DIEL PATTERNS OF INSECT HERBIVORY AND PLANT SECONDARY METABOLITES IN UNDERSTORY SHRUBS ON BARRO COLORADO ISLAND

BY

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THESIS

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ABSTRACT

Plants produce a broad diversity of secondary metabolites as defenses against herbivory. In response, herbivorous insects have evolved a diversity of behavioral and biochemical counter-adaptations to these defenses. Despite that a significant portion of leaf area removal by insects in the tropics occurs at night, virtually all studies of chemically mediated interactions between herbivorous insects and their host plants have been conducted primarily or entirely during the daytime. Accordingly, I set out to quantify if rates of herbivory differ between the day and night. I used leaf photographs and single plant herbivore exclosures on 126 individual plants of four species in the genera *Piper* and *Psychotria* on Barro Colorado Island, Panama, to quantify the timing of herbivory over the course of 56 diel cycles. I found that on young leaves, protecting plants from herbivores during the night causes significantly fewer leaves to be damaged than if plants are protected only during the day but are exposed at night. I then characterized qualitative differences in the secondary metabolite profiles of the leaves of these plant species during daytime and nighttime hours using ultra high-performance liquid chromatography (UHPLC), electrospray ionization and molecular fragmentation, and tandem mass spectrometry (MS/MS). Network analyses show that plant secondary metabolites varied greatly in their presence in leaves over the course of several hours, suggesting that ecologically significant differences exist in the overall chemical profile that herbivores would encounter in leaves during different times of a day. Whether these variations in putative defense compounds may affect the foraging times and behaviors of herbivorous insects remains unresolved.

Most larval herbivores lack an effective means for dispersing to new hostplants. Therefore, feeding strategies that maximize assimilation and growth, and minimize time to pupation, are expected to be most advantageous. However, larval lepidopterans exhibit numerous

behaviors that appear to interfere with maximizing the rate of foliage consumption. Caterpillars may limit their foraging times to specific periods of the diel due to uneven predation risk, to predictably variable abiotic (especially temperature and humidity) conditions, or to short-term variation in forage quality. While daily variation in forage nutrient levels and secondary metabolite concentrations has been well-studied in numerous systems, the influence of such variation on the daily rhythms of herbivore feeding activity has not yet been determined. I propose that the highly sensitive and specialized senses of olfaction and gustation in herbivores suit them well for limiting their feeding bouts to times of the day when plants are less well-defended (which I propose is the night). Such behavior is especially likely to occur in environments where there is relatively low variation between daytime and nighttime temperatures and humidity and where predation risk is always relatively high, or even higher during the nighttime than the day, as is likely the case in many tropical forests.

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CHAPTER I: QUANTIFICATION OF DAYTIME AND NIGHTTIME HERBIVORY RATES ON FOUR SPECIES OF UNDERSTORY SHRUBS ON BARRO COLORADO ISLAND

Introduction

The simple yet profound observation that the world is green (Hairston *et al.* 1960) is abundantly apparent in tropical forests. In this context, Feeny (1975) called herbivory "a conspicuous non-event" in that insects are rarely observed in the act of feeding. In the Neotropics, despite abundant signs of insect damage, this view of herbivory may be even more pronounced than in temperate regions. Herbivores may be particularly inconspicuous in these forests for three reasons. First, an estimated 80% of total leaf area removal by herbivores in a lowland tropical forest occurs during a relatively narrow window, *i.e.*, when a leaf is young and still expanding (Coley 1982). Second, leaves of most evergreen tropical plants are relatively long-lived, with some living up to seven years or more (Coley and Barone 1996). Third, there are abundant anecdotal observations of reclusive behaviors exhibited by herbivores that reduce the likelihood of being observed, including feeding nocturnally (*e.g.*, Windsor 1978, Reagan *et al.* 1996, Miller *et al*. 2006, Wagner 2005). In their book *100 Caterpillars*, Miller, Janzen, and Hallwachs (2006) conclude an introductory discussion of herbivory by caterpillars in a Costa Rican forest with the statement "Some species of caterpillars feed in daylight hours, but a very large number perch motionless, not feeding, during the day and feed only at night. Presumably the daytime inactivity is to avoid being seen by predators" (p. 16). Nearly all authors invoke a top-down explanation for this observation, with visually oriented predators of herbivores assumed to be a stronger force on

the evolution of feeding rhythms than circadian rhythms of plant secondary metabolites (Hairston *et al.* 1960, Hassell and Southwood 1978, Heinrich 1979).

Secondary plant metabolites known or assumed to be defenses against herbivory are often present in lower concentrations during the night (Fairbairn and Suwal 1961, Fairbairn and Wassel 1964, Robinson 1974, Wink and Witte 1984, Okolie and Obasi 1993, Morandim *et al.* 2005, Kim *et al.* 2011, Goodspeed *et al.* 2012, Goodspeed *et al.* 2013). Therefore, nocturnal herbivores might benefit from feeding during the scotophase (from Greek, *scoto-* "darkness"; *i.e.* the night of a natural diel cycle).

Potential mechanisms whereby plants could drive herbivore feeding rhythms to be nocturnally biased include some plant chemical defenses that may be compromised due to their temporally-constrained reliance on active photosynthesis (Arimura *et al.* 2008), rapid turnover or short half-lives such as in many active alkaloids (Robinson 1974, Wink and Witte 1984), or phototoxicity (Berenbaum 1995). This pattern is consistent with observations from the Luquillo Experimental Forest in Puerto Rico; as Reagan *et al.* (1996) write, "Most of the herbivory that occurs in the forest seems to be performed by nocturnal insects, but no quantitative assessment is available" (p. 469).

The longstanding assumption that more insect herbivory happens at night due to lower predation risk (Hassell and Southwood 1978, Heinrich 1979) may not apply in all communities. In New Guinea, the night is a relatively enemy-free time in terms of predation by invertebrates, but herbivore abundance is roughly three times greater on leaves during the day (Novotny *et al.* 1999). On Barro Colorado Island (BCI) in Panama, insectivorous gleaning bats exert stronger predation pressure on herbivorous insects than birds (Kalka *et al.* 2008). Collectively, these

observations suggest that factors other than predation may drive diel foraging rhythms of herbivorous insects.

Several significant gaps exist in our understanding of diel patterns of herbivory in the tropics. It is widely observed that more herbivory seems to occur during the night, but I know of no quantification of this observation in an intact natural community. Furthermore, the general assumption that this pattern exists because predation risk to herbivores is lower during the night remains relatively untested. Plants are physiologically active organisms, and their quality to an herbivore, with regard to their nutritive and anti-nutritive properties, cannot be assumed to be stable over short periods of time, such as a single diel cycle. That the timing of herbivory may be driven by plants is another relatively untested hypothesis that I sought to explore.

I examined diel patterns of herbivory on congeners in the 'species swarms' *Piper* and *Psychotria*, in association with diel patterns of plant chemistry. Because of the challenges posed to classical niche theory by the coexistence of highly speciose tropical plant 'species swarms,' these genera provide an ideal opportunity to determine whether phytochemical divergence is associated with different specialized herbivore guild dynamics, and therefore niche partitioning in plant enemy space (*sensu* Gentry 1982, and see Sedio *et al.* 2012, Sedio *et al.* 2017). By quantifying herbivory in a manipulative experiment on BCI, I tested the hypothesis that a greater proportion of herbivore damage is inflicted on foliage during nighttime hours than during daylight hours.

Materials and Methods

Study site

In March 2015, I set up a manipulative experiment on Barro Colorado Island (BCI; 9º 09' N, 79º 51'W), Panama. The site is lowland moist tropical forest, with an annual rainfall of 2612 mm and a distinctly seasonal pattern of rainfall, with 90% of the total moisture arriving in May-November (Windsor 1990). The dry season is generally from early January to late April, with the onset of heavier rains usually beginning in late April or early May (Windsor 1990).

Study species natural history

I selected small understory plants for my observations and quantification of herbivory, with sufficiently few leaves per plant (4-46 leaves/plant) so that I could number and keep track of all leaves in the study over the course of several months. Along a ~1.6 km route on pre-established trails, I searched for plants in the genera *Piper* (Piperaceae) and *Psychotria* (Rubiaceae). The two most important criteria that I used to select my focal species were that 1) it was locally abundant enough that I could survey at least three individuals of similar size within a small area (roughly 2 m radius), and 2) its growth form permitted the positioning of a camera so that images could be captured without damaging the leaves. I also spent time searching for active herbivores at various times of the day and night on plants in these genera, to improve my chances of collecting data on foliage removal rates.

Plants from two species each of two genera (24 *Psychotria marginata*, 42 *Psychotria limonensis*, 24 *Piper cordulatum*, 36 *Piper aequale*) were selected as representative understory shrubs. The *ca.* 20 species of *Psychotria s. l.* on BCI are mostly highly shade tolerant, and show variations in their drought tolerance, though all are relatively good at surviving periodic droughts. The rhythms of leaf production and flowering phenologies are driven by rainfall seasonality to

varying degrees (Wright 1991; Wright *et al.* 1992). Being an understory shrub – being slow-growing and light-limited – means that tissue loss to herbivores is predicted to be costly, so investment in appropriate chemical defenses against herbivores should be "optimized" in these plants. This has indeed been shown to be the case in the most abundant *Psychotria* on BCI, *P. horizontalis* (Sagers and Coley 1995). The coffee family (Rubiaceae), and the *Psychotria* lineage specifically, is rich in bioactive secondary metabolites, especially in alkaloids. Of the literature reviewed by Martins and Nunez (2015) (Rubiaceae phytochemical studies between 1990 and 2014), 34 species of members of the Psychotrieae tribe have been the subjects of natural products investigations, and genera in this tribe have elevated alkaloid diversity relative to other tribes in the family (Martins and Nunez 2015). Herbivores on *Psychotria* are relatively diverse, but not well characterized for the site. I observed larvae of the lepidopteran families Sphingidae (specifically Macroglossinae: *Xylophanes* spp.) and Crambidae (specifically Spilomelinae: *Desmia* spp.) to be major herbivores on *Psychotria* on BCI.

The pepper family (Piperaceae) genus *Piper* has been extensively and intensively studied, and has been thoroughly developed as a model system for tropical ecology (Dyer and Palmer 2004). On BCI, *Piper aequale* is one of the most abundant species in the genus (Thies and Kalko 2004). I have observed that on BCI, *Piper aequale* sustains relatively high amounts of folivory, mostly from larvae of Geometridae (*Eois* spp.) moths, and from weevils (Curculionidae). Both *Piper aequale* and *Piper cordulatum* are shade-tolerant understory shrubs, producing few leaves over the course of a year, but at a fairly constant rate (*i.e.* no major leaf flushing is observed for these species) (Thies and Kalko 2004). The flowering time of these plants is mostly synchronous on BCI, and coincides with the transition from the dry season into the wet season (Thies and Kalko 2004).

Leaf photographs

Every leaf on each plant was numbered, tagged, and photographed prior to the experiment for area calculation. I used a fluorescent light in a ~35x35 cm plastic box, to illuminate the leaves from below and accentuate their outlines and any internal holes. An opaque white sheet of plastic with a 1x4 cm black scale bar was held on top of this light box and then the leaf was positioned on this background. A second sheet of translucent, faintly speckled plastic was then placed on top of the leaf and the background stage. This top layer served to hold the leaf flat and coplanar with the scale bar, as well as to minimize possible glare in the photos. While all four of these components were held together with the help of an assistant, I photographed the leaf with a Nikon D3000 camera with an 18-55 mm Nikon lens.

In total, 1767 leaves were photographed prior to herbivore exclusion; 474 new leaves that grew over the two-month study period were added. At the end of that period, there were 130 fewer original leaves in the census (Table 1.1). The fate of most of these leaves was senescence (an El Niño Southern Oscillation climate pattern this summer caused drier than average conditions over the study period (Paton 2016)). In cases where insect herbivores definitively caused the complete removal of entire leaves, this loss was included in the analysis as area lost due to herbivory. For the leaves that grew during the experiment ("new" leaves), initial images do not exist, but the effect of treatment on herbivory is most clearly seen on these leaves that initially had no herbivore damage. Since most herbivory occurred on young expanding leaves, this subset of leaves that grew during the experiment is the focus of the brief analysis reported.

Herbivore exclusion treatments

I selected groups of three individual plants per species and randomly assigned each plant to one of three treatments (42 total replicates unequally distributed across 4 species). The control

treatment consisted of plants that were always open to herbivores but were shaded overhead by mosquito net mesh. Mosquito nets ("Baby Mosquito Net" purchased from El Costo, Panama) were suspended over each individual treatment plant and closed at the base, to exclude herbivores during a specific time of day. Each treatment group comprised an individual always accessible to herbivores (Control), one always inaccessible to herbivores during the night (Night exclusion), and one plant always inaccessible to herbivores during the day (Day exclusion). Each exclusion net was opened and/or closed and switched daily before dawn and during sunset, meaning that the access of herbivores to plants was always restricted to only the day or only the night.

