at different time points.

Comparisons between floral and extrafloral nectar are visualized similar to EFN interspecific sample comparisons as a non-metric multidimensional scaling (NMDS) ordination plot (Clarke 1993) with Bray-Curtis dissimilarities with 95% confidence around nectar type and PERMANOVA analysis to determine the effect of nectar type on variation seen. Comparisons between total amino acid concentration were considered for floral and extrafloral nectar across all pairs of samples. Floral nectar has been shown to have lower total amino acid concentrations than extrafloral nectar (Baker et al. 1987).

The extent of similarity for any pair of floral and extrafloral nectars can be determined by the correlation of the amino acid proportions with regression analysis plots and Pearson correlation coefficients.

Table 1.2: Data subset for floral and extrafloral nectary comparison in 6 species. Extrafloral and floral nectar was collected simultaneously when possible. The same individual was sampled if sampling occurred at different timepoints.

Genus	Subg.	species	Acc. No.	Acc. orginin	Sample type	Collection			Total AA conc (nmol/uL)	sample volume (uL)
						date	time	location		
Passiflora	*Decaloba*	*affinis*	9493	Texas	floral	10/8/15	14:00	FIELD	6.98	4.0
					laminar	10/8/15	14:00	FIELD	36.17	2.0
Passiflora	*Decaloba*	*auriculata*	8028	Costa Rica	floral	12/23/15	11:00	GH	1.12	4.0
					petiole	1/7/16	12:00	GH	144.73	4.0
Passiflora	*Decaloba*	*auriculata*	9406	French Guiana	floral	12/29/15	14:30	GH	13.23	2.0
					petiole	12/29/15	14:30	GH	348.71	4.0
Passiflora	*Decaloba*	*jatunsachensis*	9402	Ecuador	floral	11/3/15	12:00	GH	1.76	4.0
					petiole	11/3/15	12:00	GH	263.51	5.0
					floral	12/23/15	11:00	GH	9.21	3.0
					petiole	12/24/15	10:00	GH	420.51	4.0
					floral	12/29/15	13:00	GH	32.13	3.0
					petiole	12/29/15	13:00	GH	333.58	5.0
Passiflora	*Decaloba*	*rufa*	9086	French Guiana	floral	12/28/15	12:00	GH	2.53	2.0
					petiole	12/28/15	12:00	GH	375.13	2.0
					floral	11/4/15	8:00	GH	8.36	2.0
					petiole	12/29/15	13:00	GH	319.21	4.0
Passiflora	*Passiflora*	*sprucei*	9410	Ecuador	floral	9/24/15	10:30	GH	33.36	5.0
					petiole	2/28/17	11:00	GH	71.55	1.5

Variation within an individual

Variation within an individual is examined by comparison of types of EFNs within one individual as well as across timepoints for one nectary type of one individual. EFN samples were collected from multiple EFN sources if available for that species, ie. nectar from petiole nectaries, laminar nectaries, and floral bract nectaries. While the source of nectar for different EFN nectary types should be the same within a species and variation should only be related to external conditions, i.e. rates of evaporation influence individual nectary concentrations, this relationship has not been explored in previous literature. Nectary type is compared within a species for *P. auriculata* and *P. vitifolia* as an additional component of field population comparisons (Methods: VI. Data subsets: D. Variation between populations). Within an individual nectary type comparisons for subgenus *Decaloba* species *P. auriculata, P. jatunsanchensis,* and *P. rufa* are compared with regression analysis plots and Pearson correlation coefficients. For variation within an individual on small temporal scales, outgroup *Dilkea*, and *Passiflora* species *P. affinis, P. auriculata, P. rufa,* and *P. jatunsanchensis* within *Passiflora* subgenus *Decaloba* **(Table 1.3),** are compared visually due to lack of replicates for individual sampling times.

Table 1.3: Subset of *Dilkea* and *Passiflora* species individuals sampled at multiple timepoints.

Genus	Subgenera	species	Acc. No.	Sample type	Collection date	Collection time
Dilkea	NA	*Dilkea_sp*	9426	laminar tip	02/06/19	14:00
					02/25/17	13:00
					02/26/17	13:00
Passiflora	*Decaloba*	*affinis*	3083	laminar	05/06/17	11:00
					06/12/17	14:30
					08/08/17	11:00
					02/27/18	13:00
					06/18/18	11:00
Passiflora	*Decaloba*	*auriculata*	8028	petiole	09/03/15	11:00
					11/09/15	15:00
					12/21/15	17:00
					12/24/15	10:00
					09/08/16	14:00
					09/09/16	14:00
Passiflora	*Decaloba*	*rufa*	9086	petiole	12/29/15	13:00
					12/28/15	12:00
					11/09/15	15:00
					01/07/16	13:00
Passiflora	*Decaloba*	*jatunsachensis*	9402	petiole	11/03/15	12:00
					12/24/15	10:00
					12/29/15	13:00
					01/07/16	13:00

Variation between populations

Field nectar samples collected in Costa Rica for *P. vitifolia* and *P. auriculata* (**Table 1.4**) from lowland forests in the Osa Penisula on the Pacific side and Gandoca on the Caribbean side (**Figure 1.2**) provide the opportunity to compare variation in EFN amino acid composition between populations since neither of these species is adapted to

the numerous high mountain ranges in the center of the country. *P. auriculata* extrafloral nectar was also sampled from one individual in central Costa Rica near San Jose. Both of these populations for *P. auriculata* and *P. vitifolia* are subject to similar herbivore pressure in these locations due to the presence of the same *Heliconius* species. Variation in these two locations would be due to external factors such as habitat, soil nutrients, or even potentially an example of the plasticity of EFN nectar chemistry related to differences in predaceous insect populations.

For each species, sample comparisons as a non-metric multidimensional scaling (NMDS) ordination plot (Clarke 1993) with Bray-Curtis dissimilarities with 95% confidence around location and PERMANOVA analysis to determine the effect of location on variation seen. The extent of similarity between locations was determined by the correlation of the amino acid proportions with regression analysis plots and Pearson correlation coefficients.

Table 1.4: Subset of *Passiflora auriculata* and *P. vitifolia* samples from field locations. *P. auriculata* was equally sampled in both locations but *P. vitifolia* was underrepresented in sampling from Gandoca, Costa Rica.

Genus	Subgenus	Supersection	Species	n	Sample Type		EFN sample Collection Location
					laminar	petiole	
Passiflora	*Decaloba*	*Auriculata*	*auriculata*	14 17 1	X (5) X (2)	X (9) X (15) X	Osa Penisula, Costa Rica Gandoca, Costa Rica San Jose, Costa Rica
Passiflora	*Passiflora*	*Coccinea*	*vitifolia*	19	X (1) X (1)	X (5) X (12)	Gandoca, Costa Rica Osa Penisula, Costa Rica

Figure 1.2: Costa Rica with field sampling locations broadly marked.

Error Analysis - Comparison of current analysis with 1988 analysis

Previous unpublished work by Dr. Janet Lanza, carried out under Dr. Gilbert at UT in 1988, provides amino acid analysis of extrafloral nectar from *Passiflora* species allowing for larger scale temporal comparisons in six species that overlapped between this and our current study. These results were obtained through HPLC analysis carried out at the University of Texas at Austin though equipment and exact protocol are unknown. Several of these individuals are still present in greenhouses under Gilbert's supervision and were sampled in our current work. These earlier samples would have been collected under similar environmental conditions as current greenhouse conditions due to consistent greenhouse protocol being in place for ca 40 years. Soil and health of the plant could influence nectar chemistry, but these shifts should be minimal and transient. Comparisons of Dr. Lanza's samples and current samples for any given species should not be significantly different in amino acid composition due to maintenance of greenhouse conditions especially if samples compared are the same individual. Age of tissue sampled could also lead to variation and no sampling notes remain from these earlier samples, however, since young new tissue has the largest volume of nectar produces it is assumed that tissue age is roughly the same between historical and current samples. Variation for comparison of potentially different individuals of the same species will vary at the same level as would be expected within a species, however the degree of this variation in not known.

Comparison of these profiles with current samples analyzed through HPLC utilizing facilities at Texas A&M will act as an error analysis between equipment and facilities, as well as provide a platform for the hypothesis that nectar amino acid

composition can be maintained within a species while under controlled environmental conditions. As seen in **Table 1.5**, not all current samples could be matched to the exact individual sampled in 1988 due to lost records and several clones or individuals present in 1988 when sampling by Lanza occurred. Two of the six, *Passiflora menispermifolia* and *Passiflora auriculata,* are known to be the exact same individual sampled in 1988 and currently. *Passiflora pittieri* may not be the exact individual but sampled individuals from Lanza and current samples are greenhouse generated clones of the same individual collected from the P.N. Corcovado in Costa Rica. We can assess that *Passiflora vitifolia* sampled in 1988 by Lanza and currently sampled are individuals collected from the same location but are not confident it is the same individual, all *P. vitifolia* accession in the greenhouse in 1988 were collected from P.N. Corcovado in Costa Rica. *P. quadrangularis* and *P. microstipula* cannot confidently be considered the same individual or from the same location. For both species several individuals from several locations were present in the greenhouse in 1988 when Lanza collected nectar samples. In these incidences, differences between the amino acid composition of samples from 1988 and current samples could be due to being different individuals and not an accurate comparison of temporal variation in amino acid composition. Analysis included paired t-test on total concentration of amino acids present in extrafloral nectar between sample years, ordination visualization using non-metric multidimensional scaling (NMDS), and regression analysis between sample years and individual amino acids to determine variation due to HPLC system differences or actual variation between amino acid composition in EFNs between sample years.

Table 1.5: Accession numbers for species sampled in 1988 by Dr. Janet Lanza and 2018-2019 by Rees for extrafloral nectar amino acid analysis.

***Passiflora* subgenera**	***Passiflora* species**	**Lanza 1988 Sample accession #**	**Rees 2018/2019 Sample accession #**
Astrophea	*pittieri*	unknown clones 8048-8051 present	8051
Passiflora	*menispermifolia*	9045	9045
Passiflora	*vitifolia*	unknown acc. 9038-9041 present	9041
Passiflora	*quadrangularis*	unknown acc. 8054, 9056, 9057 present	9056
Decaloba	*microstipula*	unknown acc. 7010, 8018, 9271 present	9271
Decaloba	*auriculata*	8028	8028

Error Analysis - Technical variation analysis with Passiflora pittieri

To determine technical variation due to sample processing before analysis via Texas A&M University Protein Chemistry Laboratory, a pooled 10 uL sample from three nectaries on the same branch of *P. pittieri* was split into three samples for individual analysis via HPLC. Submitted samples were 3uL volumes separated the day prior to submission, dried and spun down via speedvac to eliminate movement of sample up the sides of the Eppendorf tube, and sent in same sample (2019) bundle.

Any difference between these samples that is beyond the reported standard error of the HPLC analysis will be due to sampling and preparation of samples prior to analysis. These samples will be compared using regression analysis and Pearsons correlation coefficients as pairs between pooled samples and additional *P. pittieri* samples from different sampling timepoints and batches analyzed by Texas A&M.

Statistical Analyses

Data table formatting with tidyverse and all statistical analyses were performed in R version 3.6.1 used with R studio version 1.2.5001 (R Development Core Team 2019) with additional packages listed below, and graphically visualized with ggplot (Wickham 2016) with additional formatting and layout style through cowplot (Wilke 2019). Unconstrained ordinations were determined using nonmetric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarities with the function metaMDS in R package vegan (Oksanenet al. 2019) and PERMANOVAwith the function Adonis (999 permutations, method = bray) to analyze the variance with between sample data subsets due to specific criterion for each subset. For pairwise comparisons *Passiflora* species with adequate sample size (n>2) the function pairwise.perm.manova within the R package RVAideMemoire (Herves 2020) was utilized.

When applicable, significance of differences due to specific amino acids was assessed with a Kruskal-Wallis multiple comparisons test in the FSA package (Ogle 2016) with Bonferroni p-value adjustments. Correlations were calculated using the Pearson correlation coefficient in R.

RESULTS

Interspecific variation across genus

Actual extrafloral concentration of all amino acids in nmol/uL (**Figure 1.3**) shows large difference between species and within a species where sampling occurred multiple times, but there are some species that consistently had low concentrations of EFN amino acids. *P. pittieri, P. oerstedii,* and *P. tenuiloba* had low total concentrations (<65nmol/uL) for multiple nectar samples. Nectar samples for these three species are all

petiole nectary samples but otherwise there are no shared characteristics between these species in nectar morphology or taxonomic relationships. On the other hand, the multiple samples of *Dilkea sp.* consistently had nectar concentrations (>550 nmol/uL) on the high end of all samples.

Shannon diversity measure distinguish how overall composition varies between species. Median, high, and low boundaries were set to classify individual species as high or low diversity (**Figure 1.4**). The highest diversity was seen in *P. pittieri* where individual samples had diversity in the range of 2.26-2.65. Individual lowest diversity was seen in *P. oerstedii* at 0.956. All species that would classify as high in diversity due to criteria are *P. garckei, P. misera, P. pittieri, P. suberosa, P. citrifolia.* The majority of species fall between the mid control and the lower quadrant with diversity values between 1.94-2.31. Twelve species fall below the low quadrant control and will be considered low diversity with diversity values between 1.1-1.94 (**Table 1.6**).