More specifically, every dawn for two months, I began a circuit of plant checking and exclusion net manipulation at around 4:30 am. Sunrise over the two-month experiment, from 7 April to 7 June 2015 was at 6:05 ± 7 minutes; sunset during this period was at 18:31 ± 4 minutes. Because I was unable to observe and switch treatments on 84 plants simultaneously (126 plants total, 42 day exclusion and 42 night exclusion plants needed exclusion manipulation every 12 hours), I timed my walking of the circuit such that I had switched the nets on roughly 50% of the plants by the sunrise/sunset time. Each circuit generally took between three and four hours, so the first plants to be checked in the "dawn" were actually checked and changed during the darkness of pre-dawn, and the first plants to be checked in the "dusk" were checked and changed during the light of day. For this reason, every seven days, I reversed the direction of the route I walked. In summary, the total average effect of this treatment regime, therefore, was that, for a 56-day period, each night exclusion plant was exposed to herbivore risk only during the ~12.5 hours of day (so any leaf damage that occurred on these plants is the cumulative total of herbivores feeding for 700 hours, during the day only); each day exclusion plant was exposed to

herbivore risk only during the ~11.5 hours of night (so any leaf damage that occurred on these plants is the cumulative total of herbivores feeding for 644 hours, during the night only). Although I could not always be absolutely certain that I had not missed any very small invertebrates that fed during the time that the exclusion treatment was in effect, due to the complex damage patterns on most pre-existing leaves, I monitored all newly expanding leaves (474 total over the two months) closely each 12 hours, and in only one instance was there damage that occurred on a new leaf by an herbivore that had remained inside or gained access to a closed plant (on a *Piper aequale* individual ~90% of one leaf was eaten during one night by a small katydid nymph, on the night exclusion plant).

The details of the experimental design and treatment manipulations conducted to measure the rates of herbivory during these two phases of the diel are summarized in Figure 1.1. In brief, I cleared the plant to be covered for the next ~12 hours of all flying herbivores and removed them from the immediate area. Because a major portion of the total herbivory on these plants, and on most tropical plants in general, is from larval lepidopterans (Dyer *et al.* 2007, Novotny *et al.* 2006), I devised a method for quantifying the proportion of their feeding that occurred during the day and the proportion that occurred during the night. Flightless herbivores (in nearly all cases larval Lepidoptera) found on the exclusion treatment plants at a given time were moved between the two treatment plants within a treatment group each dawn and dusk, such that the portion of nocturnal feeding by a caterpillar occurred on the day exclusion plant, and the portion of diurnal feeding by a caterpillar occurred on the night exclusion plant. At the end of the two months, each study leaf was again photographed to calculate the area change. Any larvae that were on the plants on the final day were removed, and treatment plants were kept covered until the leaf images could be taken. All final leaf photos were taken in a period of three days.

Statistical analysis

For the subset of leaves that grew during the exclusion experiment (reported as "new leaves" in Tables 1.1-1.5), I visually scored leaves for herbivory. I created binary categories of leaves that had either been damaged or remained damage-free. Since some minor leaf damage sometimes was not due to insects, but was a result of falling debris piercing a leaf, or foliar pathogens causing leaf necrosis, I also conducted a separate set of analyses on leaves that were binned into low (<10% area missing) or high (≥10% area missing) categories. I used the 'lme4' package (Version 1.1.13) in R (Version 3.3.2) to construct generalized linear mixed-effects models (Bates *et al.* 2015). I treated the time period when herbivores were excluded (treatment) as the predictor of the probability that a leaf was damaged. I used a binomial distribution, and tested the significance of the overall model by comparing it to a null model using a likelihood ratio test.

Results and Discussion

The general trend for three of the four species was for higher herbivore damage to occur at night (Figures 1.2-1.4). Herbivory was so infrequently observed on *Piper cordulatum* that no conclusions can be drawn regarding herbivory patterns on this species (Figure 1.5). As an interesting natural history note, I have spent hundreds of hours searching thousands of leaves of *Piper cordulatum*, at all hours of the day and night and in both wet and dry seasons, and have observed exactly three instances of herbivory on this plant. Though older leaves of *Piper cordulatum* are often tattered and skeletonized, this damage is almost certainly caused by a pathogen. No analysis is reported for *Piper cordulatum* for this reason.

For the other three species, leaves that grew during the experiment had a greater proportion of leaves with no damage when they were on plants that were protected from herbivores during the night, as compared to control and day exclusion plants (Figures 1.2-1.4). With a binomial distribution and the 'lme4' package for conducting GLMM analysis in R, the overall effect of exclusion treatment is significant for *Piper aequale* ($\chi^2 = 9.9638$, $p = 0.00686$, $df = 2$). The night exclusion treatment for this species reduced the probability of a leaf being eaten relative to both the control and the day exclusion ($0.17 < 0.36 < 0.62$; 95% CI for night exclusion, *versus* $0.50 < 0.76 < 0.91$; 95% CI for day exclusion, and $0.63 < 0.89 < 0.97$; 95% CI for control). This pattern was also consistent for *Psychotria marginata* – it is significantly less likely for a night exclusion leaf to experience damage ($p = 0.0064$, probability of herbivory: $0.10 < 0.21 < 0.41$) than it is if a leaf is exposed during the night ($0.26 < 0.48 < 0.71$ or all the time ($0.21 < 0.39 < 0.62$), ranges represent 95% CIs in all cases. However, the overall effect of treatment for *Psychotria marginata* is not significant ($\chi^2 = 3.0925$, $p = 0.213$, $df = 2$). No significant differences in leaf damage probability are explained by treatment for *Psychotria limonensis* ($\chi^2 = 1.9817$, $p = 0.371$, $df = 2$), and the individual probabilities of leaf damage all overlap for this species. However, when the herbivory data for *Psychotria limonensis* are re-analyzed using the more lenient categories of low or high damage, which helps to correct for small areas lost due to pathogens and small dead branches (which tend to fall from the canopy and often will puncture small holes in the very large and tender leaves of this species), the nighttime is a slightly more likely time for leaves to be damaged ($0.001 < 0.017 < 0.215$ is the probability of damage when protected during the night, *versus* $0.027 < 0.134 < 0.460$ and $0.023 < 0.118 < 0.434$ for the day exclusion and control treatments, respectively). However, the overall

effect of treatment is still not significant ($\chi^2 = 3.6398$, $p = 0.162$, $df = 2$). No results are reported for *Piper cordulatum.*

Visually, the distribution of frequencies for damage for the three species that accumulated damage during the different periods of the diel show that more leaf area gets removed during the night (Figures 1.2-1.4). For example, for *Psychotria marginata,* when newly expanding leaves are protected from herbivores during the night, 78% of leaves are undamaged, as compared to 52% and 59% undamaged in day exclusion and control leaves, respectively (Figure 1.3). Similarly, fewer leaves were severely damaged when they were protected from herbivores specifically at night (2% of leaves with major damage, compared to 14% in day exclusion plants and 17% in control) (Figure 1.3).

This study provides quantitative estimates of variation in the relative risk of herbivory to leaves of Neotropical shrubs during daytime and nighttime hours. Overall, the trends in the three species that accumulated herbivore damage on newly expanding leaves were similar across species, with all showing that the plants open to herbivores at night displayed damage patterns that closely resembled the distribution of damage levels seen on control plants that were always open to herbivores. This finding suggests that, for these three species of common understory plants, the majority of total folivory on young leaves occurs during the nighttime. This information should be of interest to ecologists because it is one of only a handful to examine diel differences in herbivory rates in an intact tropical community.

Historically, Elton (1927) suggested that the diel turnover in species that interact with each other in a community is high. However, very few studies have examined this prediction for herbivores in a tropical forest (Reagan *et al.* 1996). It is likely that herbivores face different levels of predation risk at night relative to the day (Novotny *et al.* 1999, Hassell and Southwood

1978, Heinrich 1979), and birds are most often invoked as primary drivers in pushing more herbivore feeding activity to the scotophase (Herrebout *et al.* 1963, Heinrich 1979). However, most published studies that measure diel variation in herbivore predation risk are from the temperate zone, where birds and other visual predators, such as salticid spiders (Richman and Jackson 1992), are important drivers of herbivore feeding rhythms. When the effects of insectivorous gleaning bats are partitioned out, as opposed to bird predation of herbivores, the risk of a Neotropical herbivore being eaten at night may actually be greater than during the day (Kalka *et al.* 2008).

The higher rates of herbivory at night, observed especially in the tropics (Reagan *et al.* 1996, Windsor 1978), are concordant with recent findings of clear differences in secondary metabolite profiles between day and night (*e.g.* Kim *et al.* 2011; Goodspeed *et al.* 2012, Goodspeed *et al.* 2013). In the Brassicaceae (*A. thaliana* and *Brassica oleracea*), glucosinolates are circadian-entrained and timed to periods of higher herbivore risk (Goodspeed *et al.* 2012, Goodspeed *et al.* 2013). In *A. thaliana*, both the circadian clock and jasmonate response functions are light phase-dependent, and susceptibility to herbivory is reduced when the folivore *Trichoplusia ni* (cabbage looper) is entrained out of phase with the plant clock (Goodspeed *et al.* 2012).

On Barro Colorado Island, predation risk for herbivores may be higher at night (Kalka *et al.* 2008), so high levels of nocturnal feeding may be linked more to hostplant quality factors than to avoidance of predators. One possible variable that is likely to affect plant quality in the presence and absence of sunlight is leaf content of secondary metabolites that are phototoxic; although the four species I studied have not been tested for their content of photochemically

active phytochemicals, other species in both Piperaceae and Rubiaceae are known to produce such compounds (Downum *et al.* 1991).

Larval herbivores should be expected to maximize their potential for growth by feeding constantly, with resting periods for digestion. Foraging behaviors rarely if ever follow this pattern. Caterpillars need time to digest food, may restrict foraging bouts to time periods when abiotic variables are less extreme, may restrict feeding to times when predation risk is lower, and may feed more at times of the day when foliage is more nutritious and/or less toxic (*cf.* Raubenheimer and Simpson 1996, Heinrich 1979). My observations of sphingids feeding on *Psychotria* spp. and *Eois* spp. feeding on *Piper aequale* suggest that even when the larvae remain on the leaves, at their feeding sites, they rarely eat much during the daylight. In fact, a large sphingid larva, such as the specimen presented in Figure 1.6, can eat multiple entire leaves in a single nocturnal feeding bout, and can cause the total defoliation of a small *Psychotria* plant (~30-40 total leaves) over the larva's development. This phenomenon, of entire leaves being consumed primarily at night, is rarely accounted for in estimates of total herbivory in a given community.

Multiple factors are likely contributing to the pattern discussed here, of herbivory rates varying between times of day. In addition to the biotic factors previously discussed (predators and plant secondary chemistry), abiotic conditions could also influence herbivory. However, abiotic factors that likely play a major role in the temperate zone (e.g. temperature, humidity, wind) are less variable in a tropical understory (Kira and Yoda 1989). Although temperature may regulate herbivore diel feeding rhythms in some temperate systems (Edwards 1964, Edwards 1965, Lance *et al.* 1986), recent findings on the roles of plant circadian rhythms suggests that these daily patterns may relate to herbivore feeding times as well (Goodspeed *et al.* 2012, Jander

2012, Meldau and Baldwin 2013). In response to Hairston *et al.* (1960), Murdoch (1966) argued that bottom-up effects—notably, chemical defenses—may explain at least in part why the world is green; such bottom-up effects may be especially strong in tropical forests and may help explain why herbivory is primarily nocturnal.

The extent to which chemical changes may be of developmental consequence to herbivores remains untested. More than 50 years ago, Ehrlich and Raven (1964) suggested that "Diurnal chemical cycles, influenced by exposure of the plant to sunlight, may be of prime importance in determining the habits of night-feeding groups…" (p. 587). Beyond phytochemical defenses, diurnal chemical cycles influenced by sunlight may include variation in nutritional quality that may favor nocturnal herbivory. The qualitative diel variation in secondary metabolomes of *Psychotria* spp. (Zehr *et al.* in prep.) suggests that this question is more central than previously considered. Further tests of this hypothesis must be conducted via controlled bioassays of herbivore performance, as on semi-defined diets that are treated with plant extracts from different periods of the diel. In addition, choice trials involving intact plants or freshly-collected leaves from the night *versus* the day could be useful in testing the idea that herbivore feeding behavior may be at least partially determined by time-specific nutritive aspects of plants.

Tables and Figures

Table 1.1 – Summary of plants and leaves used for quantifying folivory

	Psychotria limonensis	*Psychotria marginata*	*Piper aequale*	*Piper cordulatum*
n plants	42	24	36	24
n leaves pre	450	559	336	422
n leaves post	422	499	313	403
n new leaves	90	224	130	30
total	962	1282	779	855

Table 1.2 – *Psychotria limonensis* leaf sample sizes per treatment

	Treatment		
	Control	Day Exclosure	Night Exclosure
n plants	14	14	14
n leaves pre	156	146	148
n leaves post	147	139	136
n new leaves	28	34	28

Table 1.3 – *Psychotria marginata* leaf sample sizes per treatment

	Treatment		
	Control	Day Exclosure	Night Exclosure
n plants	8	8	8
n leaves pre	175	210	174
n leaves post	162	183	154
n new leaves	75	64	85

Table 1.4 – *Piper aequale* leaf sample sizes per treatment

	Treatment		
	Control	Day Exclosure	Night Exclosure
n plants*	11	11	11
n leaves pre	103	115	118
n leaves post	97	105	111
n new leaves	34	46	50

* For new leaves, *n* plants/treatment was 12.

Table 1.5 – *Piper cordulatum* leaf sample sizes per treatment

	Treatment		
	Control	Day Exclosure	Night Exclosure
n plants	8	8	8
n leaves pre	144	152	126
n leaves post	134	147	122
n new leaves	14	8	8

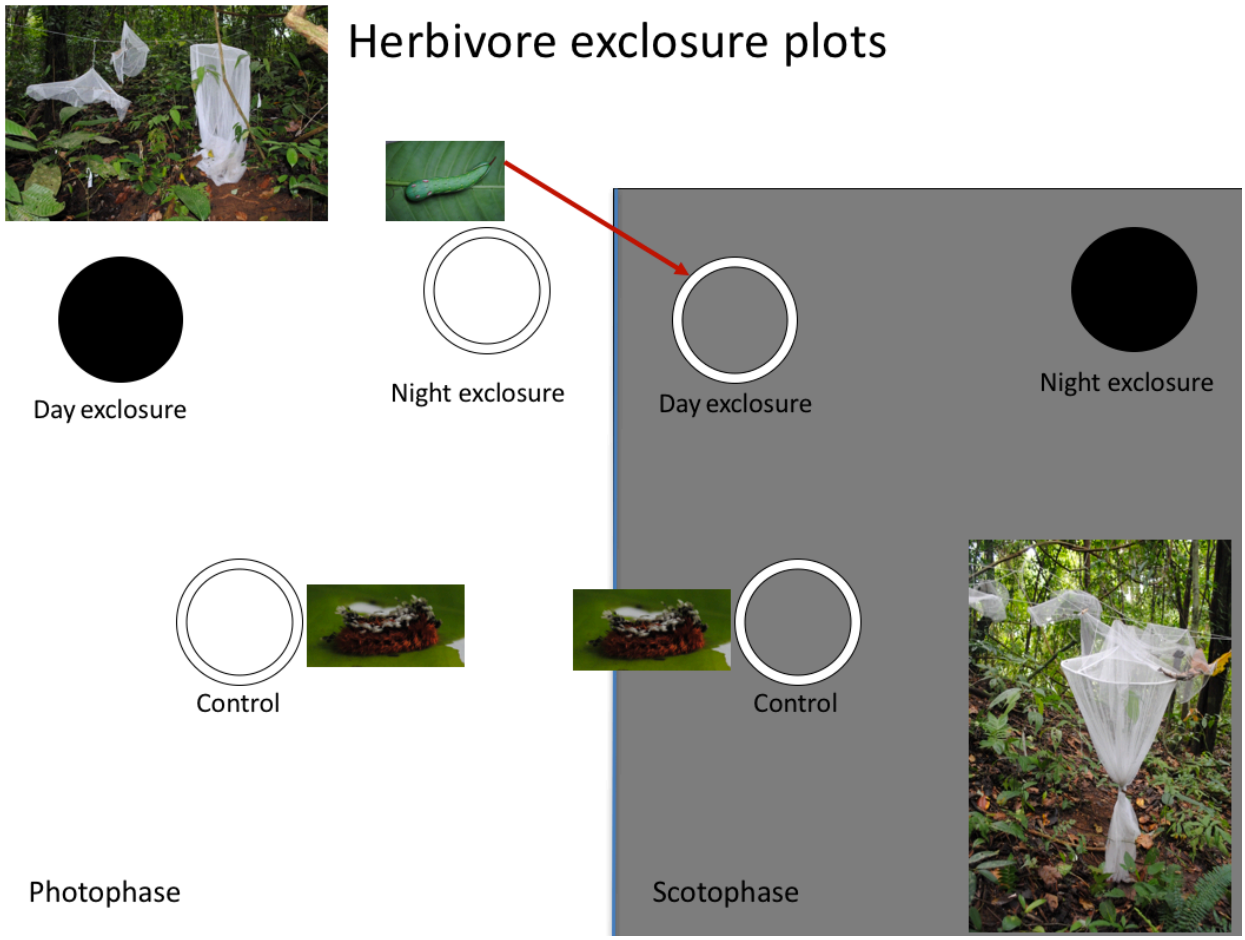

Figure 1.1 – Mosquito nets were used as single plant herbivore exclosures, which were switched in each treatment group each dawn and dusk, to partition herbivory rates into each phase of the diel. At the dawn (far left), the net was placed over and closed on the "Day exclosure" plant. This net was usually the net used to cover the "Night exclosure" plant in the preceding night. In the case that the "Night exclosure" net was not transferred to the "Day exclosure" plant in the day, it was opened and kept open above the plant for the day. Around the sunset of each day (middle of figure), the opposite was done – the "Day exclosure" net was opened and kept above the plant, and the "Night exclosure" net was lowered and closed around the plant. Again, if one net was shared between plants, a control cover was placed above the open plant. The "Control" plants were never closed to herbivores, but always had a shade cover suspended above the plant.

The second part of the manipulations of this experiment involved moving non-volant herbivores (in most cases larval Lepidoptera) between the treatment plants each dawn and dusk, to partition the portions of feeding done by caterpillars into day or night. For example, if a caterpillar was encountered feeding on a "Night exclosure" plant during the day, it was moved to the "Day exclosure" at the dusk, so that the portion of feeding that caterpillar did during the night was measured on the plant that was open to other herbivores at night. Then, in the following morning, that individual caterpillar was moved back to the "Night exclosure" plant for the day, when the "Day exclosure" plant was closed for the day.

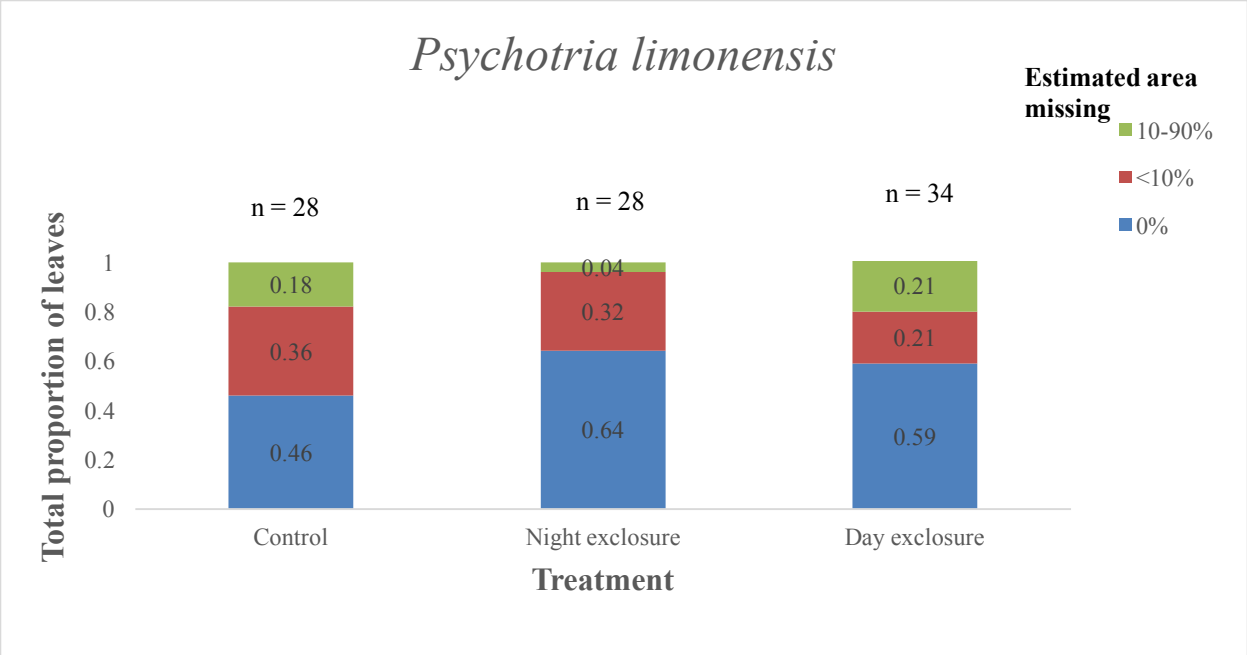

Figure 1.2 – Relative leaf area removal from new leaves, visually estimated from leaf photographs, over a two-month period of the day/night exclusion treatments.

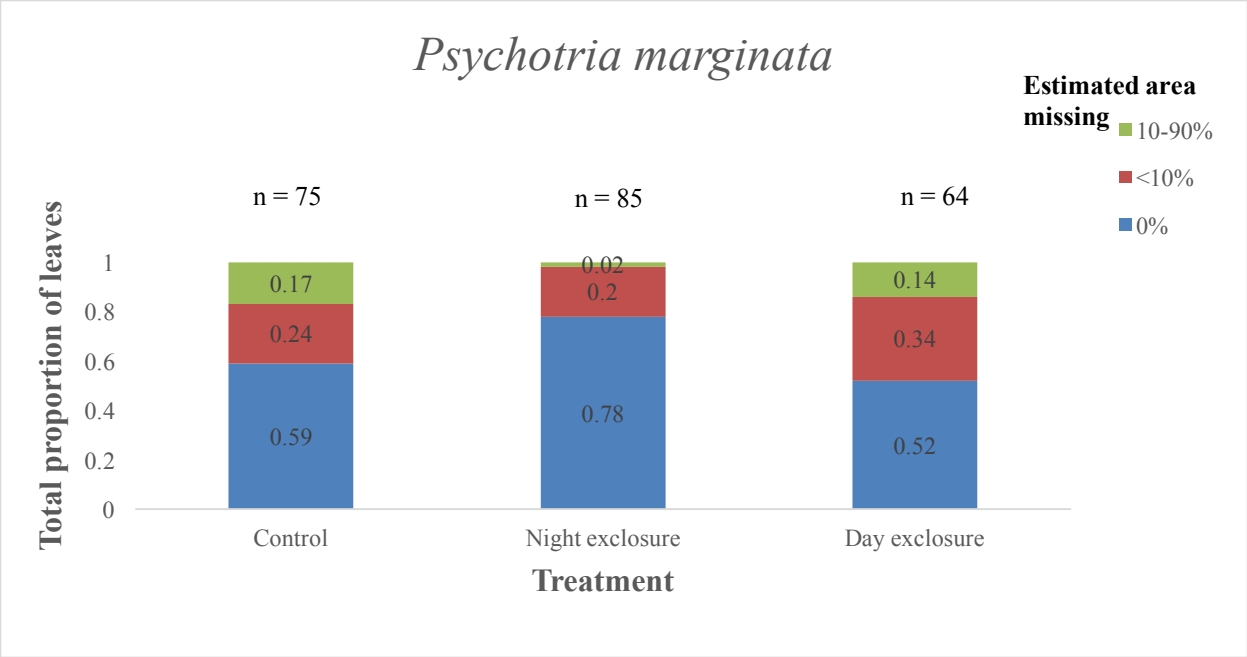

Figure 1.3 – Relative leaf area removal from new leaves, visually estimated from leaf photographs, over a two-month period of the day/night exclusion treatments.

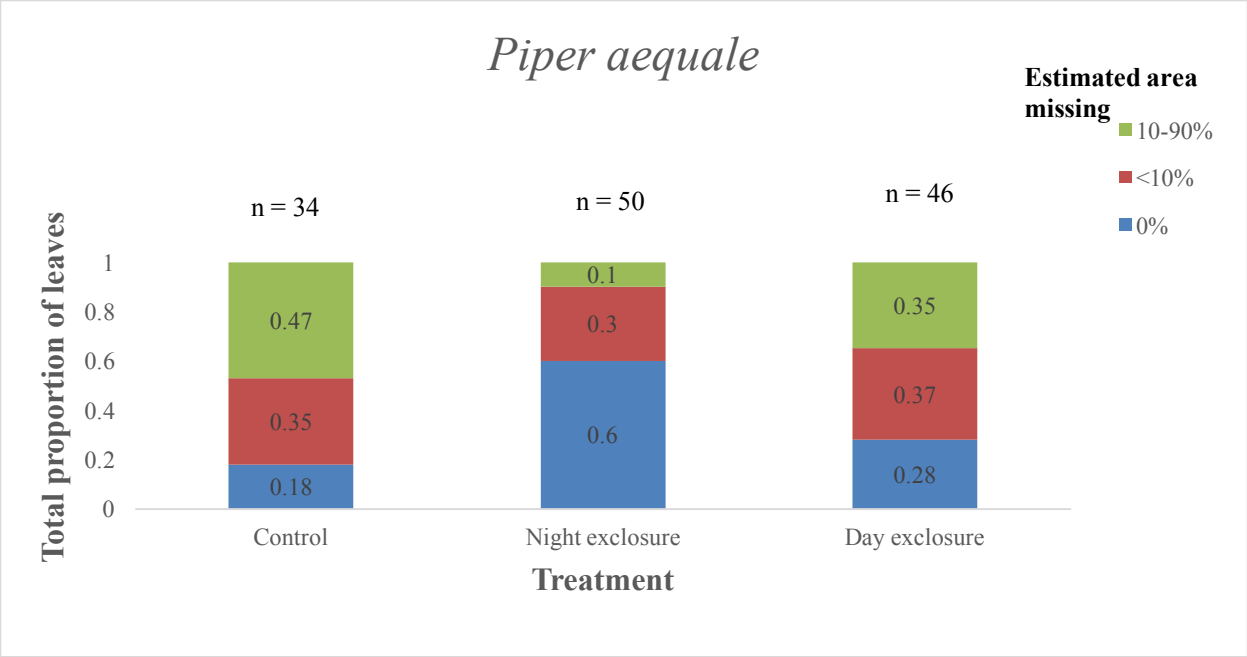

Figure 1.4 – Relative leaf area removal from new leaves, visually estimated from leaf photographs, over a two-month period of the day/night exclusion treatments.