NMDS visualization of *Passiflora* species plotted as sites and analyzed amino acids plotted as species contributing to each unique 'site' with 95% CI ellipses for subgenus (**Figure 1.5**) and species (**Figure 1.6**) where n>2. Variation between samples can be explained by subgenus (PERMANOVA, vegan (Oksanen 2019), method='bray', permutation=999, r^2=0.29933) with p=0.001, but a larger proportion of total variation is explained through species comparisons (r^2=0.52610) with p=0.001. In pairwise comparison, subgenus *Passiflora* is significantly different than subgenus *Decaloba* (p=0.006) and subgenus *Astrophea* (p=0.030) and subgenus *Decaloba* is significantly different than subgenus *Astrophea* (p=0.024); subgenus *Deidamoides* was not considered in subgenus comparisons due to the paraphyletic nature of this subgenus. Outgroup genera, *Dilkea* and *Adenia*, are not significantly different than any subgenus within *Passiflora*. In pairwise comparisons between species (n>2 sample size); several species

are significantly different than others largely due to one or two amino acids at higher concentrations than others in that species EFN amino acid composition (**Table 1.7**). *P. affinis* is significantly different than all other species in comparison besides *Dilkea* largely due to concentration of phenylalanine and additional amino acids associated with the shikimate biosynthesis pathway that are not seen in any other species. *P. auriculata* is significantly different than all species besides *P. rufa* due to high proportions of alanine and serine. *P. oerstedii* is significantly different than *P. pittieri*, *P. affinis, P. auriculata* and *P. jatunsanchensis* due to its EFN amino acid composition largely consisting of glutamine.

Kruskal-Wallis rank sum test with Bonferroni p-value adjustments was used to assess individual amino acids contribution to differences between species with n>2 sample size (**Table 1.8**). Of the 19 amino acids analyzed for in this study, 13 were significant in differences found between species.

Table 1.6: Shannon diversity measures for 19 amino acids in *Passifloraceae* species analyzed. Mid control (10 amino acids at 9.1%) and low quadrant (5 amino acids at 17.2%) controls to establish high and low diversity rankings for *Passiflora* EFN amino acid composition.

Genus	**Subgenus**	**species**	**Avg. H'**
Passiflora	*Passiflora*	*garckei*	2.54
Passiflora	*Decaloba*	*misera*	2.49
Passiflora	*Astrophea*	*pittieri*	2.47
Passiflora	*Decaloba*	*suberosa*	2.36
Passiflora	*Astrophea*	*citrifolia*	2.33
Mid control (10 AA at 9.1%, 9 trace)			2.31
Passiflora	*Decaloba*	*tenuiloba*	2.29
Passiflora	*Decaloba*	*jatunsachensis*	2.24
Passiflora	*Passiflora*	*edulis*	2.24
Passiflora	*Deidamioides*	*arbaelezii*	2.24
Adenia	*NA*	*mannii*	2.23
Passiflora	*Passiflora*	*alata*	2.23
Passiflora	*Passiflora*	*vitifolia*	2.19
Passiflora	*Passiflora*	*menispermifolia*	2.17
Dilkea	*NA*	*Dilkea sp*	2.15
Passiflora	*Decaloba*	*auriculata*	2.13
Passiflora	*Decaloba*	*microstipula*	2.12
Passiflora	*Passiflora*	*cincinnata*	2.11
Passiflora	*Decaloba*	*rufa*	2.08
Passiflora	*Passiflora*	*laurifolia*	2.05
Passiflora	*Passiflora*	*mucronata*	2.02
Low quadrant control (5 AA at 17.2%, 14 trace amount)			1.94
Passiflora	*Passiflora*	*nitida*	1.86
Passiflora	*Passiflora*	*serratifolia*	1.82
Passiflora	*Decaloba*	*affinis*	1.77
Passiflora	*Passiflora*	*sprucei*	1.77
Passiflora	*Deidamioides*	*contracta*	1.76
Passiflora	*Passiflora*	*quadriglandulosa*	1.74
Passiflora	*Passiflora*	*oerstedii*	1.56
Passiflora	*Passiflora*	*seemannii*	1.47
Passiflora	*Astrophea*	*sphaerocarpa*	1.46
Passiflora	*Passiflora*	*ambigua*	1.45
Passiflora	*Passiflora*	*actinia*	1.17
Passiflora	*Passiflora*	*eichleriana*	1.10

Table 1.7: Pairwise comparisons using permutation MANOVA on NMDS distance matrix. Small sample size in any one species leads to similarities in significance values. *P. affinis* is significantly different than all other species compared beside outgroup *Dilkea*. Besides *P. affinis, P. pittieri* is significantly different than *P. oerstedii* and *P. auriculata*. *P. oerstedii* is in turn significantly different than *P. auriculata. P. auriculata* is significantly different than both *P. jatunsachensis* and *P. tenuiloba.*

	Dilkea sp.	***P. pittieri***	***P. oerstedii***	***P. affinis***	***P. jatunsachensis***	***P. auriculata***	***P. rufa***
P. pittieri	1.000	-	-	-	-	-	-
P. oerstedii	0.560	0.028	-	-	-	-	-
P. affinis	0.112	0.028	0.028	-	-	-	-
P. jatunsachensis	0.868	0.252	0.056	0.028	-	-	-
P. auriculata	0.028	0.028	0.028	0.028	0.028	-	-
P. rufa	0.532	0.196	0.084	0.028	0.336	1.000	-
P. tenuiloba	0.532	0.196	0.084	0.028	0.308	0.028	0.308

Table 1.8: Kruskal-Wallis rank sum test with Bonferroni p-value adjustments of individual amino acids contribution to differences between all species in Table 1.7. Of the 19 amino acids analyzed for in this study, 13 were significant in differences found between *Dilkea sp., P. pittieri, P. oerstedii, P. affinis, P. jatunsachensis, P. auriculata,* and *P. rufa*.

Amino Acid	**3-letter code**	**Bonferroni**
Alanine	ALA	8.83E-09
Leucine	LEU	4.80E-08
Phenylalanine	PHE	2.02E-07
Serine	SER	3.57E-07
Arginine	ARG	8.39E-07
Tryptophan	TRP	1.23E-06
Proline	PRO	2.43E-06
Methionine	MET	2.77E-06
Tyrosine	TYR	2.52E-05
Lysine	LYS	3.63E-05
Glutamine	GLN	0.00024738
Glutamic Acid	GLU	0.00262724
Asparagine	ASN	0.0061147
Valine	VAL	0.05833124
Threonine	THR	0.11818857
Glycine	GLY	0.23584922
Aspartic Acid	ASP	0.35556483
Histidine	HIS	1
Isoleucine	ILE	1

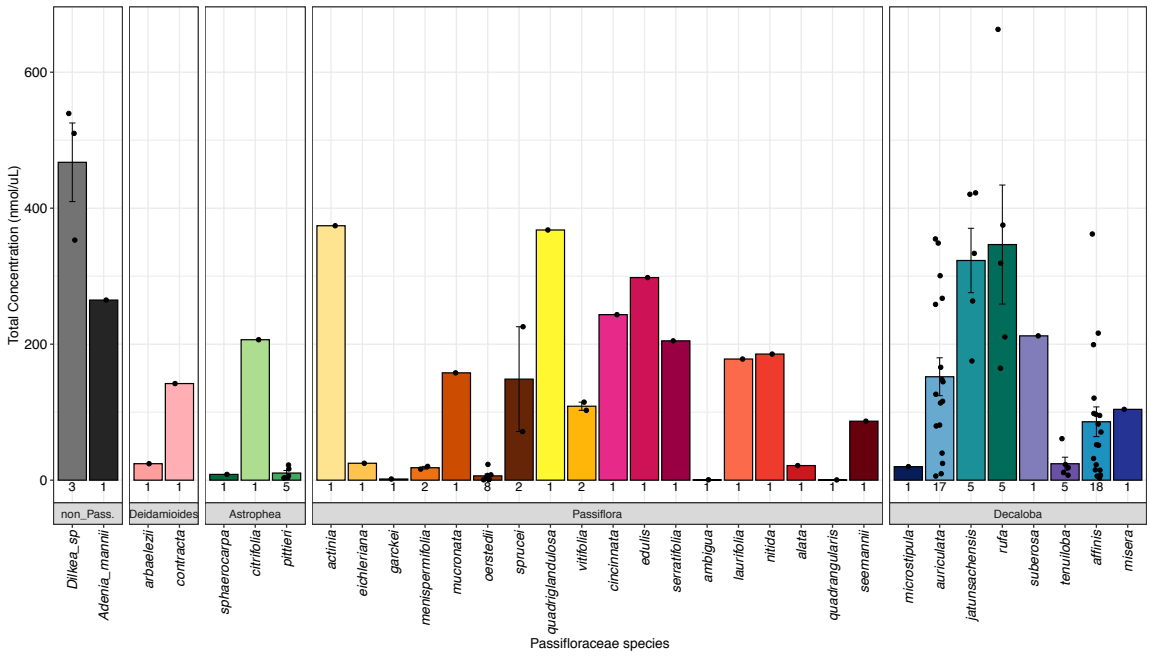

Figure 1.3: Total amino acid concentrations (nmol/uL) for greenhouse sampled species in genus *Passiflora* and outgroup genera *Dilkea* and *Adenia.* Nectar sample size for each species indicated by value below each bar. Error bars represent standard error.

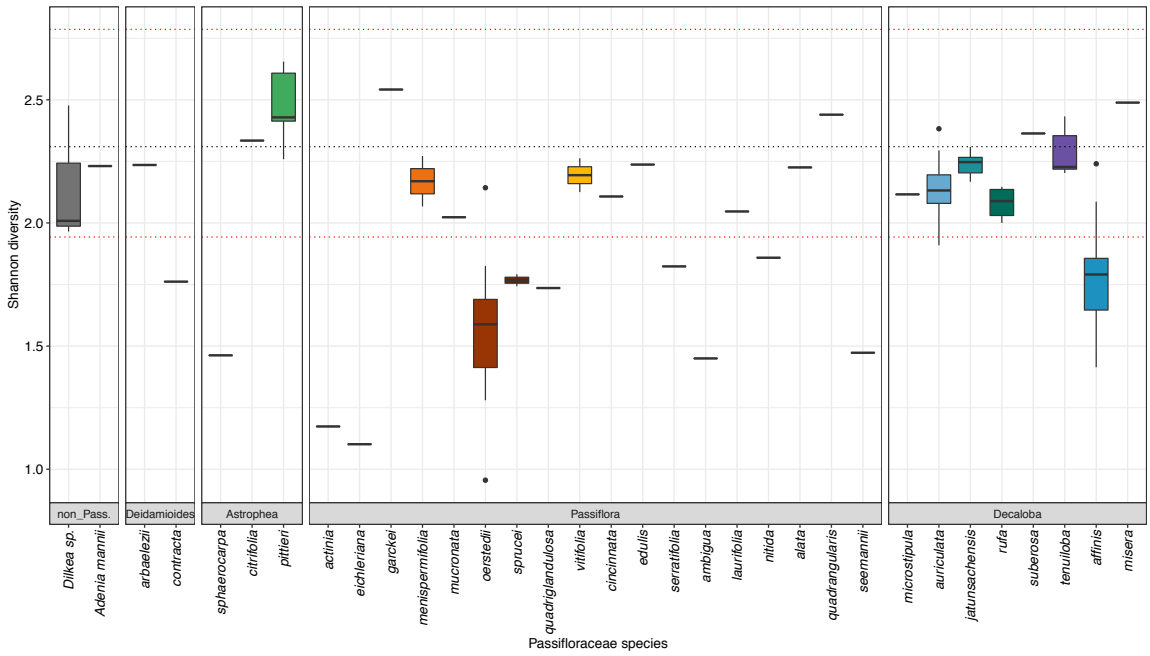

Figure 1.4: Shannon diversity measures of *Passifloraceae* species (genus *Dilkea, Adenia,* and *Passiflora*). Maximum diversity possible is all 19 amino acids analyzed for in this study were equal in proportion at 5.263% (H'=2.944). A median diversity with 10 amino acids at 9.1% (H'=2.310, black dotted line). High and low quadrants for diversity were set at 15 amino acids at 6.8% (H'=2.79) and 5 amino acids at 17.2% (H'=1.94) (red dotted lines).