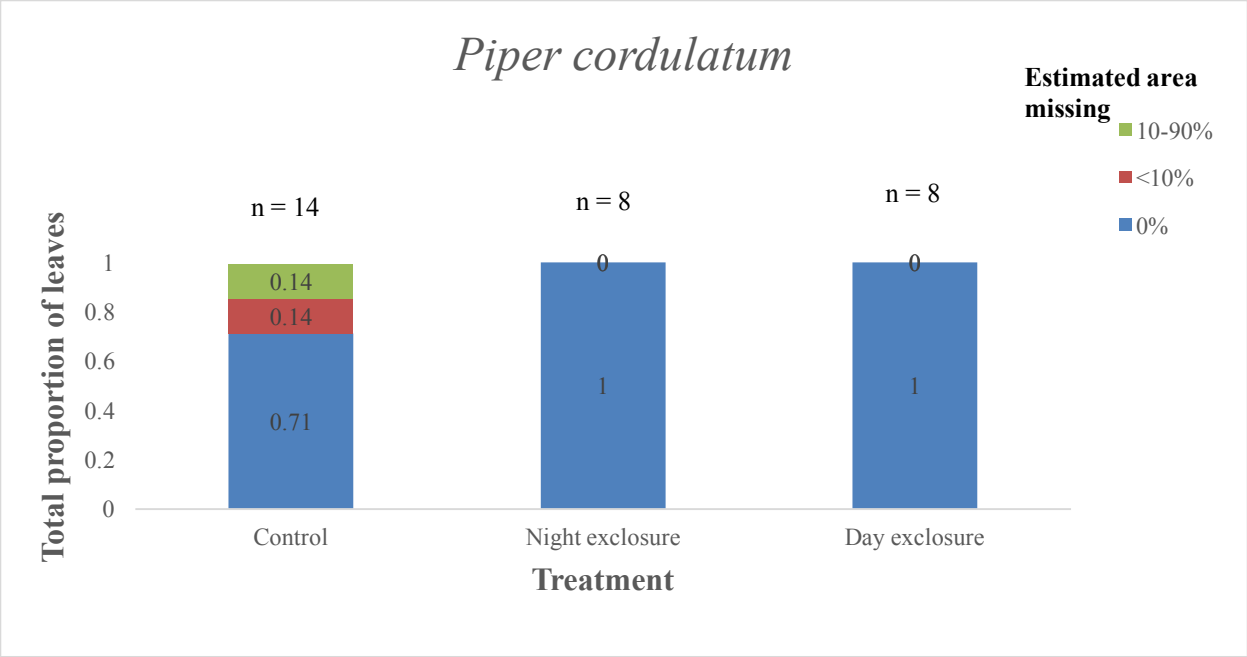

Figure 1.5 – Relative leaf area removal from new leaves, visually estimated from leaf photographs, over a two-month period of the day/night exclusion treatments.

Figure 1.6 – A frequently encountered *Psychotria* herbivore that was observed to feed almost exclusively nocturnally, *Xylophanes chiron* (Drury, 1771) (Sphingidae: Macroglossinae), shown here on the abaxial surface of a *Psychotria limonensis* leaf.

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CHAPTER II: TEMPORAL VARIATION IN SECONDARY METABOLITE PROFILES OF TROPICAL FOLIAGE: THE DIFFERENCE IN TASTE TO AN HERBIVORE MAY BE AS DIFFERENT AS DAY FROM NIGHT

Introduction

"Also that some roots should be gathered at night, others by day, and some before the sun strikes on them…for the properties of these plants are harmful; they take hold, it is said, like fire and burn…" (Theophrastus, transl. A. F. Hort 1916, pp. 256-257).

The 24-hour cycle of photoperiod and its corresponding changes in temperature comprise the most predictable and global abiotic variations to which terrestrial organisms adapt and respond. Diel rhythmicity in the behavior and physiology of animals and plants is a readily apparent phenomenon, but nocturnal interactions between plants and herbivores have received considerably less attention than diurnal ones (Elton 1927, Hassell and Southwood 1978, Reagan and Waide 1996, Saunders 2002). Because, over an individual's lifetime, plants are sessile, they have evolved diverse and robust mechanisms for coping with abiotic stresses and fending off biotic threats, particularly herbivores (*e.g.*, Rosenthal and Berenbaum 1992, Strauss and Agrawal 1999, Karban and Myers 1989, Paré and Tumlinson 1999, Herms and Mattson 1992).

The herbivory risk to a plant should not be assumed to be constant over the diel cycle, because the foraging activities of herbivores are in many cases primarily diurnal or nocturnal, circadian-controlled, or otherwise non-homogeneous temporally (Saunders 2002, Hassell and Southwood 1978). Furthermore, the composition of the communities of herbivores active during the night may differ greatly from that of the daytime community; this pattern is especially

evident in some Neotropical forests (Reagan and Waide 1996). Abundant anecdotal evidence exists that suggests that a majority of insect herbivory may occur during the night in the Neotropics, but little to no quantitative information on this pattern exists (Reagan and Waide 1996, Zehr *et al.* in prep., Miller *et al.* 2006, Windsor 1978). Although the assumption that this apparent pattern of higher herbivory rates at night is top-down regulated (i.e., lower predation risk to herbivores feeding at night) is a predominant one in the literature (Hassell and Southwood 1978, Heinrich 1979, Novotny *et al*. 1999, Berger and Gotthard 2008), the idea remains relatively untested and is challenged by somewhat inconclusive (Novotny *et al.* 1999) or contradictory (Kalka *et al.* 2008) findings.

That plants are physiologically active over the diel cycle with regard to the nature and concentrations of secondary metabolites present in their tissues is well known (Robinson 1974, Wink 1998, Kim *et al.* 2011, Goodspeed *et al.* 2012, Meldau and Baldwin 2013, Higashi *et al.* 2016). In *Nicotiana attenuata*, metabolites show a high degree of cyclical fluctuations that are tissue-specific; in leaf extracts, 72% of metabolites that had diel patterns peaked during the day and troughed at night (Kim *et al.* 2011). This finding together with the report that herbivores (*Trichoplusia ni*) on *Arabidopsis* show diel foraging rhythms that correlate with circadian-regulated plant defense cycles (Goodspeed *et al.* 2012) have led to suggestions that the timing of herbivory may relate to plant secondary metabolite diel changes (Jander 2012, Chapter 1).

Plant metabolomics, especially for chemically hyperdiverse and understudied tropical plants such as species in the genera *Piper* and *Psychotria*, have great potential for advancing understanding of plant-insect interactions, pharmacologically active compounds, and tropical species diversity (Sedio 2017, Sedio *et al.* 2017, Dyer *et al.* 2014, Kuhlisch and Pohnert 2015, Richards *et al.* 2015). I chose these chemically diverse plant genera to characterize the degree of

turnover in the secondary metabolome of the leaves over one diel cycle. I hypothesized that, because plants are physiologically and biosynthetically constrained during the night (Arimura *et al.* 2008, Greenham and McClung 2015) and because phototoxicity likely is important even for these understory plants (Downum *et al.* 1991, Downum and Wen 1995), the increased herbivory rates observed during the night may be reflective of a more bottom-up regulated rhythmicity that occurs in many tropical plants. I predicted that the quality and quantity of secondary metabolites present in leaf tissues would differ significantly over the diel cycle; more specifically, I hypothesized that lower concentrations of the metabolites would be present in the leaves during the night, as has been observed in several other plants, including at least one species of *Piper* (Morandim *et al.* 2005).

Materials and Methods

Study site and focal species

I collected leaves from 8 species from the plant genera *Piper* and *Psychotria* on Barro Colorado Island (BCI; 9º 09' N, 79º 51'W), Panama to measure diel variation in plant secondary metabolites (see Leigh *et al.* 1982, Foster and Brokaw 1982, Gentry 1990, and Windsor 1990 for good descriptions of the climate and natural history of BCI). The site is classified as moist, semi-deciduous lowland tropical forest (Holdridge *et al.* 1971). More than 1300 species of plants coexist on this 15.6-km^2 island (Croat 1978, Foster and Brokaw 1982), with 356 species of tree (Foster and Brokaw 1982) or 409 species of trees and shrubs listed (Kress *et al.* 2009).

I chose to study plants in the genera *Piper* (Piperaceae) and *Psychotria* (Rubiaceae) because of their prominence in tropical forests, as abundant and diverse genera (Gentry 1982, Leigh *et al.* 1982, Dyer and Palmer 2004, Kress *et al.* 2009, Sedio *et al.* 2012). The genus *Piper*

has been extensively studied as a model system for chemically mediated plant-insect interactions, phytochemical diversity and insect herbivores as drivers and maintainers of plant diversity, and species coexistence (*e.g.* Marquis 1984, Dyer and Palmer 2004, Dyer *et al.* 2010, Richards *et al.* 2015, Glassmire *et al.* 2016, Salazar *et al.* 2016). Similarly, the genus *Psychotria* is hyperdiverse and chemically distinctive (Matsuura *et al.* 2013, Martins and Nunez 2015). The lineage of Rubiaceae that contains *Psychotria* is more alkaloid-rich than other lineages at the same taxonomic level (Martins and Nunez 2015), and members of both the Piperaceae and the Rubiaceae are known to have phototoxic properties (Downum *et al.* 1991).

Leaf collection

In 2015, I collected leaves from *Psychotria limonensis, Psychotria marginata, Piper cordulatum,* and *Piper aequale.* One specific location on BCI, along a trail but not in a major light gap, was selected where all four species were growing within an 18-m radius, in sufficient numbers that leaves could be collected from five individuals of each of the four species. Furthermore, I selected plants that had at least 10 leaves that were of an intermediate age, based on their color, texture, and location on the plant, so that leaves used for the analysis were not yet fully toughened, but had mostly completed expansion. Some of the leaves used for chemical analysis had slight herbivore damage, but none had more than 10% of original leaf area removed (visually estimated).

I randomly selected and tagged 10 leaves on each of the 20 plants in the late afternoon preceding leaf collection. On 23 July 2015, beginning at 2:02 am, I collected the leaves from the plants by plucking them at the base of the petiole, wrapping the five leaves from a plant into one piece of aluminum foil to serve as the pooled leaf tissue sample, and placing each sample directly onto ice in a cooler. All leaves for the "night" sample were harvested between 2:02 am

and 3:08 am and frozen at -80ºC at 3:18 am. Leaves were kept shielded from light using aluminum foil to minimize the chemical changes in leaves.

The leaves comprising the "day" sample were harvested from the same 20 plants from which the "night" samples had been collected roughly 6 hours before, with the same method. Although there were intermittent clouds early this morning, the leaves had been exposed to the diffuse light normally reaching the understory for at least 1.5 hours prior to harvest. The "day" sample leaves were harvested on 23 July 2015, between 8:05 and 8:54 am, and were frozen at -80ºC at 9:04 am. No attempt was made to remove epiphylls (which likely contribute to the secondary metabolites present in a sample, see Coley and Kursar 1996) from leaf blades, but visible foreign material such as fallen detritus and arthropods were removed from the leaf samples. Leaves were extracted within one to two days post-harvest.

In July 2016, we sampled four other species, all in the genus *Psychotria.* These four species used were *Psychotria acuminata*, *Psychotria deflexa*, *Psychotria hoffmannseggiana*, and *Palicourea guianensis.* (*Palicourea guianensis* was considered a congener of *Psychotria* spp. *sensu lato*, following Kress *et al.* 2009 and Sedio *et al.* 2017). The leaf sampling protocol was slightly different than in 2015. In order to obtain a more fine-grained time-series of diel changes in the plant metabolome, I collected leaves directly into liquid N_2 in the forest, at four times over the diel cycle. Collection times and samples are shown in Appendix A. For this analysis, three leaves were pooled into one sample, and five individuals of each four species were sampled at each of the four time points, named as "pre-sunset, post-sunset, pre-dawn, and post-dawn." Leaf samples were collected into small paper envelopes and frozen in liquid N_2 within one minute of harvest. Exposure of leaves to light from headlamps was minimized as much as possible during the leaf collections during the night.

Leaf extractions

For the entire extraction procedure, leaf material was kept as cold and as dark as possible, to minimize potential chemical changes that can occur in complex chemical samples, especially at higher temperatures and in the presence of light. Leaf samples were removed from the -80ºC freezer, but kept on dry ice, as a small square from a haphazardly selected area near the center of each leaf was excised using a sterilized scissors (submerged in ethanol and flamed between each sample). For each sample, an equal area from each of the five leaves in a pooled sample was homogenized (see below). This area was roughly 0.2 cm^2 per leaf, so that the total fresh mass to be homogenized was 0.100 g (±0.0015 g). An Ohaus Adventurer SL balance (OHAUS, Parsippany, NJ, USA) was used to weigh leaf samples. Leaf tissue was chopped and introduced into a lyser tube with two steel grinding beads, and 700 µL of freezing 90% methanol at pH 5 was added to each sample tube. A ball mill (Qiagen/Retsch TissueLyser, Hilden, Germany) was used to homogenize the leaf samples and extract plant secondary metabolites. Samples were chilled using liquid N_2 in the lyser trays during the extraction procedure, extracted at 20 revolutions/second for 2 minutes, chilled again with liquid N_2, and extracted again at 20 rps for 2 minutes, and then were extracted for 10 minutes at 20 rps. Samples that had not been visibly homogenized after this procedure were subjected to tissue lysing for repeated rounds of 10 minute cycles at 20 rps until they were homogenized.

Once homogenized, the samples were centrifuged at 12000 rpm for 3 minutes (Eppendorf Centrifuge 5804 R, Eppendorf AG, Hamburg, Germany). The ~700 µL of supernatant was pipetted from each sample, and introduced to individual Eppendorf tubes, and these primary extraction solutions were frozen at -20ºC. The pellet was re-extracted with 500 µL of freezing 90% methanol at pH 5, samples were vortexed to disperse the pellet, and then centrifuged a

second time, at 12000 rpm for 3 minutes. This second extraction, of ~500 µL of supernatant, was then added to each respective ~700 µL primary sample, and this total sample was then centrifuged at 12000 rpm for 3 minutes, and stored at -20ºC until it was diluted prior to analysis. Four blanks were prepared using all of the same described methods, so that any foreign substances from materials used with the leaf samples could be subtracted from resulting mass spectra (e.g., Sharpie® markers, aluminum foil, potential contaminants from the methanol and laboratory environment, and plastics from the extraction tubes and nitrile gloves).

Chemical analysis

Samples were diluted by a factor of 10, using 70 µL of the extract prepared as described above, and 630 µL of freezing 90% methanol at pH 5, vortexed, and then stored at -80º C until they were transported on dry ice to Instituto de Investigaciones Científicas y Servicios de Alta Technología (INDICASAT AIP). The diluted extracts were filtered as they were injected into standard GC-MS vials, using Millex-LG Filter Units (with 0.22 µm low protein binding hydrophilic PTFE membranes, EMD Millipore, Billerica, MA, USA) on a 500 µL Hamilton syringe, which was rinsed with three successive portions, each of ~100 µL pure HPLC-grade methanol (Sigma-Aldrich) between each sample filtration.

Samples were analyzed with reverse phase ultra high-performance liquid chromatography (UHPLC, Agilent Technologies, Santa Clara, CA), electrospray ionization and molecular fragmentation, and tandem mass spectrometry (MS/MS). The UHPLC was conducted with a flow rate of 0.5 mL/min at 25°C. A previously optimized solvent gradient was used, which includes a 25-min gradient going from 5% to 100% acetonitrile followed by 8 min of isocratic 100% acetonitrile, using a Kinetex C18 UHPLC column (length = 100 mm, internal diameter = 2.1 mm, and particle size = 1.7µm) (Phenomenex, Torrance, CA) in order to achieve optimal

separation of metabolites across a wide range of polarities. Each solvent also included 0.1% formic acid to facilitate protonation.

Following UHPLC, samples were separated using tandem mass spectrometry (MS/MS) detection using electrospray ionization (ESI) in positive mode on a Bruker micrOTOF-QIII quadrupole time-of-flight mass spectrometer (Bruker Daltonics, Fremont, CA). Collision energy, acquisition time, and other parameters had previously been optimized for similar plant metabolomics investigations (see Sedio *et al.* 2017) in order to fragment and detect molecules representing as wide a range in the mass-to-charge ratio (m/z) of the parent compound as possible, from ~150 m/z to over 1,600 m/z.

Molecular network analysis

The online molecular data pipeline hosted by the University of California at San Diego, Global Natural Products Social (GNPS) Molecular Networking, was used to visualize the structural relationships of putative compounds among and between samples (gnps.ucsd.edu, Wang *et al.* 2016). The details of this data processing can be found in Sedio *et al.* (2017). In brief, the MS/MS spectra of all fragments detected in my samples are clustered into "consensus spectra," each of which represents a single, unique chemical structure. These putatively unique structures will hereafter be referred to as "compounds." Because these samples have a high number and diversity of secondary metabolites, the vast majority of which have not been isolated and characterized previously, I used the comparison of the *m/z* ratio of the fragments of two molecules to infer their degree of structural similarity. This inferential process is based on the understanding that molecules of similar structures fragment in similar and repeatable manners in mass spectrometry, producing similar fragments with similar *m/z* ratios. Following the procedures outlined in Wang *et al.* (2016), I quantified the structural similarity between each pair

of compounds with a cosine ≥ 0.6, with the angle of interest being the one formed by the vectors of the *m/z* ratio of the fragments that comprise that pair of compounds.

Statistical analysis

Using the Smithsonian Hydra Cluster supercomputer, we calculated the Chemical Structural and Compositional Similarity (CSCS) for each pairwise combination of the 80 samples, following the methods described in Sedio *et al.* 2017. We used the 'vegan' package in R (Oksanen *et al.* 2009), to conduct permutation tests on the CSCS matrix by species and by treatment (time of day of leaf collection). All pairwise combinations of molecules were used to calculate an index of chemical structural and compositional similarity (CSCS) for each pair, as described by Sedio *et al.* (2017). This procedure treats a consensus MS/MS spectrum as a vector and measures the cosine of the angle between two consensus spectra to quantify the amount of structural similarity between compounds. It also accounts for the concentration (ion intensity) of compounds detected, and weights their proportional representation in different samples. Permutation tests were conducted in RStudio (Version 1.0.136) to test for a significant effect of time of day of leaf collection on within-treatment similarity versus between-treatment similarity. Using "time of leaf collection" as the treatment, I randomized the assignment of treatment to samples, calculated the distributions of all possible differences between within-treatment and between-treatment similarity, and concluded that, if the observed difference is greater than 0.95 of the distribution of possible differences, there is a significant effect of time of day on overall CSCS of the secondary metabolome (Sedio *et al.* 2017).

Results

The consensus mass spectra from 80 samples of leaves from four species of Rubiaceae, sampled four times over a diel cycle, suggest that more than 11,000 unique secondary metabolites are present in the leaves of these plants. I found evidence that 11,261 secondary metabolites are found in at least one of the four species. In molecular networks constructed on the GNPS server, using network parameters as described in Sedio *et al.* (2017), 2,420 of these compounds were structurally similar to at least one other compound in the network; these are linked by edges. This leaves 8,841 compounds that were sufficiently structurally novel that they did not share any edges with any other compound in the network (shown in Figure 2.1).

Although not all species tested show a consistent pattern, when all four species from the 2016 leaf samples are pooled, there is a significant ($p = 0.0145$) effect of time of day on the overall composition of secondary metabolome of these plants (Table 2.1). However, a Non-Metric MDS plot of these samples shows that the diel effect is subtle – species are clearly different in their secondary metabolite profiles, but the time of day of leaf collection is less important than the species (Figure 2.2). In an attempt to visualize consistency in the diel changes in secondary metabolites, I separated the MDS analysis into separate plots for each species (Figure 2.3). I also grouped the four time points into two categories of "night" and "day," in an attempt to reduce the complexity of the analysis to aid in interpretation. However, this analysis actually obscured the significant effect of the time of day on CSCS of the leaf samples, suggesting that the diel changes in the metabolome occur on a finer time scale than 12-hour day/night cycles, or not consistently in a circadian pattern (Table 2.2).

The leaf samples from 2015, taken from *Piper aequale*, *Piper cordulatum*, *Psychotria limonensis*, and *Psychotria marginata*, did not reveal any consistent pattern in the effect of time

of day of leaf collection on CSCS. Even though the network models of CSCS for these four species suggest that there is diel variation in the secondary metabolites present in leaves collected during the night versus leaves collected during the day from individual plants (Figures 2.4-2.7), that pattern was not consistent between individuals of the same species across time (Table 2.3).

I had also predicted that, due to diel patterns of secondary metabolites seen in other plant species, and based primarily on the physiological constraints imposed on plants during the nighttime due to lack of sunlight, overall concentrations of secondary metabolites would be lower during the night. I did not find support for this hypothesis. The four species sampled in 2015 did not segregate into groups in the ordination analysis as the species sampled in 2016 did (Figure 2.8), and no significant effect of time of day on CSCS changes was detected (Table 2.3).

For the species sampled in 2016, the total ion intensity, a proxy for metabolite concentration, did not differ significantly over the diel cycle. Permutation tests on the total ion intensity for these species showed no effect of time of day on overall ion intensity. Additionally, simple comparisons (ANOVAs) of the total ion intensity between different times of the diel cycle revealed no significant differences (data not shown). In the cases where a species-specific trend might exist, based on a visualization of these results (Figure 2.9), the pre-dawn time point may trend toward the time with the highest overall concentration of metabolites, contrary to my predictions. However, this suggests that chemical compositional differences over the diel are not due to differences in compound intensities in samples, but are in fact real compositional differences.

Efforts to match the identities of the compounds in my networks with known compounds, using the MS-MS spectra libraries available on GNPS have not yielded many meaningful

matches. This is likely because GNPS mostly has data on pharmaceuticals; the Prestwick Phytochemical Library, containing only 300 plant secondary metabolites, is the only phytochemical reference library currently available on GNPS. However, some preliminary inferences may be made using library matches from samples analyzed in 2015, and presented in Table 2.4.

Discussion

Abundant evidence shows that plants are physiologically active organisms that respond to biotic and abiotic stressors in their environment. Diel changes in plant primary metabolism and secondary metabolism are controlled by extrinsic factors (most notably photoperiod) and intrinsic factors (circadian regulation of gene expression) (Greenham and McClung 2015, Atamian and Harmer 2016, Niinemets *et al.* 2013, Higashi *et al.* 2016). Plants have likely evolved to optimize their responses to predictable fluctuations in their environment, such as temperature and light availability (Meldau and Baldwin 2013). To the extent that risk of herbivore attack varies with the photophase, whether due to intrinsic diurnal/nocturnal behavior of the herbivore or other potential drivers that likely vary with photophase, such as predation risk to the herbivores or diel changes in the plant's physiological state, I predicted that plants in natural systems show rhythms that optimize their defenses against diel variation in herbivore damage.

Secondary metabolite profiles of the leaf samples over a 24-hour period were highly variable and a substantial number of phytochemicals were restricted to nocturnal intervals. Daily changes in secondary metabolites are known from numerous previous studies, but the extent to which such cycles may affect the timing of feeding behavior of herbivores has not been well

documented (Kim *et al.* 2011, Jander 2012). For example, Jander (2012) states, "if diurnal peaks in antiherbivore defenses, which have been observed in both *Arabidopsis* and *Nicotiana attenuata* … are a more general phenomenon in plants, nocturnal feeding may also be an adaptation to reduced nighttime plant defenses."

Some of the compounds summarized in Table 2.4 are phototoxic. Downum and Wen (1995) specifically report on some lignans from the genus *Piper* that show phototoxicity. Additionally, furochromones and pheophorbide A are phototoxic (Downum and Wen 1995). The presence of pheophorbide A in the sample analysis, however, seems problematic, because primary metabolites have mostly been discounted in my analysis. The presence of pheophorbide A suggests that it is not present in all plant samples that have been uploaded to GNPS, and against which these samples were mapped.

I did frequently encounter leaf-tying and leaf-rolling larvae on both species of *Psychotria* in Chapter 1. In fact, the single observation of an herbivore feeding on one of the *Piper cordulatum* plants that was studied intensively for Chapter 1 was a minute leaf-rolling larva. Sagers (1992) has reported that a leaf-rolling larva improves the quality of its *Psychotria horizontalis* hostplants (reduces toughness and tannins) when it constructs its rolls and feeds in the shade. This behavioral phenotype – constructing shaded microhabitats on a phototoxic plant, thereby circumventing phototoxicity to the larva – was first proposed and demonstrated by Berenbaum (1978).

I have observed one of the most abundant leaf-rolling *Desmia* sp. on *Psychotria* spp. on BCI to feed constantly over the day and night, suggesting that it might also have circumvented any phototoxic effects that might harm less-specialized herbivores during the day. This could be one potential hypothesis to test, based on my observations on BCI: more generalists feed more at

night, but specialists can feed more constantly (*e.g.* melolonthine scarabs, especially *Phyllophaga* spp. that are voracious generalists at night, on many plants commonly thought to be toxic; tettigoniids that feed on plants ranging from *Piper darienense* to *Monstera* spp. at night; pseudostigmatid grasshoppers that feed on *Psychotria* at night; phasmids, which tend to be so cryptic that their diet breadth in the tropics is very poorly known, that I have observed feeding on several putative toxic plants during the night, etc.) (L. Zehr, pers. obs.).

Although I did find a significant effect of time of day of leaf collection on the secondary metabolome of four species of *Psychotria*, I am unable to determine which specific compounds contribute to this pattern. Additionally, I found no support for the hypothesis that plants may be less well chemically defended at night – a pattern consistent with my findings that herbivory risk is greater at night (Chapter 1). However, it likely is not necessarily true that a higher diversity of secondary metabolites is an index to efficacy of chemical defense.

If plants consistently face higher risk of herbivory at night, it might actually be the case that plants, at least in the tropics, are in some ways better defended at night. I did see a substantial proportion of night-specific putative defenses, although there was no time period during which the plants had significantly higher total ion concentration. Diel changes in plant secondary metabolites are suspected of driving herbivore feeding rhythms (Goodspeed *et al.* 2012, Jander 2012, Zehr *et al.* in prep., and references in Slansky 1993), but, the extent to which diel changes in overall plant nutrition might affect herbivore preference and performance has not been well-tested. The one study of which I am aware that aimed to test the hypothesis that plant tissue is more nutritious to an herbivore at one period of the day versus another was performed using aphids. Cull and van Emden (1977) found that aphids increased in moisture content more during the night, likely because the phloem sap was more dilute with respect to dissolved

carbohydrates, possibly benefiting aphids by helping them cope with water balance stress during the day. Further experiments that test the performance of herbivores on foliage from the night versus foliage from the day should be useful in testing the ideas presented here in a more direct and conclusive manner.