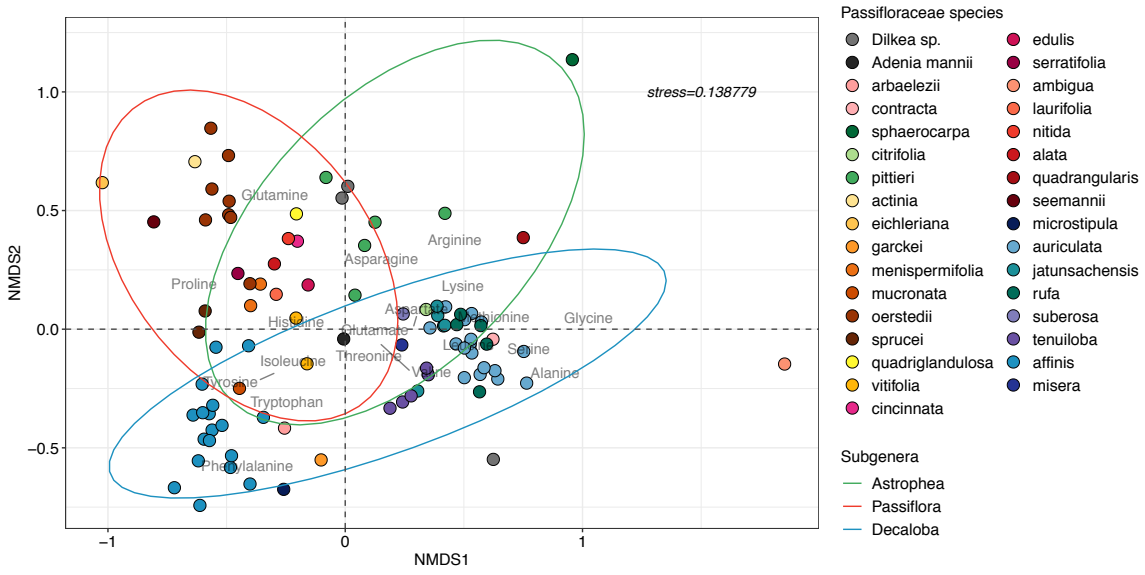

Figure 1.5: NMDS visualization with 95% CI ellipses around Subgenera. 30% (r^2=0.29933) of the sum of the squares can be explained by subgenus, $p<0.001$. There is a significant difference in amino acid composition between subgenera within genus *Passiflora*.

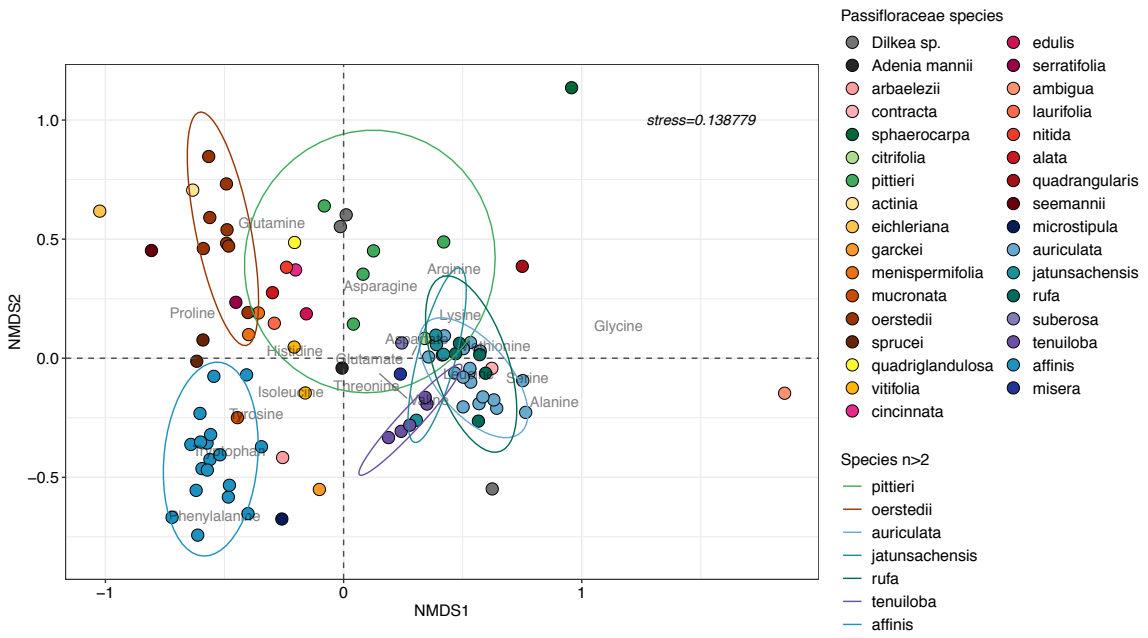

Figure 1.6: NMDS visualization with 95% CI ellipses around species with n>2. 53% (r^2=0.52610) explained by species, p<0.001. There is a significant difference in amino acid composition between species in genus *Passiflora*.

Floral and extrafloral nectar comparison

Floral and extrafloral nectar was sampled for a subset of species (**Table 1.2**) and compared for total concentration and nectar amino acid composition. Total concentration of amino acids in nectar is significantly higher in extrafloral nectar than floral nectar across subset of species (p=0.00081) in a paired t-test (**Figure 1.7A**). NMDS and PERMANOVA analysis revealed that floral and extrafloral nectar are significantly similar due to nectar type (p=0.008) with 20.9% percent of the variation of this sample subset explained by nectary type (**Figure 1.7B**). Only *P. jatunsanchesis* (n=3) had multiple floral and extrafloral nectar pairs for comparison at different sampling timepoints. Total concentration of amino acids significantly different than floral nectar

(p=0.0018) when considering this species alone (**Figure 1.8B**). Individual amino acid concentration comparisons reveal that several amino acids are significantly different between the floral and extrafloral nectar samples. Isoleucine, aspartate, and glutamate are in higher concentration in extrafloral nectar and alanine is at higher concentration in floral nectar for *P. jatunsachensis* (**Figure 1.8A**).

Due to *P. jatunsachensis* being the only species in the floral and extrafloral sample subset with multiple sample points, all other species were compared through regression analysis to determine degree of correlation between floral and extrafloral nectar samples (**Figure 1.9**). Correlations between sample type vary from close to no correlation (R=-0.071) in *P. auriculata* (collected in Costa Rica) to high correlation (R=0.96, R=0.97) in *P. auriculata* (collected in French Guiana) and *P. sprucei.* Comparisons of nectar amino acid composition and total amino acid concentration for *P. sprucei* and *P. auriculata* (French Guiana) show similarity in composition but concentration of amino acids is >3x higher in the extrafloral nectar sample for both species (**Figure 1.10**).

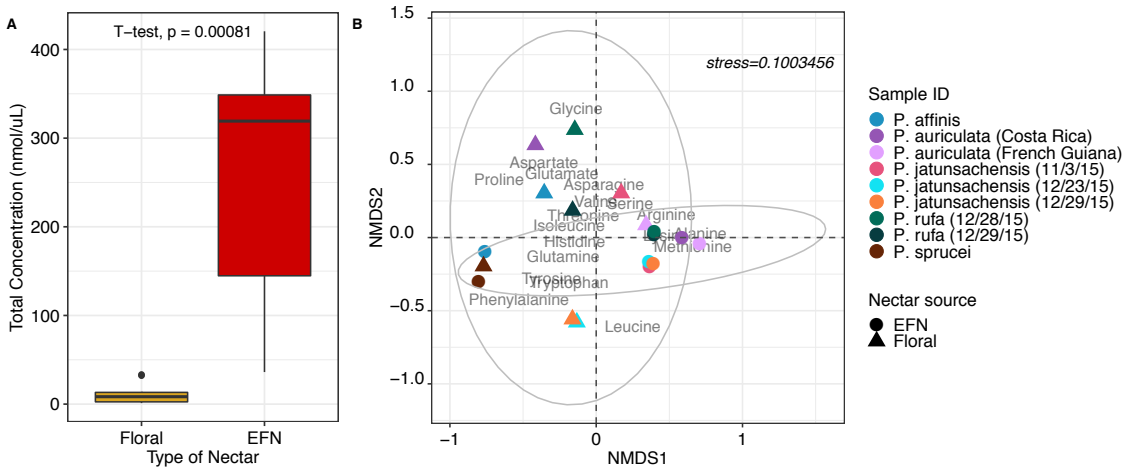

Figure 1.7: **A**. Total concentration (nmol/uL) of amino acids in floral and extrafloral nectar of all species in floral/extrafloral nectar comparison. **B**. Unconstrained ordination with 95% confidence intervals of floral versus extrafloral nectar sample subset based on Bray-Curtis dissimilarities of nectar type (stress 0.1003456).

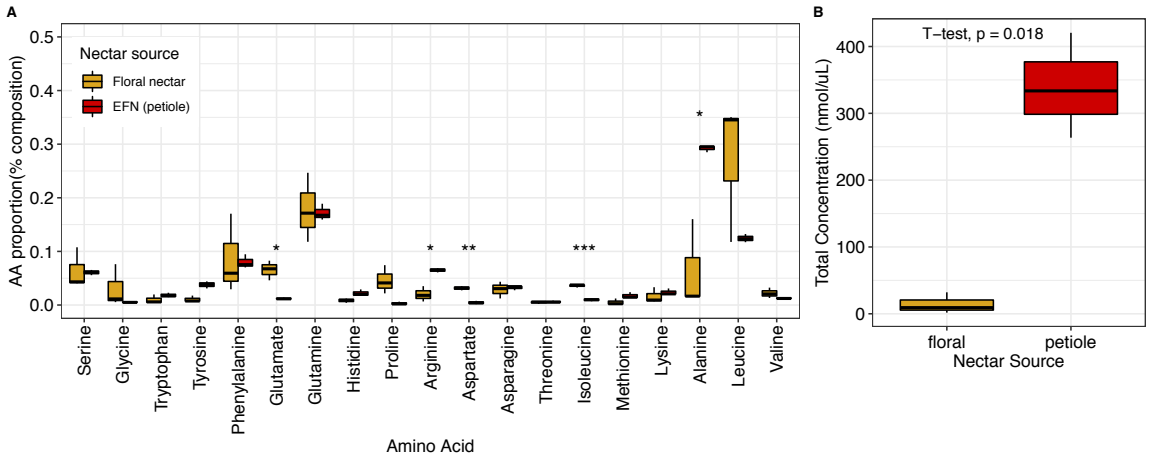

Figure 1.8: **A**. Composition of amino acids in floral and extrafloral nectar in *P. jatunsachensis* (n=3) with paired t-test for individual amino acids (* = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$). **B**. Total concentration (nmol/uL) of floral and extrafloral nectar in *P. jatunsachensis.*

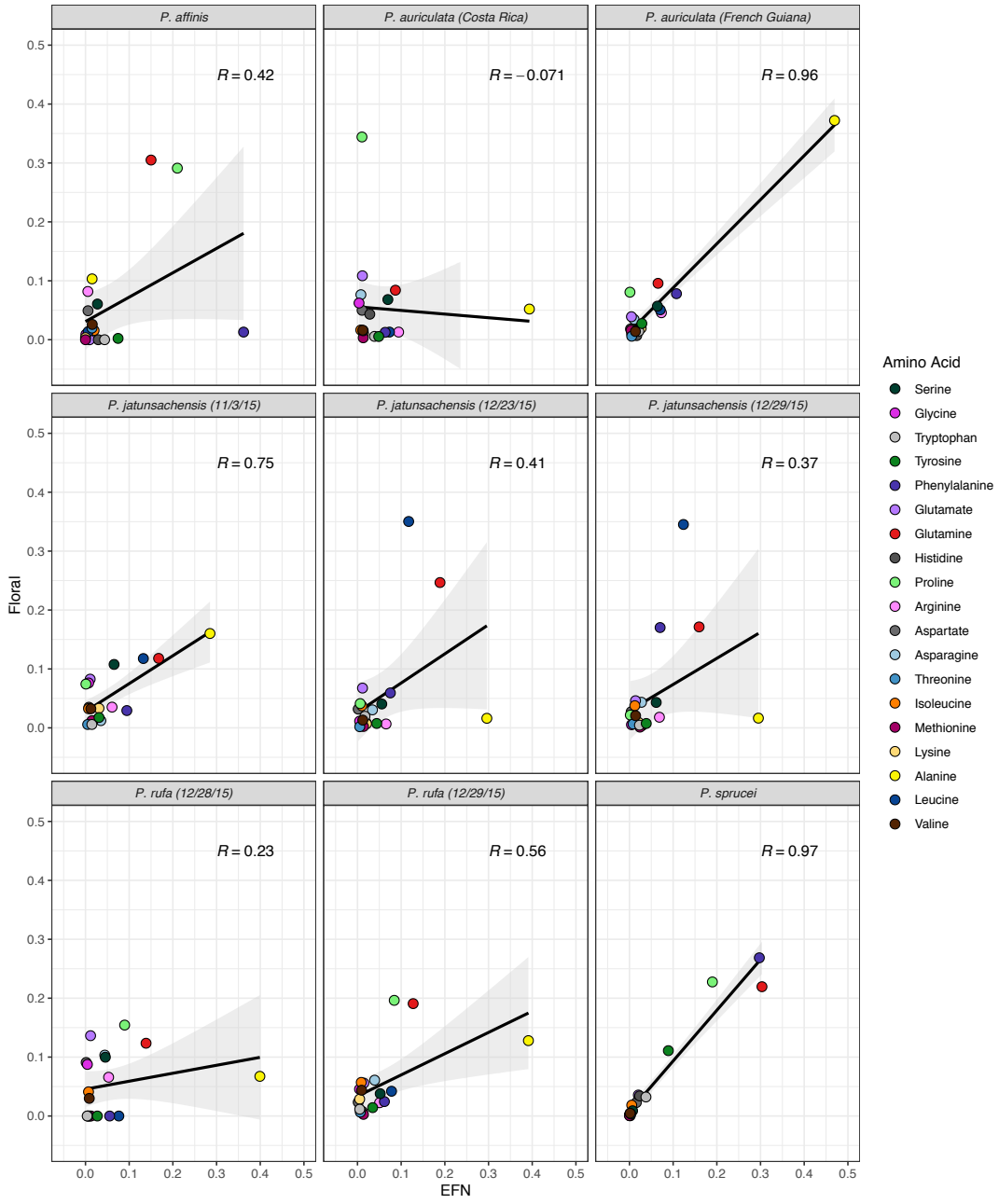

Figure 1.9: Pearson correlations for *Passiflora* species floral and extrafloral comparisons with linear regression to measure degree of similarity.