Tables and Figures

Table 2.1 – Permutation test results on the effect of time of day on chemical structural-compositional similarity (CSCS) of four species of '*heteropsychotria*'

Treatment:	CSCS			
Diel time	Within	Between	Difference	p
Pa. guianensis	0.10	0.09	0.01	0.055
Ps. acuminata	0.26	0.20	0.05	**0.001**
Ps. deflexa	0.06	0.04	0.02	**0.002**
Ps. hoffmannseggiana	0.12	0.11	0.01	0.096
Four species combined	0.13	0.11	0.02	**0.0145**

Table 2.2 – Permutation test results on the effect of time of day, grouped into day vs. night, on chemical structural-compositional similarity (CSCS) of four species of '*heteropsychotria*'

Treatment:	CSCS			
Day/night	Within	Between	Difference	p
Pa. guianensis	0.096	0.091	0.005	0.190
Ps. acuminata	0.212	0.205	0.007	0.191
Ps. deflexa	0.043	0.038	0.005	0.119
Ps. hoffmannseggiana	0.114	0.106	0.008	0.134

Table 2.3 – Permutation test results from the four species sampled at two time points, in 2015

Treatment:	CSCS			
Diel time	Within	Between	Difference	p
Pi. aequale	0.02	0.02	0.00	0.32
Pi. cordulatum	0.01	0.01	-0.00	0.96
Ps. limonensis	0.01	0.02	-0.01	0.92
Ps. marginata	0.01	0.02	-0.00	0.71

Table 2.4 – GNPS library ID matches for compounds found in the four species sampled in 2015

Library match	Chemical class
2-O-rhamnosyl-swertisin	Flavonoid
Chrysin	Flavonoid (flavone)
Grandisin	Lignan
Janthielamide	Lipoamide
5,7-dihydroxy-2-(4-hydroxyphenyl)-6-[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]-8-(3,4,5-trihydroxyoxan-2-yl)chromen-4-one	Chromone
4-(2,4-dimethoxy-3,6-dimethylbenzoyl)oxy-2-hydroxy-3,6-dimethylbenzoic acid	Benzoic acid
5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-7-[3,4,5-trihydroxy-6-[(3,4,5-trihydroxy-6-methyloxan-2-yl)oxymethyl]oxan-2-yl]oxychromen-4-one	Oxychromone
4-hydroxy-9-(2-methylbut-3-en-2-yl)furo[3,2-g]chromen-7-one	Furochromone
7-hydroxy-2-(4-methoxyphenyl)chromen-4-one	Chromone
Nordihydroguaiaretic acid	Lignan
Pheophorbide A	Photosensitzer (chlorophyll degradation product)
Physostigmine	Pyrroloindole alkaloid
Piperine	Piperidine alkaloid
Vicenin-2	Flavonoid glycoside
Xanthoquinodin A3	Xanthoquinodin

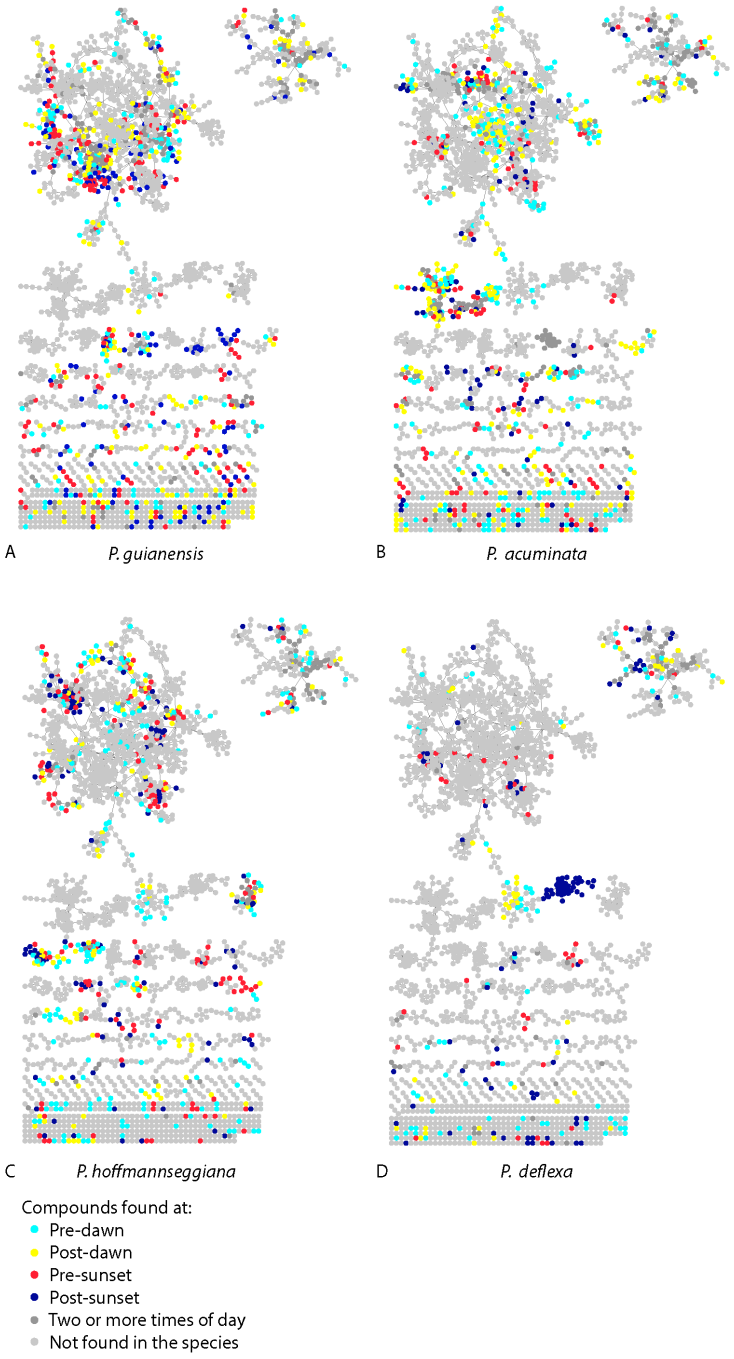

Figure 2.1 – Molecular network showing secondary metabolite diversity within and between four species of *Psychotria*, with significant diel turnover in the presence and absence of unique secondary metabolites. Included are 2420 metabolites linked to at least one other putative compound by a cosine score of ≥0.6. Each node is a distinct metabolite; edges represent chemical structural similarity between metabolites. (Note – *P. guianensis* is still named as *Palicourea guianensis*, but molecular phylogenies confirm its monophyly with the 'heteropsychotria' clade. I include it in *Psychotria* here).

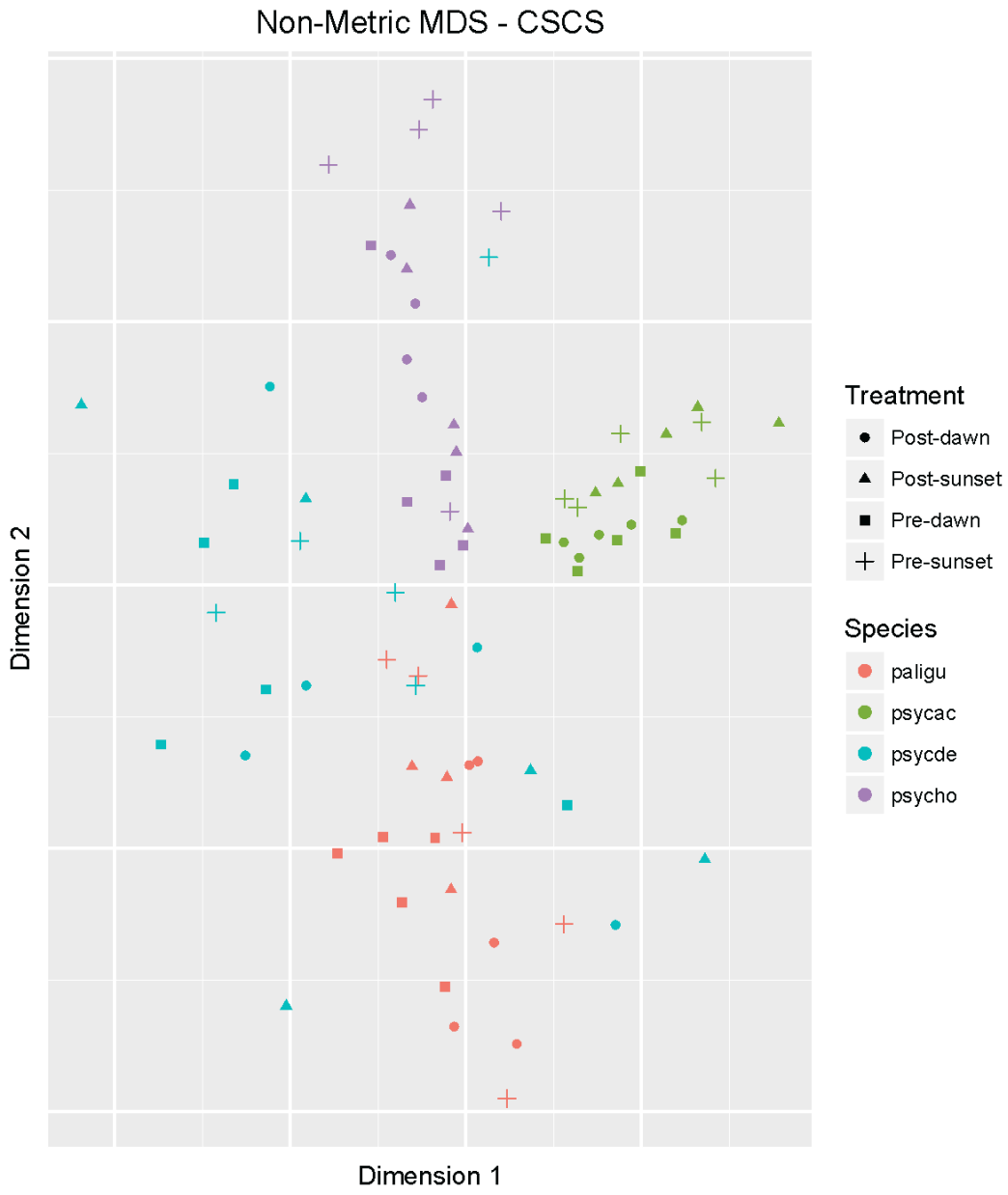

Figure 2.2 – Non-metric Multidimensional Scaling analysis reveals that species show fairly consistent segregation from each other in the CSCS of their secondary metabolome, but the effect of time of day on chemical compositional changes in the plants is unclear.

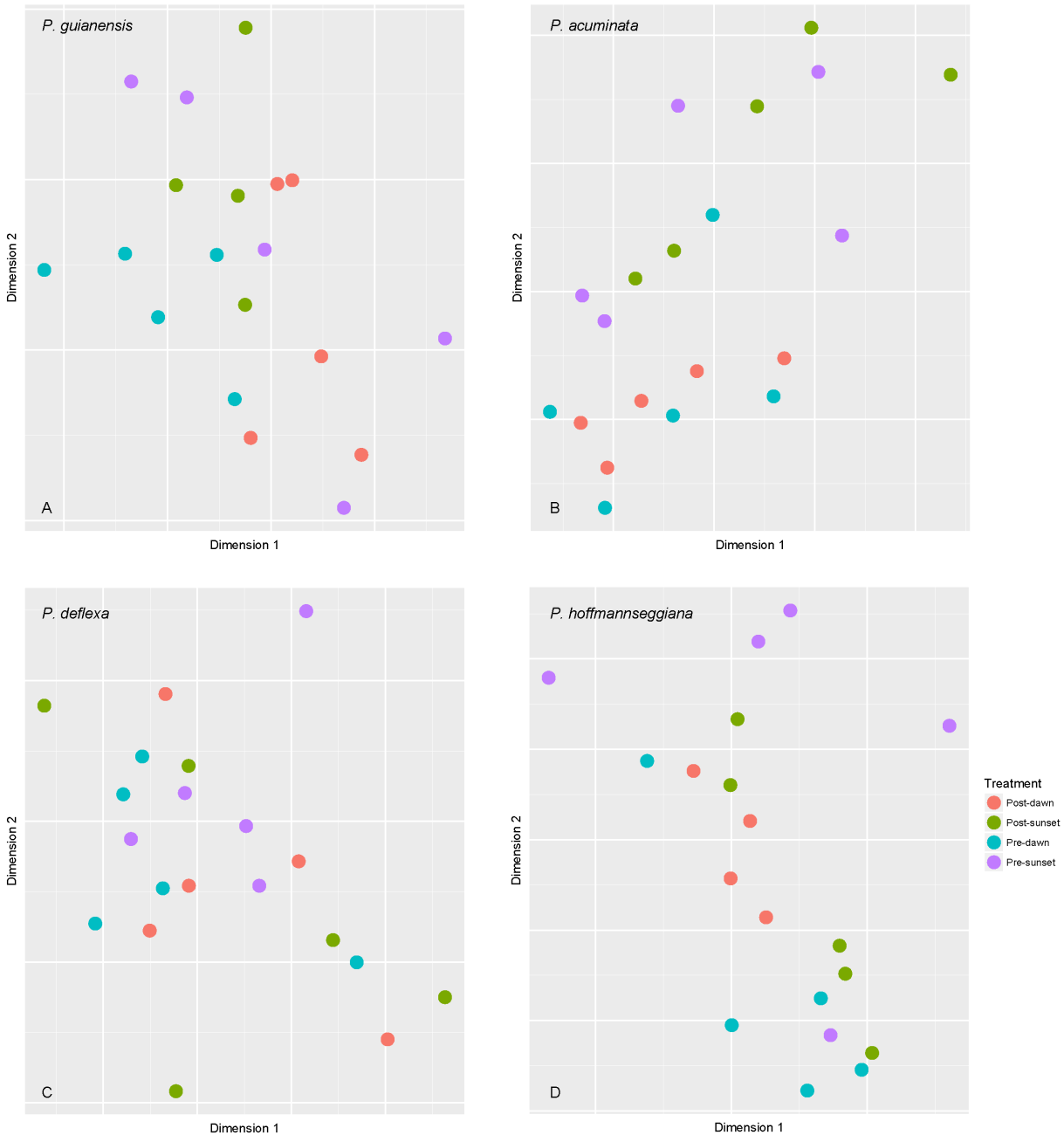

Figure 2.3 – Non-metric Multidimensional Scaling analysis for the CSCS diel variation of each species separately.

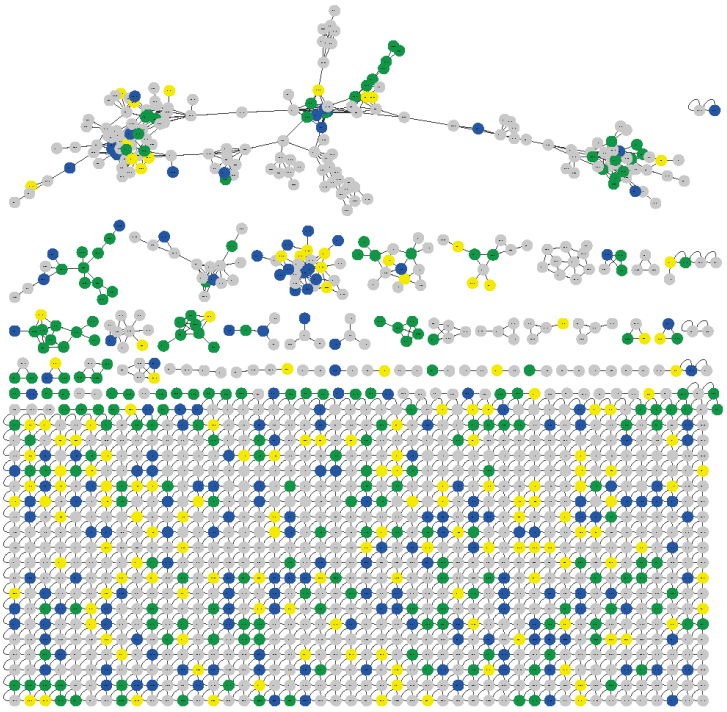

Figure 2.4 – Network view of the secondary metabolites present in *Piper aequale*. Each node is a distinct metabolite; edges link compounds that share structural similarity. Green dots represent metabolites present during in samples from both collection times, blue dots represent compounds present only during the night, yellow dots represent compounds present only during the day. Gray dots are compounds that were present in at least one of the other three species used in this analysis, but not in *Piper aequale*.

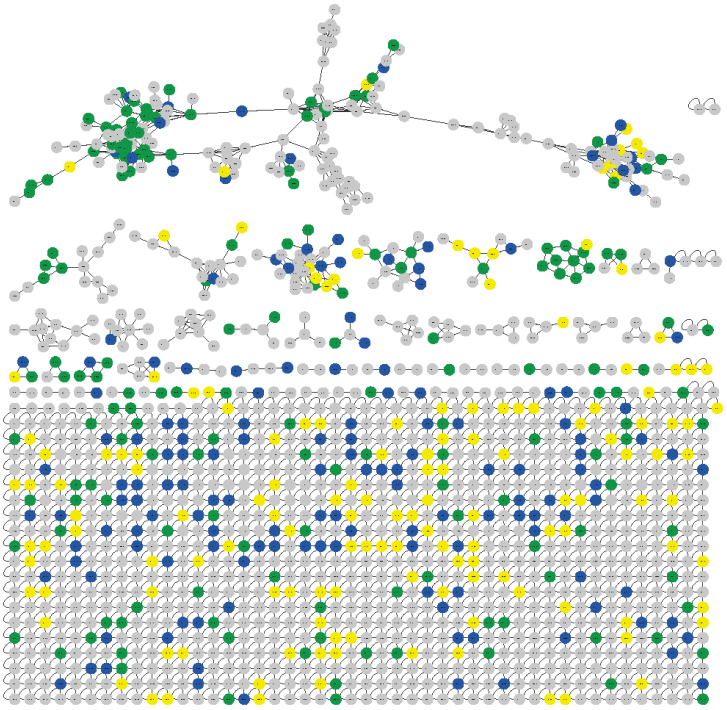

Figure 2.5 – Network view of the secondary metabolites present in *Piper cordulatum*. Each node is a distinct metabolite; edges link compounds that share structural similarity. Green dots represent metabolites present during in samples from both collection times, blue dots represent compounds present only during the night, yellow dots represent compounds present only during the day. Gray dots are compounds that were present in at least one of the other three species used in this analysis, but not in *Piper cordulatum*.

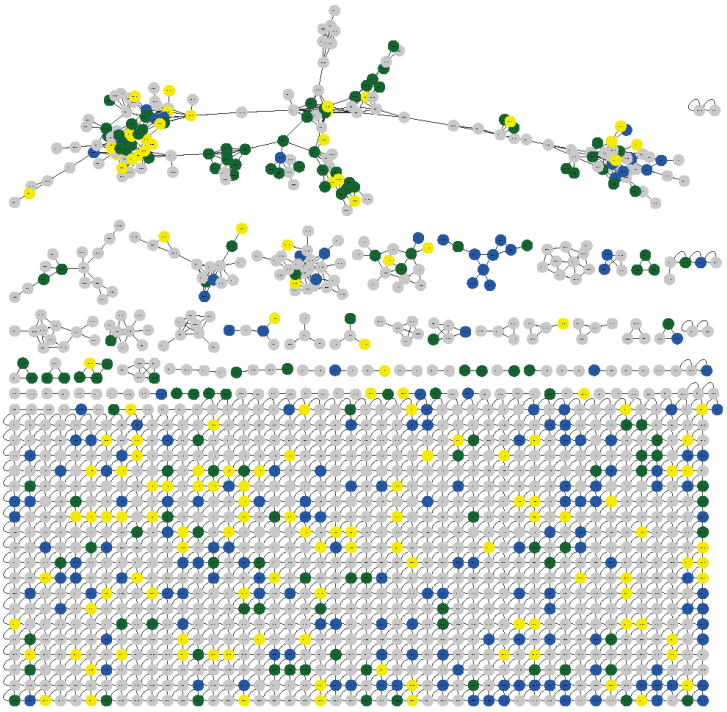

Figure 2.6 – Network view of the secondary metabolites present in *Psychotria limonensis*. Each node is a distinct metabolite; edges link compounds that share structural similarity. Green dots represent metabolites present during in samples from both collection times, blue dots represent compounds present only during the night, yellow dots represent compounds present only during the day. Gray dots are compounds that were present in at least one of the other three species used in this analysis, but not in *Psychotria limonensis*.

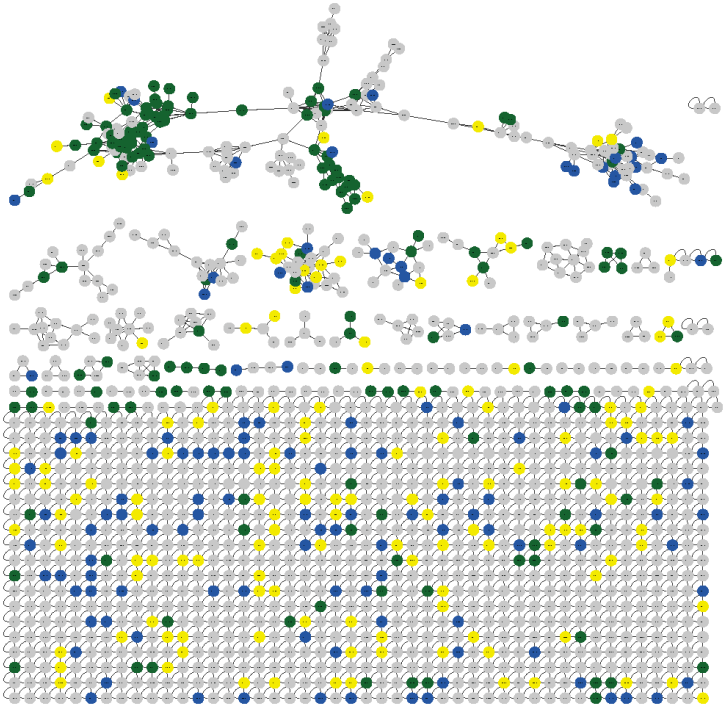

Figure 2.7 – Network view of the secondary metabolites present in *Psychotria marginata*. Each node is a distinct metabolite; edges link compounds that share structural similarity. Green dots represent metabolites present during in samples from both collection times, blue dots represent compounds present only during the night, yellow dots represent compounds present only during the day. Gray dots are compounds that were present in at least one of the other three species used in this analysis, but not in *Psychotria marginata*.

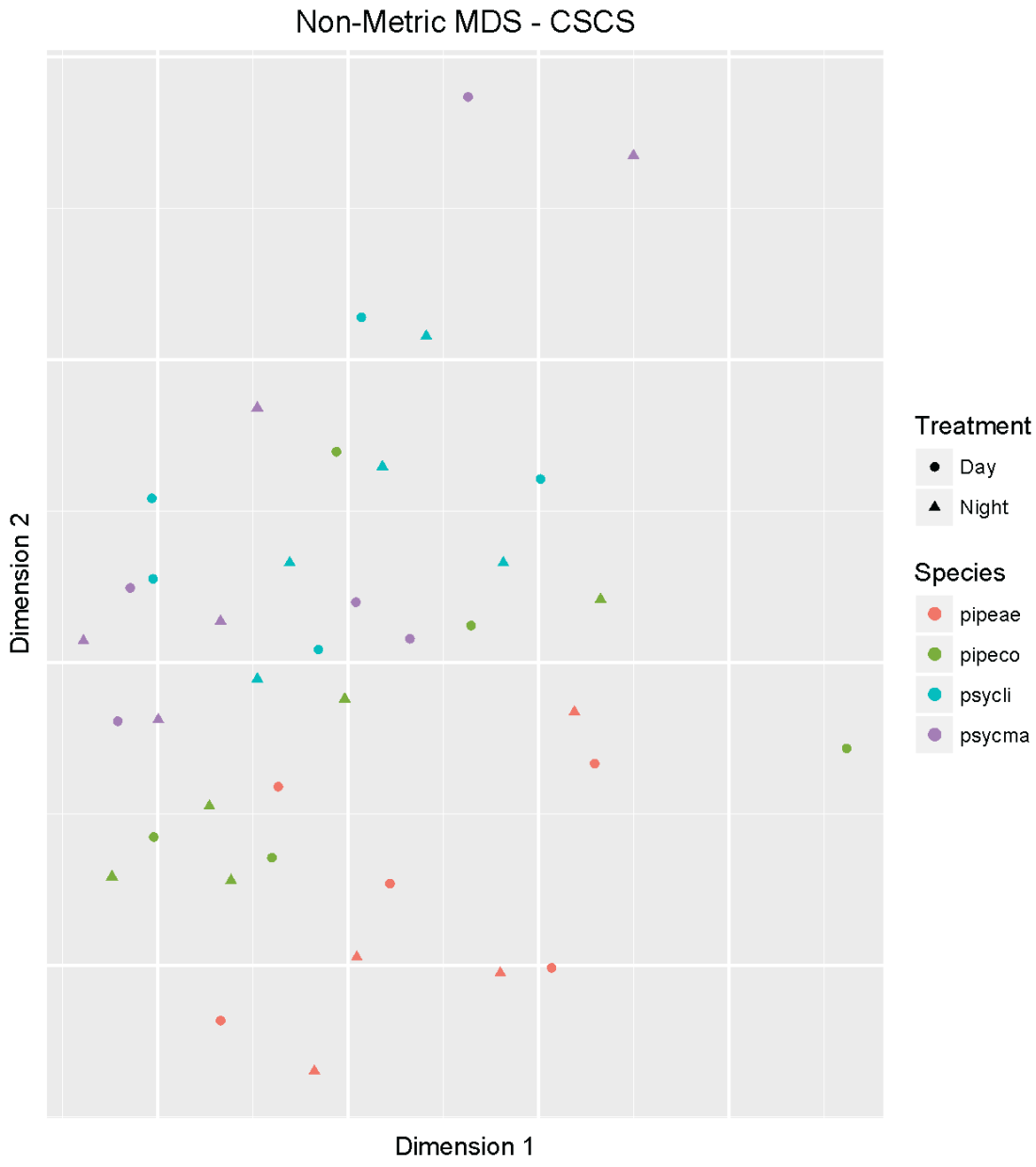

Figure 2.8 – Non-metric Multidimensional Scaling analysis of the CSCS for the four species sampled in 2015. No pattern of species or time of day is evident.

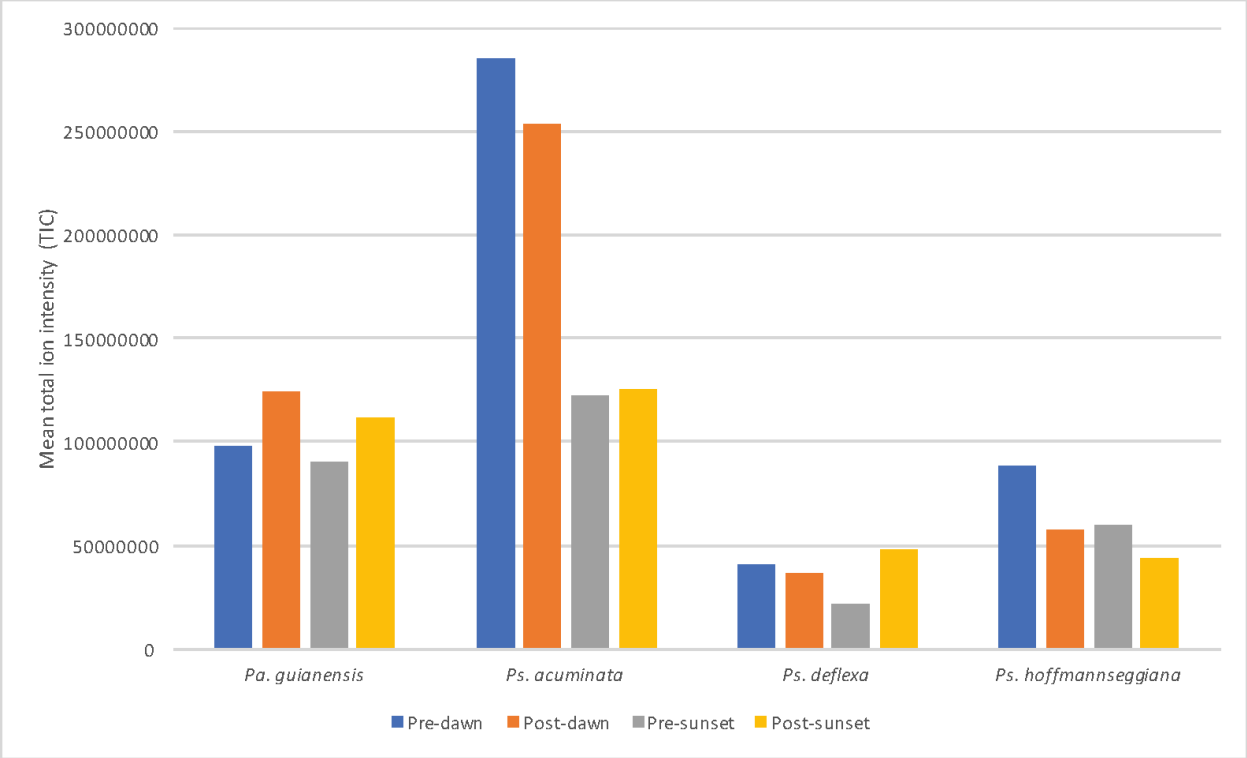

Figure 2.9 – Total ion chromatogram (TIC) for the four species of 'heteropsychotria,' grouped by species, and showing time of leaf collection.