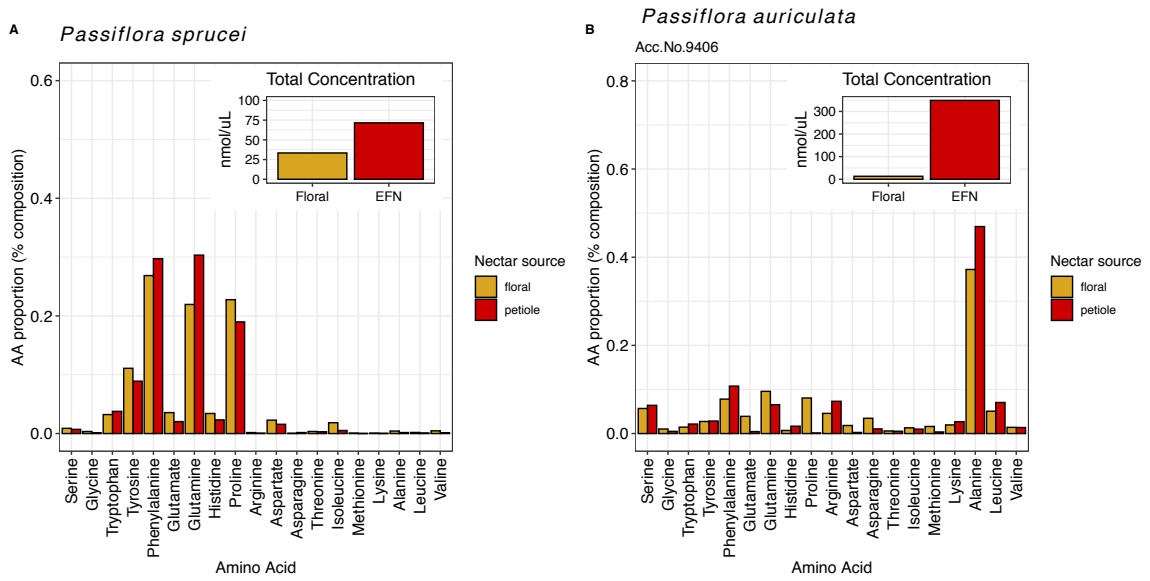

Figure 1.10: Floral and extrafloral nectar composition bargraphs for (**A**) *P. sprucei* and (**B**) *P. auriculata* (Accession number 9406) with inset of total amino acid concentration for both species. Both species showed high correlation between floral and extrafloral nectar composition but differ in total amino acid concentration for nectar types.

Variation within an individual

Variation within an individual is compared through nectary type within species *P. rufa, P. jatunsachensis,* and *P. auriculata* (Acc. 8028). All species show high correlation between nectary type for these species (R=0.83-0.98) (**Figure 1.11**). Variation within an individual is cannot be statistically calculated, but is illustrated visually. *P. jatunsachensis* (**Figure 1.12A**) and *P. rufa* (**Figure 1.12B**) show little to no variation for any amino acid between the four time points sampled for each. Both individuals were sampled in the same time frame of 2 months at the end of 2015 and beginning of 2016. *P. auriculata* (Acc. 8028) (**Figure 1.12C**) was sampled at the same time points, early in 2015, and one year later in 2016. *P. auriculata*, sampled 09/08/2016 shows a dramatic

increase in glutamate with an equal decrease in glutamine. *P. affinis* (**Figure 1.12D**) was sampled in 2017 and 2018 with the two 2018 sampled (02/27/18 and 06/18/18) showing a decrease in phenylalanine proportions along with slight decrease in additional aromatic amino acids related to the shikimate pathway, tryptophan and tyrosine, with an increase in glutamine in the 02/27/18 sample and an increase in proline in both 2018 samples (02/27/18 and 06/18/18). The outgroup species from genus *Dilkea* (**Figure 1.12E**) appears to differ dramatically in the sample collected from 02/25/17 then sample collected the next day on 02/26/17 and 2 years later on 02/06/10. The sample from 02/25/17 shows low glutamine with slight increases in several other amino acid common to the amino acid composition of the other *Dilkea* samples but no dramatic increase in any one amino acid.

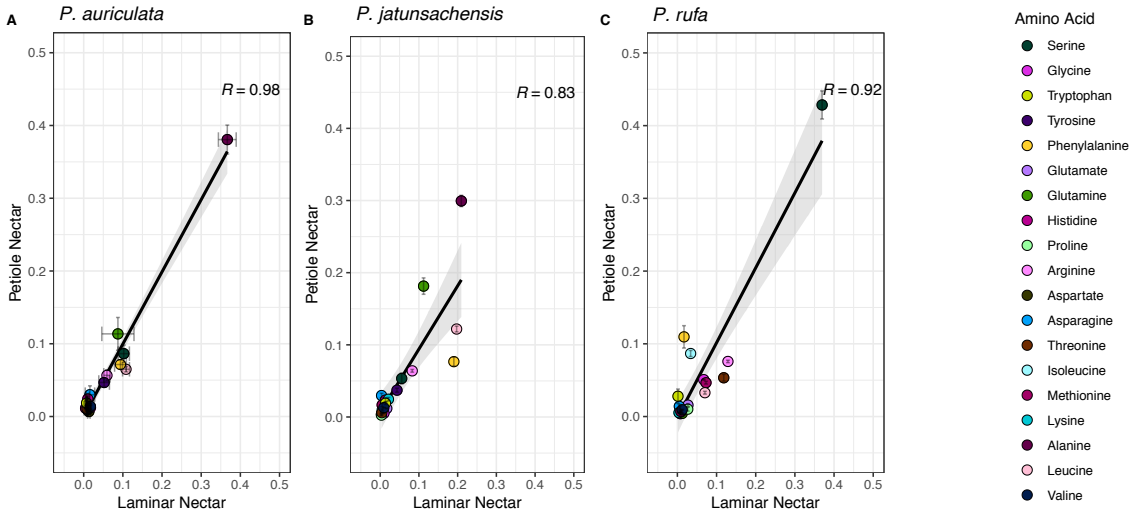

Figure 1.11: Pearson correlations extrafloral nectar comparisons between nectary type, laminar and petiole, with linear regression to measure degree of similarity for *Passiflora auriculata* (**A**), and *P. jatunsachensis* (**B**), *P. rufa* (**C**), from subgenus *Decaloba.* Error bars represent standard errors.

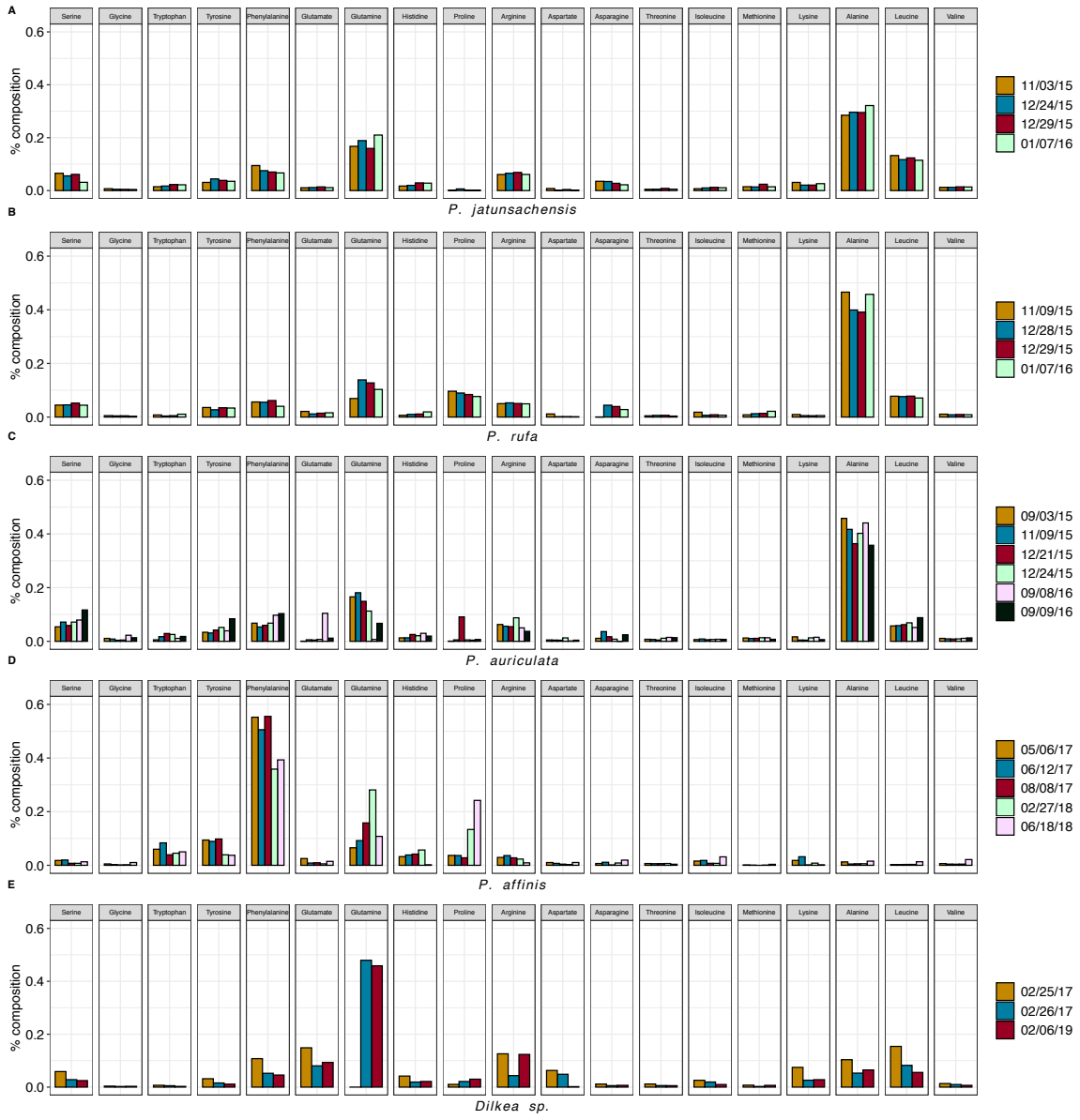

Figure 1.12: Variation within an individual across timestamps across several years. *P. jatunsachensis* (**A**), *P. rufa* (**B**), *P. auriculata* (**C**), *P. affinis* (**D**) from subgenus *Decaloba* and outgroup species comparisons for genus *Dilkea* (**E**).

Variation between population

Passiflora vitifolia and *P. auriculata* were sampled in the field from two distinct populations in Costa Rica, the Osa peninsula on the Pacific side and Gandoca on the Carribean side. *Passiflora auriculata* also has one sample from central Costa Rica near San Jose. NMDS visualization with 95% confidence intervals around locations shows high overlap, no significant difference is measured between locations (p=0.21) and less than 9% of the sum of squares can be explained by locations of samples through PERMANOVA analysis (**Figure 1.13B**) Regression analysis shows high correlation between locations (**Figure 1.13A**). *P. vitifolia* shows more variation between locations in NMDS visualization (**Figure 1.14B**) but no significant different between samples(p=0.09) due to sampling location and less than 12% of the sum of squares can be explained by location (**Figure 1.14A**). *P. auriculata* has a pair of large cup-like petiole nectaries and conspicuous yellow laminar nectaries. *P. vitifolia* has 2 or more oval cup petiole nectaries and additional nectaries along the leaf tooth edge which we will consider laminar nectaries. Petiole and laminar nectar was collected from individuals of *P. vitifolia* and *P. auriculata* in the field, nectary type was also not significant in variation seen within a species for either *P. vitifolia* (r^2=0.025, p=0.67) or *P. auriculata* (r^2=0.04, p=0.254). Extrafloral nectar amino acid composition was highly correlated and not significantly different between populations of either *P. vitifolia* or *P. auriculata* from the Atlantic and Pacific coastal lowland forests of Costa Rica.