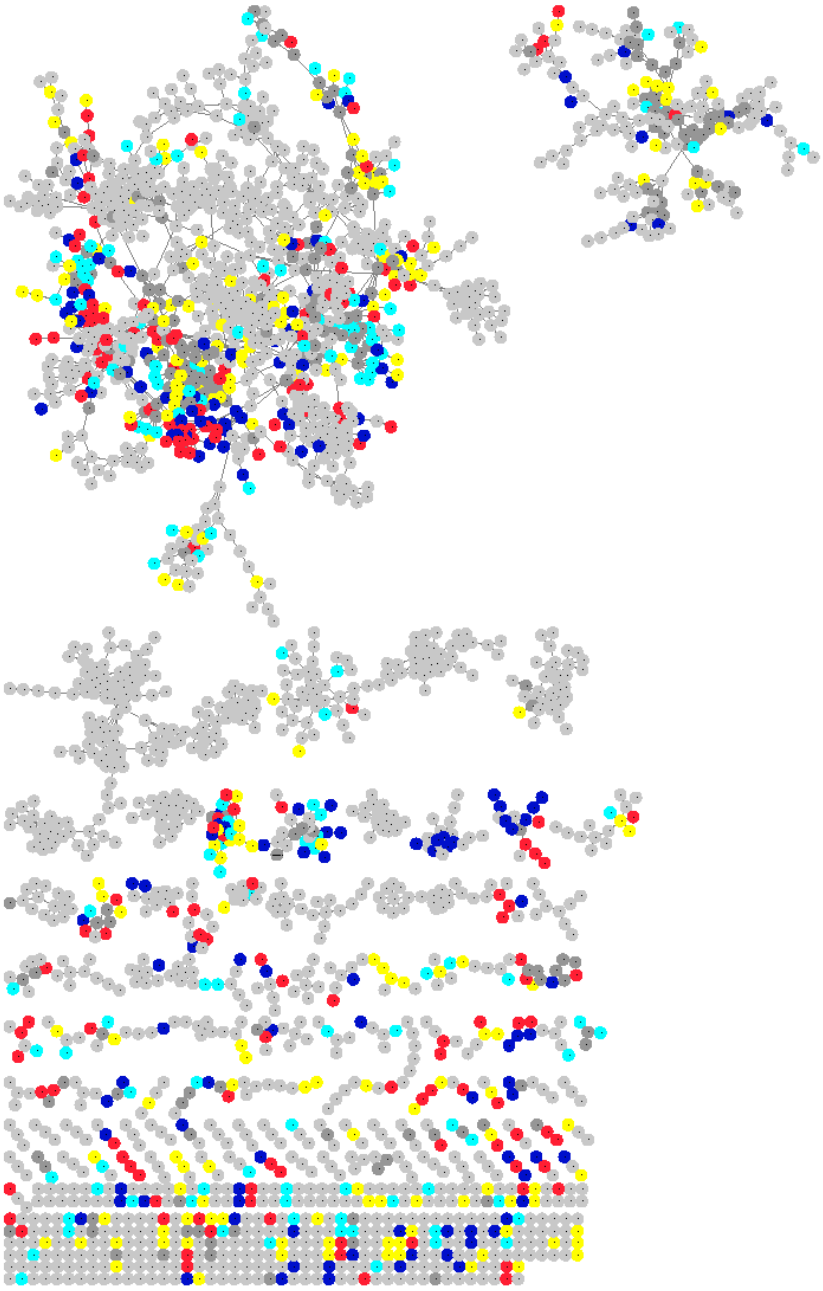

Figure 2.10 – Molecular network for CSCS of *Palicourea guianensis*, showing the only GNPS library match in the data from 2016 – Pheophorbide A, a photosensitivity-inducing photosynthesis degradation product.

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APPENDIX A: LEAF SAMPLE COLLECTION INFORMATION

Table A.1 – Leaf sample collection times for 2016 diel variation in plant metabolome

Sample	Species	Treatment	Time collected	Time in -80ºC	Date
A1.1	*Psyc. acuminata*	Pre-sunset	16:33	18:10	9-Jul-16
A1.2	*Psyc. acuminata*	Post-sunset	21:21	23:00	9-Jul-16
A1.3	*Psyc. acuminata*	Pre-dawn	3:46	4:51	10-Jul-16
A1.4	*Psyc. acuminata*	Post-dawn	8:57	10:47	10-Jul-16
A2.1	*Psyc. acuminata*	Pre-sunset	16:54	18:10	9-Jul-16
A2.2	*Psyc. acuminata*	Post-sunset	21:40	23:00	9-Jul-16
A2.3	*Psyc. acuminata*	Pre-dawn	4:00	4:51	10-Jul-16
A2.4	*Psyc. acuminata*	Post-dawn	9:21	10:47	10-Jul-16
A3.1	*Psyc. acuminata*	Pre-sunset	17:10	18:10	9-Jul-16
A3.2	*Psyc. acuminata*	Post-sunset	21:49	23:00	9-Jul-16
A3.3	*Psyc. acuminata*	Pre-dawn	4:06	4:51	10-Jul-16
A3.4	*Psyc. acuminata*	Post-dawn	9:34	10:47	10-Jul-16
A4.1	*Psyc. acuminata*	Pre-sunset	17:46	18:10	9-Jul-16
A4.2	*Psyc. acuminata*	Post-sunset	22:34	23:00	9-Jul-16
A4.3	*Psyc. acuminata*	Pre-dawn	4:30	4:51	10-Jul-16
A4.4	*Psyc. acuminata*	Post-dawn	10:15	10:47	10-Jul-16
A5.1	*Psyc. acuminata*	Pre-sunset	17:48	18:10	9-Jul-16
A5.2	*Psyc. acuminata*	Post-sunset	22:37	23:00	9-Jul-16
A5.3	*Psyc. acuminata*	Pre-dawn	4:32	4:51	10-Jul-16
A5.4	*Psyc. acuminata*	Post-dawn	10:17	10:47	10-Jul-16
D1.1	*Psyc. deflexa*	Pre-sunset	17:02	18:10	9-Jul-16
D1.2	*Psyc. deflexa*	Post-sunset	21:46	23:00	9-Jul-16
D1.3	*Psyc. deflexa*	Pre-dawn	4:04	4:51	10-Jul-16
D1.4	*Psyc. deflexa*	Post-dawn	9:29	10:47	10-Jul-16
D2.1	*Psyc. deflexa*	Pre-sunset	17:16	18:10	9-Jul-16
D2.2	*Psyc. deflexa*	Post-sunset	21:55	23:00	9-Jul-16
D2.3	*Psyc. deflexa*	Pre-dawn	4:11	4:51	10-Jul-16
D2.4	*Psyc. deflexa*	Post-dawn	9:56	10:47	10-Jul-16
D3.1	*Psyc. deflexa*	Pre-sunset	17:21	18:10	9-Jul-16
D3.2	*Psyc. deflexa*	Post-sunset	21:58	23:00	9-Jul-16
D3.3	*Psyc. deflexa*	Pre-dawn	4:13	4:51	10-Jul-16
D3.4	*Psyc. deflexa*	Post-dawn	9:59	10:47	10-Jul-16
D4.1	*Psyc. deflexa*	Pre-sunset	17:24	18:10	9-Jul-16
D4.2	*Psyc. deflexa*	Post-sunset	22:11	23:00	9-Jul-16
D4.3	*Psyc. deflexa*	Pre-dawn	4:16	4:51	10-Jul-16
D4.4	*Psyc. deflexa*	Post-dawn	10:05	10:47	10-Jul-16
D5.1	*Psyc. deflexa*	Pre-sunset	17:37	18:10	9-Jul-16
D5.2	*Psyc. deflexa*	Post-sunset	22:15	23:00	9-Jul-16
D5.3	*Psyc. deflexa*	Pre-dawn	4:19	4:51	10-Jul-16

Table A.1 (cont.)

D5.4	*Psyc. deflexa*	Post-dawn	10:07	10:47	10-Jul-16
G1.1	*Pali. guianensis*	Pre-sunset	16:43	18:10	9-Jul-16
G1.2	*Pali. guianensis*	Post-sunset	21:28	23:00	9-Jul-16
G1.3	*Pali. guianensis*	Pre-dawn	3:53	4:51	10-Jul-16
G1.4	*Pali. guianensis*	Post-dawn	9:08	10:47	10-Jul-16
G2.1	*Pali. guianensis*	Pre-sunset	16:45	18:10	9-Jul-16
G2.2	*Pali. guianensis*	Post-sunset	21:31	23:00	9-Jul-16
G2.3	*Pali. guianensis*	Pre-dawn	3:55	4:51	10-Jul-16
G2.4	*Pali. guianensis*	Post-dawn	9:11	10:47	10-Jul-16
G3.1	*Pali. guianensis*	Pre-sunset	17:55	18:10	9-Jul-16
G3.2	*Pali. guianensis*	Post-sunset	22:44	23:00	9-Jul-16
G3.3	*Pali. guianensis*	Pre-dawn	4:38	4:51	10-Jul-16
G3.4	*Pali. guianensis*	Post-dawn	10:34	10:47	10-Jul-16
G4.1	*Pali. guianensis*	Pre-sunset	17:55	18:10	9-Jul-16
G4.2	*Pali. guianensis*	Post-sunset	22:46	23:00	9-Jul-16
G4.3	*Pali. guianensis*	Pre-dawn	4:39	4:51	10-Jul-16
G4.4	*Pali. guianensis*	Post-dawn	10:35	10:47	10-Jul-16
G5.1	*Pali. guianensis*	Pre-sunset	16:47	18:10	9-Jul-16
G5.2	*Pali. guianensis*	Post-sunset	21:35	23:00	9-Jul-16
G5.3	*Pali. guianensis*	Pre-dawn	3:57	4:51	10-Jul-16
G5.4	*Pali. guianensis*	Post-dawn	9:13	10:47	10-Jul-16
H1.1	*Psyc. hoffmannseggiana*	Pre-sunset	16:51	18:10	9-Jul-16
H1.2	*Psyc. hoffmannseggiana*	Post-sunset	21:37	23:00	9-Jul-16
H1.3	*Psyc. hoffmannseggiana*	Pre-dawn	3:58	4:51	10-Jul-16
H1.4	*Psyc. hoffmannseggiana*	Post-dawn	9:17	10:47	10-Jul-16
H2.1	*Psyc. hoffmannseggiana*	Pre-sunset	16:55	18:10	9-Jul-16
H2.2	*Psyc. hoffmannseggiana*	Post-sunset	21:43	23:00	9-Jul-16
H2.3	*Psyc. hoffmannseggiana*	Pre-dawn	4:01	4:51	10-Jul-16
H2.4	*Psyc. hoffmannseggiana*	Post-dawn	9:23	10:47	10-Jul-16
H3.1	*Psyc. hoffmannseggiana*	Pre-sunset	17:40	18:10	9-Jul-16
H3.2	*Psyc. hoffmannseggiana*	Post-sunset	22:20	23:00	9-Jul-16
H3.3	*Psyc. hoffmannseggiana*	Pre-dawn	4:25	4:51	10-Jul-16
H3.4	*Psyc. hoffmannseggiana*	Post-dawn	10:10	10:47	10-Jul-16
H4.1	*Psyc. hoffmannseggiana*	Pre-sunset	17:51	18:10	9-Jul-16
H4.2	*Psyc. hoffmannseggiana*	Post-sunset	22:40	23:00	9-Jul-16
H4.3	*Psyc. hoffmannseggiana*	Pre-dawn	4:34	4:51	10-Jul-16
H4.4	*Psyc. hoffmannseggiana*	Post-dawn	10:19	10:47	10-Jul-16
H5.1	*Psyc. hoffmannseggiana*	Pre-sunset	17:53	18:10	9-Jul-16
H5.2	*Psyc. hoffmannseggiana*	Post-sunset	22:42	23:00	9-Jul-16
H5.3	*Psyc. hoffmannseggiana*	Pre-dawn	4:37	4:51	10-Jul-16
H5.4	*Psyc. hoffmannseggiana*	Post-dawn	10:33	10:47	10-Jul-16