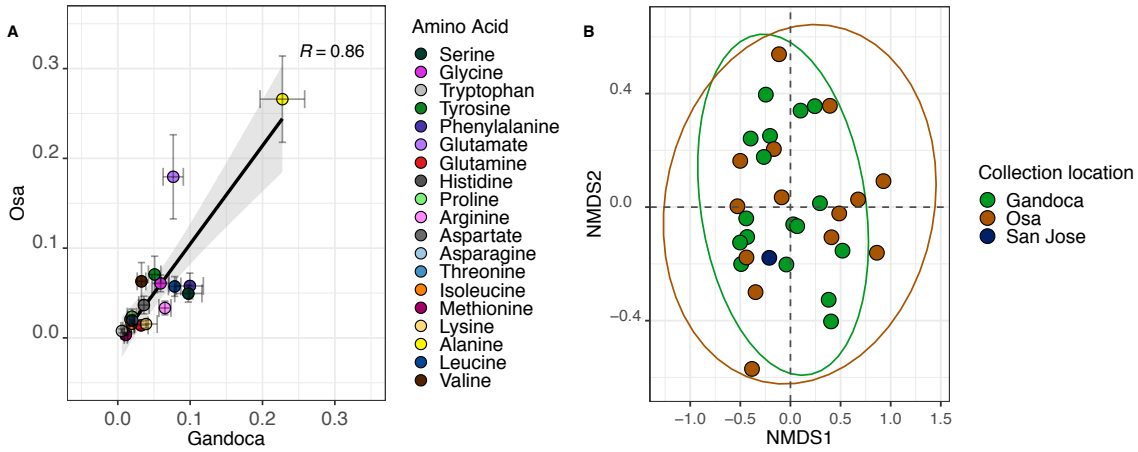

Figure 1.13: **A**. High correlation (R=0.86) between samples from Gandoca, Costa Rica and the Osa Penisula, Costa Rica for *P. auriculata*. Error bars represent standard error. **B**. Less than 9% (r^2= 0.08359) of the sum of the squares can be explained by location of samples, failed to reject the null hypothesis that there no is a difference in amino acid composition between populations of *Passiflora auriculata*.

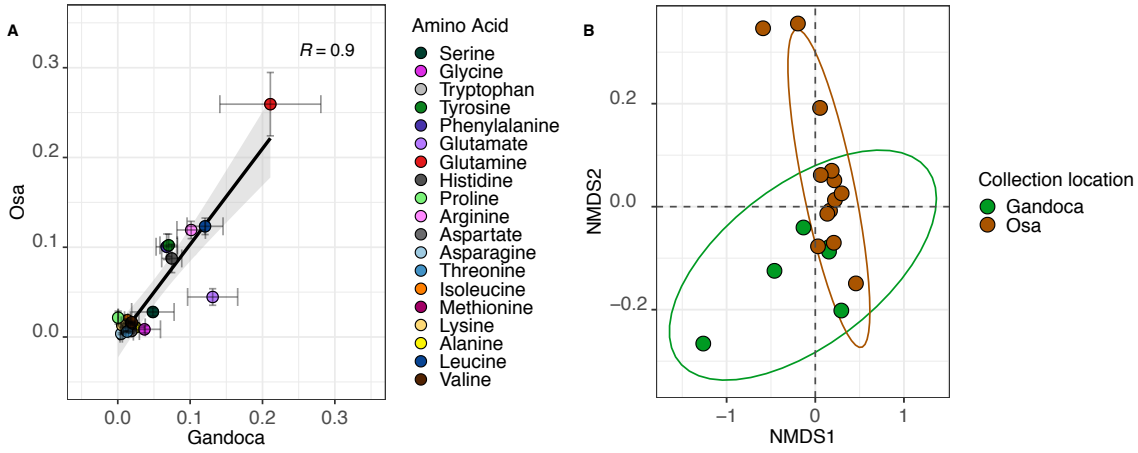

Figure 1.14: A. High correlation (R=0.9) between samples from Gandoca, Costa Rica and the Osa Penisula, Costa Rica for *P. vitifolia.* Error bars represent standard error. **B**. less than 12% (r^2= 0.11151) of the sum of the squares can be explained by location of samples, failed to reject the null hypothesis that there is no difference in amino acid composition between populations of *Passiflora vitifolia*.

Error Analysis - Comparison of current analysis with 1988 analysis

Comparison of EFN amino acid results obtained in 1988 by Dr. Janet Lanza of *Passiflora* species housed in UT greenhouses with current results of the same individual, or species, act as an error analysis between equipment and facilities, as well as provide a platform for the hypothesis that nectar chemical profiles will be fairly consistent within an individual and within a species. Grouped total amino acid concentration in extrafloral nectar for past and current samples were not significantly different (p=0.68, **Figure 1.15A**). Individual species do show differences in total concentration in 2018/2019 compared to 1988 (*P. vitifolia* and *P. auriculata* have higher total concentrations, *P. microstipula* has lower total concentration, while *P. menispermifolia, P. quadrangularis, P. pittieri* have similar total concentrations

Ordination plot of extrafloral nectar composition for individuals sampled in 1988 and current samples (**Figure 1.15B**) shows clustering by species and not by sampled date, further statistical analysis of clusters cannot be performed due to sample size. The two species with the same known accession numbers sampled in 1988 and current, *P. auriculata* and *P. menispermifolia,* are closely grouped in the NMDS plot (**Figure 1.15B**) as well as show strong correlation when plotted against each other (R=0.96 and R=0.94, **Figure 1.15C**). *P. pittieri* (R=0.84) and *P. microstipula* (R=0.97) also show strong correlation sampling dates are plotted against each other (**Figure 1.15C**). *P. quadrangularis* (R=0.3) has low correlation when sample time points are plotted against each other and visually has the greatest distance between samples on an NMDS plot (**Figure 1.15B**). *P. quadrangularis* accessions present in the greenhouse in 1988 were collected from different geographic regions (8054 from Parque National Corcovado, Costa Rica and 9056/9057 from Porto Alegre in the Rio Grande do Sul state of Brazil) (**Table 1.4**) .

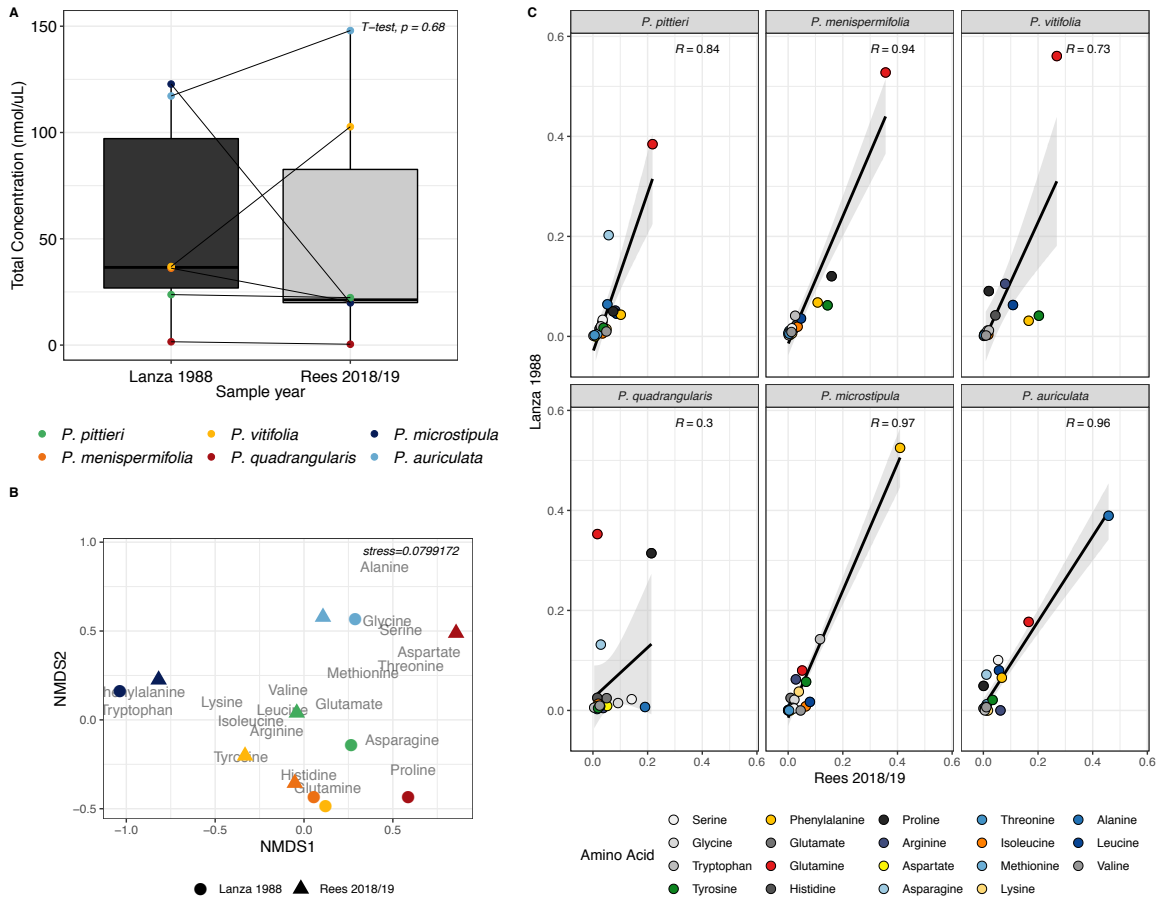

Figure 1.15: Comparison of Lanza 1988 and Rees 2018/2019. **A**. paired t-test of total amino acid concentration in EFN (nmol/uL). **B**. Ordination visualization via non-metric dimensional scaling of amino acid proportions in EFN for each species. Colors represent species and shape represents sample year. **C**. Correlation analysis between sample years for each species.

Error Analysis - Technical variation analysis with *Passiflora pittieri*

Pooled *P. pittieri* samples #1 and #3 show a strong correlation in EFN amino acid composition to each other (pearson correlation coefficient, R=0.99) and similar correlations to the 2015 sample of *P. pittieri* accession #9258 (pearson correlation

coefficient, R=0.79 and R.0.8) (**Figure 1.16A-C**). Unfortunately sample contamination appears to have occurred with pooled *P. pittieri* sample #2 since there is no correlation between it and pooled samples #1 (pearson correlation coefficient, R=0.2) and #3 (pearson correlation coefficient, R=0.24) or the 2015 sample (pearson correlation coefficient, R=0.18) (**Figure 1.16D-E**). *P. pittieri* pooled sample #2 also had double the total amino acid concentration of pooled sample #1 and #3 at 6.54 nmol/uL compared to 3.74 nmol/uL and 3.23 nmol/uL (**Figure 1.17**). The source of this potential error cannot be traced to mixed up samples on our end or at Texas A&M facilities. This sample is high in serine and glycine and low in glutamine and arginine which is found in higher proportions in other EFN *P. pittieri* samples. Comparisons between this sample and field samples collected from the Osa Penisula, where accession #9258 was collected show high similarity in EFN amino acid composition between filed and greenhouse samples and pooled sample #2 as an extreme outlier (**Figure 1.18**).

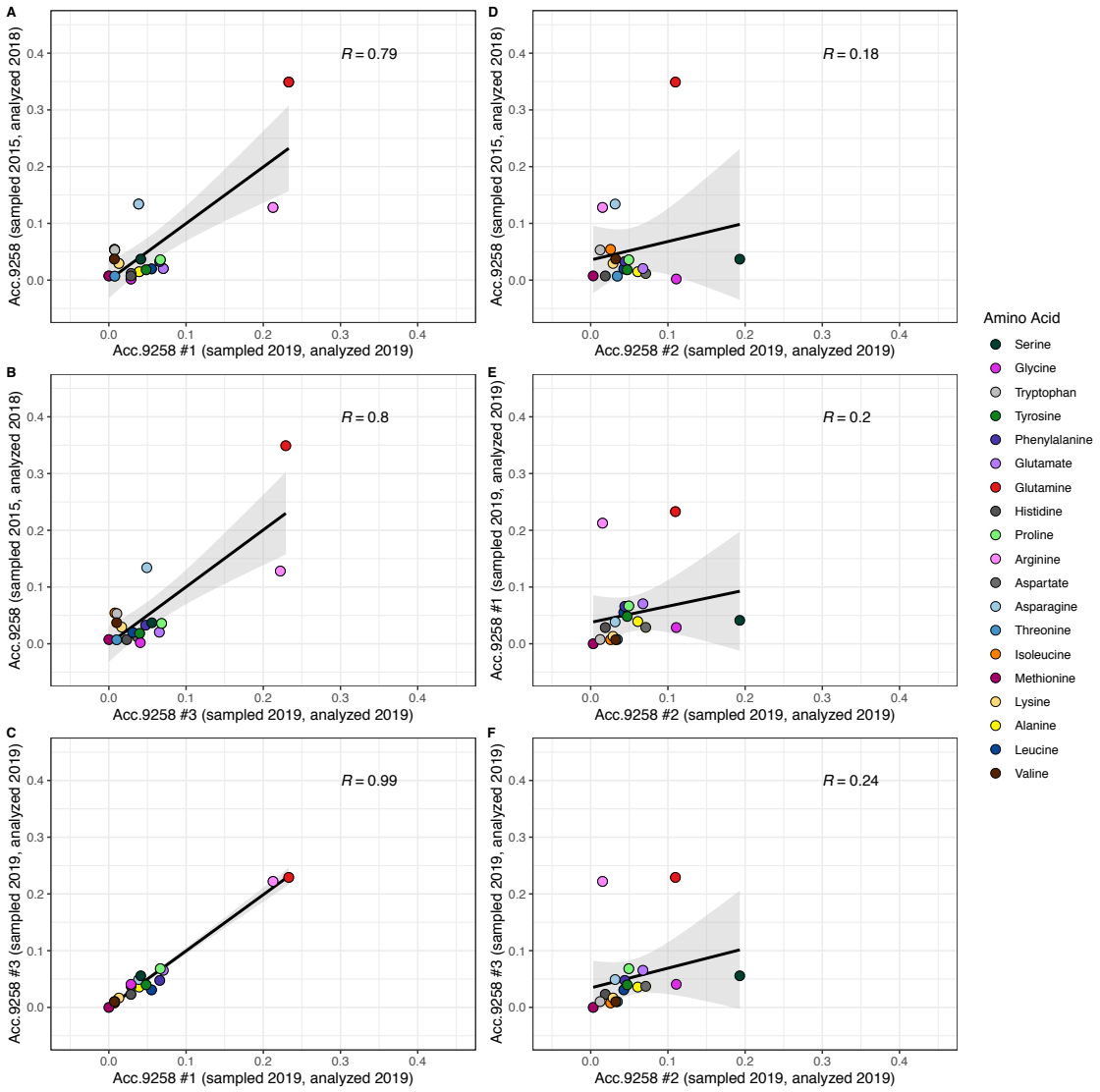

Figure 1.16: Correlation analysis between pooled *P. pittieri* (Acc. 9258) samples (1-3) and pooled samples individually compared to Acc. 9258 sampled at other timepoints. *P. pittieri* pooled samples #1 and #3 are highly similar (**C**) (R=0.99) and both sample #1 (**A**) (R=0.79) and sample #3 (**B**) (R=0.8) are similar to *P. pittieri* (Acc. 9258) sampled in 2015. However, *P. pittieri* pooled sampled #2 is not similar to either pooled sample #1 (**E**)(R=0.2) or #3 (**F**)(R=0.24) or *P. pittieri* Acc. 9258) sampled in 2015 (**D**)(R=0.18)

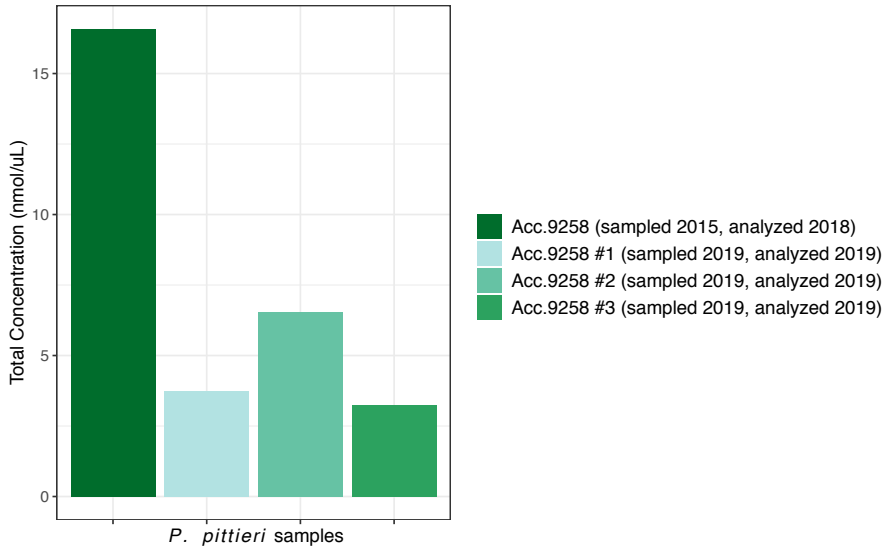

Figure 1.17: Total concentration (nmol/uL) for the *P. pittieri* pooled samples and *P. pittieri* accession #9258 analyzed in 2015. *P. pittieri* pooled #1 and #3 have roughly the same concentration, however *P. pittieri* pooled #2 is very different in total concentration further supporting conclusions that *P. pittieri* pooled sample is not part of the technical replicate samples and an error occurred.

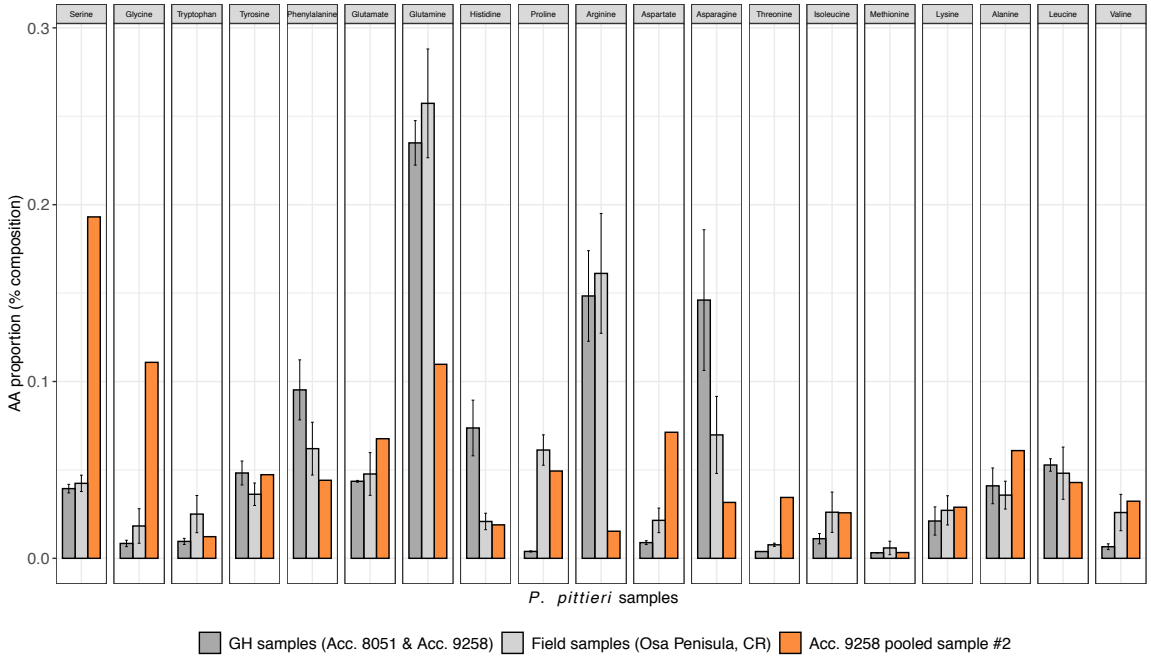

Figure 1.18: EFN amino acid composition for grouped greenhouse samples (*P. pittieri* pooled samples (acc. 9258), acc 9258 from 2015, acc 8051 from 2015) and *P. pittieri* field samples collected from the Osa Penisula, Costa Rica compared to *P. pittieri* (Acc. 9258) pooled sample #2 highlighting difference in composition for this sample that is dissimilar from any *P. pittieri* sample analyzed in this study.

DISCUSSION

Other studies have shown that EFN amino acid composition varies between species (Baker & Baker 1978) and that changes in nectar amino acid concentration can shift as a response to herbivory (Smith 1990), but that composition remains relatively constant (Gardener and Gillman 2001). In this study we reinforced these concepts with an in-depth examination of variation in EFN amino acid composition in genus *Passiflora.* Analysis of EFN amino acid composition within an individual over small timescales, comparison of two distant time points within a maintained environment, and comparisons

of distinct populations shows that EFN amino acid composition is unique for a species and for some species appears to be highly conserved.

Extrafloral nectar amino acid composition is different than floral nectar as has been shown as well as hypothesized due to different function. Side by side comparison in this study illustrate how different these two nectar compositions can be within a species, but also showed that this stark difference is not universal and should not be assumed for all species. *P. sprucei* showed high correlation between floral and extrafloral nectar for amino acid composition (**Figure 1.9**), the only difference noted between these samples is total concentration was 3x higher in the extrafloral nectar sample (**Figure 1.10A**), which is in line with other floral versus extrafloral comparisons made in this study. *P. sprucei* is understudied and a hypothesized host for only a few species of *Heliconius* that are single egg layers (Brown 1981, Penz 1995). This similarity in extrafloral and floral nectar could illustrate that only pollinator selection pressure is driving nectar amino acid composition and extrafloral nectaries do not function as a defense mechanism in this species, however this is speculative since little is known about the ecology of this species. In insectaries, *H. atthis,* an Ecuadorian species that specialized on *P. sprucei* is heavily attracked by egg parasitoids, thus it is likely that in nature this weak EFN attracts and supports small parasitoids but not ants.

P. auriculata (Acc. 9406) originally collected in French Guiana also shows high correlation between floral and extrafloral nectar composition (**Figure 1.9)** and again only deviates in total concentration (**Figure 1.10B**). In this case, the floral nectar is highly correlated to the extrafloral nectar of this individual as well as other *P. auriculata* EFN samples in this study with high proportions of alanine present. The floral sample for *P. auriculata* (Acc. 8028) does show similar high alanine composition and shows no correlation to *P. auriculata* (Acc. 8028) extrafloral nectar (**Figure 1.8**). It is possible that

P. auriculata (Acc. 9406) floral sample is actually a extrafloral sample that was also low in total amino acid concentrations, further sample of this individual would reveal whether an error occurred or floral nectar in this individual is actually highly correlated to extrafloral nectar composition for *P. auriculata.* Results for the pooled *P. pittieri* sample to determine technical error did illustrate that sampling error can result in disparate amino acid compositions due to contamination, this type of error is difficult to pinpoint if only one sample is representing a species or nectar type

Amino acid composition in *Passiflora* extrafloral nectaries varies across the genus and reflects the high diversity of EFN morphology in *Passiflora*. There are similarities in composition with closely related species (**Figure 1.5**) with 30% of the variation between species explained through subgenera classification, however it is unknown if further analysis of additional species within these subgenera would increase clustering by subgenus. There are species within each subgenus high in one amino acid leading to low EFN amino acid diversity that is potentially driving variation found between subgenera; glutamine in *P. oerstedii,* arginine in *P. sphaerocarpa*, and phenylalanine in *P.affinis and P. microstipula.*

The large portion of *Passiflora* greenhouse specimens originally collected in Costa Rica along with well documented *Passiflora-Heliconius* relationships with extensive field studies led to a focus on oviposition comparisons for this region. By sampling *Passiflora* species with known *Heliconius* species relationship (**Figure 1.1**), we are confident that the extremes of variation due to *Heliconius* oviposition pressure are captured in this study. In Costa Rica, *P. pittieri, P. vitifolia,* and *P. auriculata* are all host to *Heliconius* species that lay clusters of eggs on shoot meristems leading to high selection for attracting mutualistic defenders. Additionally, all of these species have documented high ant attendance in various field studies (Longino 1984, Smiley 1985,

Apple and Feener 2001). Few similarities between these 'highly selected' species in EFN amino acid composition (**Figure 1.6**) exist but all have medium to high diversity of that composition (**Table 1.6, Figure 1.4**). *P. pittieri* EFN composition is highest in glutamine and arginine, *P. auriculata* is highest in alanine and glutamine, and *P. vitifolia* has glutamine, phenylalanine, and tyrosine in the highest proportions. Only phenylalanine is categorized as stimulating sugar cells in insect chemoreceptors and therefore associated with an increased feeding response (Gardener and Gillman 2001), however, these taste classifications were originally described in flies. Both ant species-specific amino acid preferences (Blüthgen 2004) and preference for diversity of amino acid composition over specificity of composition (Blüthgen 2004, González-Teuber and Heil 2009) has been demonstrated. Diverse EFN amino acid composition with species-specific combinations of amino acids could lead to unique assemblage of defender communities for *P. pittieri, P. vitifolia,* and *P. auriculata*. On the other end of the spectrum*, P. oerstedii* is host to *Heliconius* species with single egg laying oviposition strategies (Benson et al. 1975) representing a species that can defend new shoots cheaply by attracting egg parasitoids. Supporting this categorization, *P. oerstedii* has been shown to have low ant attendance (Smiley 1978, Apple & Feener 2001). Glutamine was common in most *Passiflora* species EFN amino acid compositions however *P. oerstedii* had much higher proportions of glutamine leading to extremely low diversity of EFN amino acid composition in this species. This species also has low total EFN amino acid concentrations (**Figure 1.3)** however, so does *P. pittieri* and surprisingly but as stated before concentration can be highly variable. (Gardener and Gillman 2001) Since all conditions influencing concentration could not be controlled for in this study, fully elucidated the potential role of EFN amino acid concentration in mutualistic defender attraction cannot be considered.

Subgenus *Decaloba* has two clusters in the ordination plot with the majority of the species sampled in this study (*P. auriculata, P. jatunsachensis, P. rufa, P. misera, P. suberosa, P. tenuiloba*) grouping due to contributions of alanine, leucine, and serine to their individual compositions. Of these amino acids, leucine is the only one classified as stimulating a chemoreceptor response in insects as a sweet stimulatory (Gardener and Gillman 2002). These six species within subgenus *Decaloba* all exhibit high diversity in EFN amino acid composition. *P. affinis* and *P. microstipula* are unique in subgenus *Decaloba*, as well as within the *Passiflora* genus, with high proportions of amino acids derived from the shikimate pathway, (phenylalanine, tryptophan, and tyrosoine) in their EFN amino acid composition. These two species are not closely related within the subgenus *Decaloba* and differ in nectary type and habitat, the only commonality is they are both host to solitary egg laying primitive *Heliconinii* species. *P. affinis* has small laminar nectaries and is found in river valleys of Texas and northeastern Mexico (Ulmer and MacDougal 2004) where it is host to *Agraulis vanillae*. *P. microstipula* with 6-8 large paired petiole nectaries and inconspicuous laminar nectaries is found in humid, tropical forests of southern Mexico (Gilbert 2000) where it is host to *Euides lineata* (Mallet and Longino 1982). An EFN amino acid composition high in products of the shikimate pathway is of interest due this biosynthetic pathway only being present in bacteria and plants, where the aromatic amino acid products are precursors to secondary metabolites (Herrmann 1995), and classification of aromatic pathways as essential in animals due to lack of pathway. Also, phenylalanine has been shown to elicit ‘sweet cells’ in insect chemoreceptors (Gardener and Gillman 2002) and is the most abundant amino acid in floral nectar of bee-pollinated plant species and a noted phagostimulant (Inouye 1984, Petanidou 2007). However, these *Passiflora* species being hosts to single egg laying *Heliconius* with no record of strong associations with mutualistic defenders or

visitors to nectaries leaves several questions about the drivers leading to this composition in these species.

Species classification significantly influenced grouping of samples and explained 53% of the variation between samples. *Passiflora* species sampled were all maintained under greenhouse conditions and only consist of clones or individuals from only a few original locations. Therefore this species grouping may be artificial and not fully capture variation within a species across its range. Field sampling of two disjunct populations of *P. vitifolia* (**Figure 1.14**) and *P. auriculata* (**Figure 1.13**) offers a more in-depth analysis of potential intraspecific variation. In both *P. auriculata* and *P. vitifolia,* sampling location failed to explain variation between samples and both species showed high correlation between populations. These patterns may reflect the similarity of herbivore interactions for both *Passiflora* species between Atlantic and Pacific lowlands of Costa Rica. *Heliconius sara* is the primary herbivore of *P. auriculata* across its range. It's group egg laying strategy, without mutualistic defenses of its host plant, has the potential to greatly reduce seed production. *P. vitifolia* in Costa Rica utilized by oligophagous *Heliconius* single egg-laying speices but occasionally attracts masses of eggs from the heliconiine *Dione juno* (Smiley 1978, Gilbert, personal observations). Comparisons of these field samples with greenhouses samples of the same species also show high correlation, however, greenhouse individuals for these species were collected in the same region. Additional sampling of individuals of one species over their entire range would lead to stronger conclusions that support EFN amino acid composition as species-specific, which has already been demonstrated for floral nectar across genera (Gardener and Gillam 2001).

Variation within an individual between nectary types was low in species where this could be examined (**Figure 1.11**). This is not surprising due to shared developmental

pathways, physiology, and nectar production for all EFNs of a species. However, variation within an individual's EFN amino acid composition across small time-scales was higher than expected. *P. auriculata* (acc. 8028) sampled on 12/21/15 showed an increase in proline and on 09/08/16 an increase in glutamate. However these increases seem ephemeral in nature due to samples on 12/24/15 and 09/09/16 not capturing these increases. Variation seen in *P. affinis* (**Figure 1.12D**) may capture more permanent shifts in composition due to plant age. This sample was grown from seed in late 2016. Samples from 02/27/18 and 06/18/18 show the decrease in aromatic amino acids, specifically phenylalanine, countered with an increase in glutamine in 02/27/18 and proline in 06/18/18. *Dilkea* had the largest variation in composition between two consecutive time points, 02/25/17 and 02/26/17. Nectaries for this species are extremely short lived and only present on the leaf tip of newly developing leaves. Confirmation that this smaller time scale variation is capturing true EFN amino acid composition fluctuations and are not a product of error would require consecutive sampling over set time frames. Less variation was seen at more distant sampling time points in comparison of samples from 1988 and current samples (**Figure 1.15**). The outlier in these comparisons, *P. quadrangularis,* showed extremely different compositions when sampled in 1988 by J. Lanza than our analyzed sample. The individual sampled for this species in 1988 could have originally been collected in either Costa Rica or Brazil based on accessions located in the greenhouse at that time. The individual sampled for this work was collected in Brazil. This species was also noted as having low ant attendance regardless of having fairly large nectaries (Smiley 1985). Disparate results for *P. quadrangularis* could be error in one of the analyses, true variation of species across its geographic range, or that higher EFN variability is present in species where function isn't related to defense. The other 5 species comparisons across this 30-year timespan show high correlation despite

potential differences in sensitivity of HPLC analyses due to technological advances. Higher variability in small time-scale comparisons could capture the amount of variability that possible in an individual but the high correlation between individual samples from a larger time scale reinforces that these shifts are transitional and that selection pressures and genetic processes (Baker and Baker 1977) will maintain a specific EFN amino acid composition for a species.

Chapter 2: Ant trials with artificial nectar based on known *Passiflora* species EFN amino acid compositions

INTRODUCTION

As stated in Chapter 1, EFNs are indirect defense utilized by numerous plant species (McLain 1983, Rudger 2004, Rudgers & Gardener 2004, Oliveira & Freitas 2004, Heil & McKey 2003, Sendoya et al. 2009) to attract mutualistic defenders that will deter oviposition or actively remove herbivore eggs and larvae. The magnitude of effect of this defense can vary with the intensity of herbivore pressure (de la Fuente et al. 1999), environmental conditions (Heil & McKey 2013), composition of local defender community and overall abundance of defenders (Bentley 1976, Koptur 1984, Heil 2004). Components of nectar chemistry (amino acids and sugar ratios), as well as interactions between these components could be driving the type of mutualistic defender attracted to the EFN, as well the strength of that attraction.

Extrafloral nectaries are similar to floral nectaries in both structure and biosynthetic pathways, but vary in function and chemical composition. Floral nectar is associated with pollination and generally differs in sugar type present, type of amino acids present, and overall concentration of amino acids (Baker et al. 1978). Floral nectar tends to be high in a single amino acids, proline is attractive to bees and essential for the energy requirements of flight (Inouye 1994, Teulier 2016), and lower than extrafloral nectar in concentration of total amino acids(Baker et al. 1978, Koptur 1994, Escalante-Pérez 2012a).

Within P*assiflora*, extrafloral nectary morphology is diverse in relation to size of nectaries, number of nectaries, placement of nectaries, etc. In Chapter 1, we illustrated

that extrafloral nectar amino acid composition is as varied as EFN morphology. *Passiflora* species varied in amino acids at higher concentrations within their nectar as well as overall evenness of composition of the nectar. Several species analyzed in Chapter 1 have well documented associations with specific *Heliconius* species and mutualistic defenders. Apple and Feener (2001) surveyed ant abundance on *P. oerstedii* , *P. biflora* and *P. auriculata* in paired association on successional strips at La Selva Biological Station in Costa Rica. Termite bait placed on individual plants to be found by patrolling ants were infrequently recovered on *P. oerstedii*, which possess small numerous paired petiole nectaries and had less observed visitors overall. In contrast, high visitation rates were recorded for *P. auriculata* with active removal of termites and fluctuation in visitation when extrafloral nectaries were blocked (Apple and Feener 2001). Smiley (1978) characterized ant attraction for *P. auriculata, P. biflora,* and *P. oerstedii* as high, medium, and low based on ant visitor frequency observations also performed at La Selva. Based on field observations, most single eggs laid on *P. oerstedii* are killed by parasitoids (Gilbert, personal obs.). Additionally, Smiley (1985) observed lower ant attendance on *P. quadrangularis* compared to *P. vitifolia*, but both of these species are vines with large, conspicuous petiole nectaries. Smiley postulated that this difference in ant attendance could be due to *P. vitifolia* having higher nectar concentration of total amino acids or perhaps a different composition of amino acids.

Artificial nectar with amino acid compositions similar to *P. auriculata, P. vitifolia,* and *P. oerstedii* were constructed based on Chapter 1 results and presented to ant species known to visit or defend extrafloral nectaries of these species in the field determine if EFN amino acid composition is a driver behind variation in visitation of these species.

METHODS

Species of interest

Crematogaster, acrobat ants, are known visitors to extrafloral nectaries and actively forage *P. auriculata* at higher rates than *P. oerstedii* (Apple and Feener 2001). Foragers of this species are known to recruit nearby ants when prey is located. *Pseudomyrmex* are solitary foragers and will actively return to a known food source but will not recruit nestmates.

Colony structure

Colonies for *Pseudomyrmex gracilis* were collected alive by relocated nests found in bamboo ends or dead limbs in greenhouses at UT campus and Brackenridge Field Laboratory in Austin, Texas. *Crematogaster laeviuscula* colonies were collected by placing bamboo segments with a hole drilled in one end near a known colony. *Crematogaster* species are polydomous nest builders, so workers will actively move larvae into introduced suitable alternate domiciles. These bamboo domiciles were placed on greenhouse beams and in nooks of oak trees at Brackenridge Field Laboratory, Austin, TX, USA, near identified active nests. Collection of these bamboo domiciles occurred 1-2 weeks after initial use [awareness] by the colony to allow for maximum occupancy and normalized colony behavior. Some species of *Crematogaster* ants have an alternative colonization strategy where larger female workers are capable of producing unfertilized eggs that develop into males in colonies that do not have a queen. This ensures normal colony behavior once these bamboo domiciles have been collected and placed in the lab. All colonies were given a two-week adjustment period before inclusion in trials. Colonies were removed from further trials if colony size was low and abnormal behavior or low response to trials was observed.

All ants were housed in Fluon™ lined plastic containers in the lab with their collected natural nesting material or the introduced bamboo shoot as a permanent nesting chamber. A feeding chamber was constructed with an aluminum artificial bridge connected to a second plastic container were trials occurred to mimic natural foraging behavior. In the feeding container, ants were provided ad libitum access to a 20 % sugar water solution in a cotton-plugged test tube replaced once per week, colonies were also fed a dead cricket once per week. Colonies varied in size, both between and within species.

Trial set-up

Trials occurred three times per week with 1-2 days between trials to decrease over-consumption of a particular solution skewing behavior and solution choice. According to Blüthgen 2004, preference for amino acid test mixtures shifted if same mixture was presented over a short time period. All colonies received the same trial for any given trial date to minimize between colony shifts in preference due to previous trial or external factors. For each trial, a colony received 2 solution choices along with a sugar solution control in the lid of an Eppendorf tube placed in a petri dish. Each lid was labeled A-C for each colony sample plate with solutions were randomized each trial. Sample plates allowed 4cm separation between solutions. Each labeled lid was weighed before and after the addition of solution.

Trials occurred during hours of diurnal activity (9am-5pm), with each trial consisting of counts of individuals at each solution for each sample plate at 15 minutes intervals for 2 hours. Sample plates were introduced 30 minutes prior to start of trial to allow stabilization of visitation to nectaries. Solutions were weighed after the trial providing a measurement of consumption. Evaporation was assumed to affect all

solutions equally, as shown by Rathman et al. (1990) and was calculated with a control of each solution placed alongside the trial containers.

Experimental solutions were built from EFN amino acid composition determined for specific *Passiflora* species in Chapter 1. Trial solutions (**Figure 2.1A)** were determined by variation in EFN visitation to *Passiflora* species from previous studies (Apple and Feener 2001, Smiley 1986) with (high/low) EFN amino acid solutions based on concentrations for *P. vitifolia* and *P. oestedii* (**Figure 2.1D)** collected in Chapter 1, and deconstruction of amino acid composition of *P. auriculata* due to known strong association with mutualistic defenders (**Table 2.1)**. In Chapter 1 amino acid nectar chemistry ranged from less than 10 up to 450 nmol/uL or mM. Both Lanza (1993) and González-Teuber (2009) found that amino acid concentration influenced attraction of ant species and low amino acid concentrations resulted in a lack of preference, leading to a target concentration of ~60 nmol/uL in this study except in the specific low conc. trial (**Table 2.1**). Artificial solutions mimicking *P. auriculata, P. oerstedii,* and *P. vitifolia* were confirmed for amino acid composition through HPLC analysis through Texas A&M Protein Chemistry Lab alongside samples from Chapter 1 (**Figure 2.1A**) .

Statistical analysis

Data table formatting with tidyverse and all statistical analyses were performed in R version 3.6.1 used with R studio version 1.2.5001 (R Development Core Team 2019) with additional packages listed below, and graphically visualized with ggplot (Wickham 2016) with additional formatting and layout style through cowplot (Wilke 2019). Pairwise Wilcoxon comparisons on percent of nectar removed for each trial type were computed for the two ant species using ggpubr (Kassambara 2019) function compare_means. All colonies for both ant species were given the same trial at the same

time. Comparisons between trials or between ant species was not suitable due to variation in colony size and differences in foraging behavior between species.

RESULTS

At concentrations similar to *P. vitifolia* from Chapter 1 EFN amino acid concentrations, *Crematogaster* showed preference for artificial nectar similar in composition to *P. vitifolia* over a plain sugar solution (p=0.0114) as well as preference for *P. vitifolia* solution over the *P.oerstedii* solution though not significantly (p=0.081) (**Figure 2.2**). No preference any solution containing amino acids was detected when concentrations were similar to those found in *P. oerstedii* EFN concentration from Chapter 1. *Crematogaster* also preferred artificial nectar composed of 4 amino acids similar to *P. auriculata* over a plain sugar solution (p=0.0096) (**Figure 2.2)**; as well as, the *P. auriculata* mixed amino acid solution over just alanine at concentrations similar to *P. auriculata* (p=0.0224)(**Figure 2.2**). *Pseudomyrmex* strongly preferred artificial nectar solution similar in composition to *P. vitifolia* over a plain sugar solution (p=9.1e-6) (**Figure 2.2**) and artificial nectar solution similar in composition to *P. oerstedii* (p=3.9e-6) (**Figure 2.2**) at concentrations seen in *P. vitifolia* (Chpt 1). *Pseudomyrmex* also didn't show any preference when amino acid solutions were at concentrations seen in *P. oerstedii* (Chpt 1). *Pseudomyrmex* showed little to no preference for solutions containing alanine regardless of the number of amino acids present in the solution.

CONCLUSIONS

Evidence of relationships between *Passiflora* species and specific mutualistic defenders had been shown (Smiley 1986, Apple and Feener 2001) but the drivers behind those relationships were unknown. These preference trial experiments show how EFN amino acid composition could be responsible for these relationships.

As seen in **Figure 2.2**, *Crematogaster* shows preference towards artificial nectar closely resembling EFN nectar of *P. auriculata* in amino acid composition over a control sugar solution and artificial solution containing only the main amino acid found in *P. auriculata* solution. These results confirm similar outcomes obtain in Gonzalez-Teuber and Heil (2009) that a richer composition of several amino acids is preferred over solutions of 1 or 2 amino acids. We found that actually a single amino acid (alanine) or two amino acids (alanine + glutamate) at concentrations similar to those found in *P. auriculata* failed to elicit any preference over the control sugar solution in either *Crematogaster* or *Pseudomyrmex*. Interestingly, *Pseudomrymex* showed little to no preference to any solution containing components similar to *P. auriculata* regardless of richness or number of amino acids present in the solution, but this is in-line with Apple and Feener (2001) who reported low counts of *Pseudomyrmex* visitations to *P. auriculata* in the field, however they accounted that to foraging behavior.

Artificial nectar similar in composition to EFN of *P. vitifolia* was significantly preferred over just sugar by both *Pseudomyrmex* and *Crematogaster* when at higher concentrations. Both ant species also appears to favor the artificial *P. vitifolia* over the *P. oerstedii* nectar but only significantly by *Pseudomyrmex* (**Figure 2.2**). Glutamine is the dominant amino acid in extrafloral nectar of both *P. vitifolia* and *P. oerstedii* but differ in proportion of each species total amino acids. In P. *oerstedii*, glutamine is ~60% with proline as the second highest at 20% with most other amino acids at trace amounts

leading to a low EFN nectar composition richness (Shannon diversity index=1.56) (**Figure 2.1A, C**). Glutmaine in *P. vitifolia* is about ~20% of the total composition with several other amino acids (phenylalanine, tyrosine, arginine, leucine) at 10-15% leading to a more even composition (Shannon diversity index=2.19) (**Figure 2.1A, C**). Both the artificial *P. oerstedii* (5.01nmol/uL) and *P. vitifolia* (4.71 nmol/uL) nectar at low concentrations nectar failed to elicit a preference response from *Pseudomyrmex* or *Crematogaster* when at concentrations similar to those found in actual *P. oerstedii* (6.36 nmol/uL) nectar samples (**Figure 2.1D**). When artificial *P. oerstedii* was presented at concentrations similar to other *Passiflora* known to have associations with mutualistic defenders (*P. vitifolia*), there was only a slight preference over the sugar control by *Crematogaster* (**Figure 2.2**). Apple and Feener (2001) and Smiley (1978) had both noted low ant attendance of *P. oerstedii* but couldn't expand on whether this was due to nectar morphology, secretion rates, or nectar composition. Our results show that the low concentration and low diversity of composition of *P. oerstedii* extrafloral nectar contribute to the failure of *P. oerstedii* extrafloral nectaries to attract ant species as mutualistic defenders. Smiley (1978) suggested that these nectaries may be attracting parasitoid insects instead of ants, the low total amino acid concentration and high glutamine could support this hypothesis since glutamine has been shown to significantly increase late instar and pupal formation in lab reared egg parasitoid, *Edovum puttleri* (Hu 2000).

Table 2.1: Artificial nectar solutions for ant preference trials. Sugar control is a 25% sugar solution based on *Passiflora* EFN sugar concentrations and ratios (Lanza 1988). All sugar, amino acid, and total values are nmol/uL (mM). Low conc. for *P. vitifolia* and *P. oerstedii* are based on actual EFN amino acid concentrations found in *P. oerstedii* in Chapter 1.

	sugar control	**vitifolia**		**oerstedii**			**auriculata**	
		avg conc.	**low conc.**	**avg conc.**	**low conc.**	**4 AA**	**ALA only**	**ALA + GLU**
sucrose	315.51	315.51	315.51	315.51	315.51	315.51	315.51	315.51
glucose	499.56	499.56	499.56	499.56	499.56	499.56	499.56	499.56
fructose	294.18	294.18	294.18	294.18	294.18	294.18	294.18	294.18
alanine	-	-	-	-	-	27.72	36.48	27.84
glutamine	-	23.22	1.39	56.64	3.40	-	-	-
arginine	-	10.56	0.63	-	-	-	-	-
tyrosine	-	9.60	0.58	8.39	0.50	5.24	-	-
phenylalanine	-	9.38	0.56	3.75	0.23	7.63	-	-
leucine	-	11.81	0.71	-	-	-	-	-
histidine	-	7.67	0.46	-	-	-	-	-
glutamate	-	6.25	0.38	-	-	8.84	-	8.84
proline	-	-	-	11.64	0.70	-	-	-
tryptophan	-	-	-	3.08	0.18	-	-	-
serine	-	-	-	-	-	-	-	-
total_aa	-	78.50	4.71	83.51	5.01	49.43	36.48	36.74
total	1109.25	1187.75	1113.96	1192.76	1114.26	1158.68	1145.73	1145.99

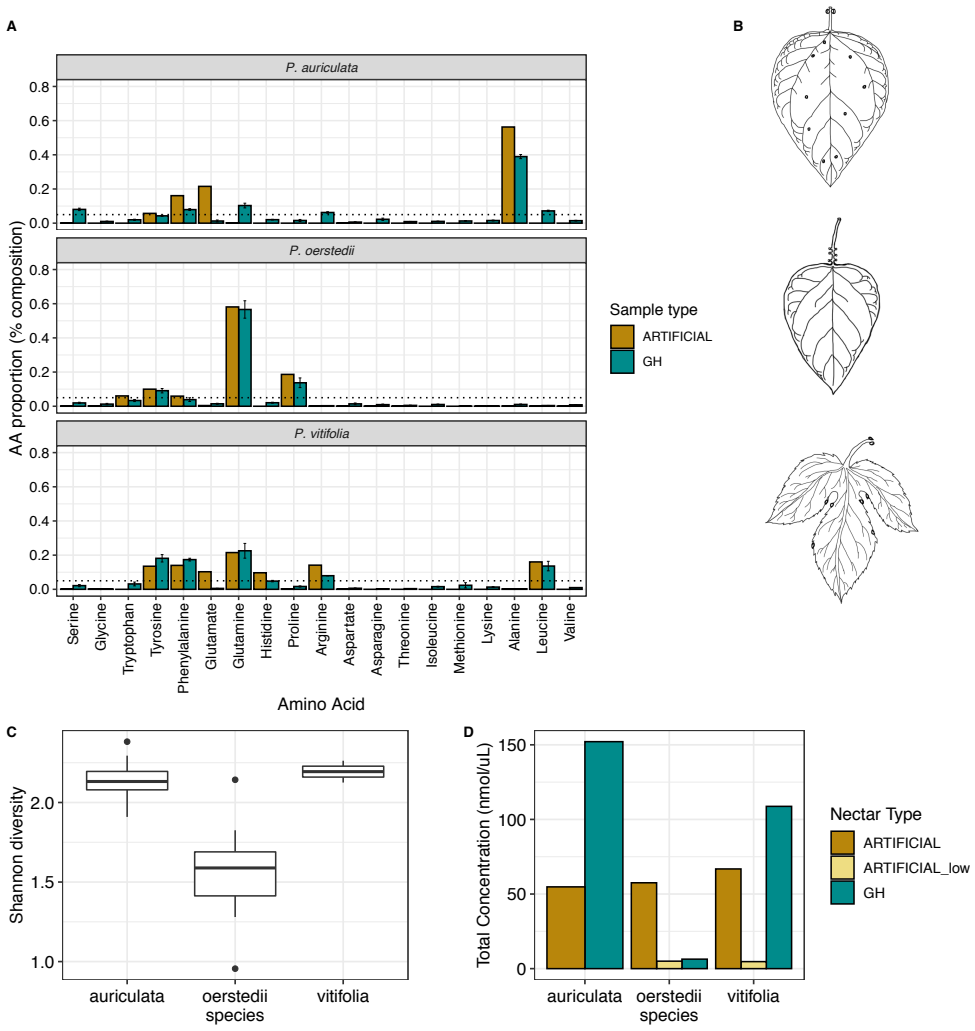

Figure 2.1: **A**. Amino acid composition for artificial nectar and actual nectar collected from greenhouse specimens for Chapter 1. Artificial solutions were analyzed through HPLC to confirm composition. Error bars represent standard error. **B**. Line drawing of nectary locations for *P. auriculata, P. oerstedii,* and *P. vitifolia.* **C**. Shannon diversity indices for *P. auriculata, P. oerstedii,* and *P. vitifolia* calculated in Chapter 1. **D**. Total nectar amino acid concentration for artificial samples compared to actual nectar amino acid concentration collected from greenhouse specimens for Chapter 1, additional low concentration for *P. oerstedii,* and *P. vitifolia.*

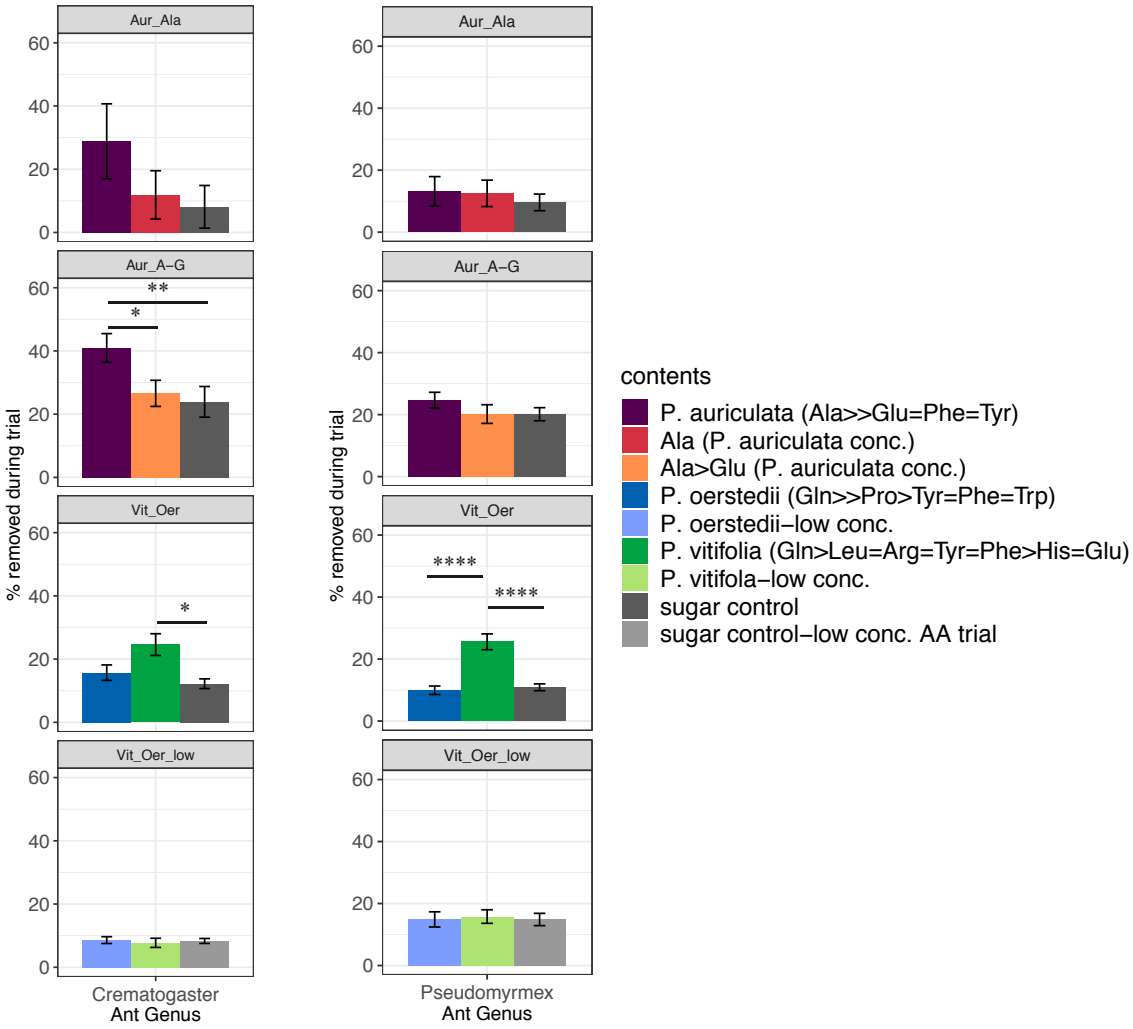

Figure 2.2: Ant preference trials with artificial nectar solutions on species *Crematogaster* and Pseudomrymex. Significance of pair-wise Wilcoxon signed-rank test (* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$).

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