Evolution and ecological consequences of diverse traits in tropical pitcher plants (Nepenthes)

Evolution and ecological consequences of diverse traits in tropical pitcher plants (*Nepenthes*)

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Evolution and ecological consequences

of diverse traits in tropical pitcher plants (*Nepenthes*)

Abstract

Plants play a major role in Earth's terrestrial biodiversity, and by extension so do their interactions with other organisms. The role of plant traits in influencing their interaction partners is well-studied in the case of pollination and herbivory, but plant symbiosis is somewhat less well-understood. One example is the class of interactions occurring in phytotelmata, or plant-held water bodies, in which diverse communities of organisms live. These systems have often been utilized as models for community ecology, but less has been done on the direct interactions between the plants themselves and the phytotelm community. As phytotelmata are essentially aquatic islands surrounded by terrestrial habitat, the aquatic inhabitants are completely reliant upon their host plant, so plant traits have strong potential to mediate interactions with symbionts. This is true of tropical pitcher plants (*Nepenthes*) whose pitchers (modified leaves) have evolved morphological and physiological traits to aid in prey capture and digestion, however there may also be selective influence on the pitchers exerted by symbionts in the phytotelm—as well as by herbivores and external abiotic conditions. So, *Nepenthes* present an excellent system for investigating how multiple biotic and abiotic interactors can shape and be shaped by plant diversity.

In this dissertation, I take a broad view of the *Nepenthes* system in order to examine potential adaptive explanations of trait variation for the autecology of the plants as well as how those traits influence symbionts and other interactors. In Chapter One, I combine field observations of a single polymorphic species (*Nepenthes gracilis*) with phylogenetic comparative analysis of 85 species across the genus to investigate correlations between color polymorphism and ecological factors including altitude, light environment, and herbivory. I find that pitcher traits appear to be remarkably labile, and largely lack

phylogenetic signal. However, stochastic character mapping shows that the evolution of color polymorphism is biased towards more red-pigmented lower pitchers as opposed to other trait states. In *N. gracilis*, color does not correlate with prey-capture and symbiont colonization, but red pitchers experience less herbivory. This work highlights ecological correlates of the vast phenotypic diversity of this group of tropical plants and points to a need for future work examining herbivores of *Nepenthes* and experimental investigations on color polymorphism.

For Chapters Two and Three of my thesis, I examine the influence that inter- and intra-specific pitcher trait variation has on the bacterial and eukaryotic phytotelm community through metabarcoding. Recent work has shown convergence in communities between the convergently evolved pitcher plant genera *Nepenthes* and *Sarracenia*. This shows that pitcher plants have specific characteristic communities. What is less well known is whether different species of pitchers actively cultivate their own characteristic communities. In Chapter Two of my thesis, I demonstrate that *Nepenthes* pitchers have the ability to filter their communities in species-specific ways and that certain pitcher traits, especially pH, can explain many aspects of community differences. I use a common garden experiment with 16 *Nepenthes* species in cultivation that were never exposed to their native environment, all reared in the same controlled greenhouse, and all filled with pH 6.5 water from a common source. The different *Nepenthes* species differentially altered the abiotic characteristics within their pitchers, including pH and viscosity. In Chapter Three, I examine how altitude and intraspecific variation influences communities in the pitchers of *N. mindanaoensis* collected from an altitudinal transect on Mount Hamiguitan, Mindanao, the Philippines. I show differences in patterns of community assembly for bacteria and eukaryotes, despite their living together in the same aquatic microhabitats. Community similarity of eukaryotes, but not bacteria, are significantly influenced by altitude. On the other hand, pitcher dimorphism has an effect on eukaryotes but not bacteria, while variation in pH levels strongly influences both taxa. Additionally, I show that arthropod abundance in this system follows the classical trend of decreasing with elevation, and I point to some differences in the patterns of abundance for living inquiline insects as opposed to insect prey, in relation to intraspecific plant trait variation.

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Introduction

Plant-animal interactions play a major role in Earth's terrestrial biodiversity. The most obvious and well-studied plant-animal interactions are those in which plants entice animals to aid in their reproduction (pollination and seed dispersal) and those in which insects attack plant tissues, leading to the evolution of defenses in the plants and an ensuing coevolutionary arms race. A more often overlooked class of plant-animal interaction, which may nevertheless be of considerable ecological importance given its prevalence, is a symbiotic relationship in which an animal uses a plant as habitat for its survival and growth—a symbiosis that can be mutualistic, parasitic, or commensal. Ant-plant associations are a classic example of a mutualistic symbiosis, where certain plant species produce special structures that house ant colonies in exchange for their protection against herbivores. These strong ecological interactions can also show evidence of coevolution. Another example is provided by gall-forming arthropods that coerce plants into forming a microhabitat and food source for them. Plants are typically harmed by this interaction, making it parasitic.

Yet another fascinating class of plant-animal symbiosis is the microhabitat known as the phytotelm, or plant-held water body. Unlike galls, the properties of this microhabitat are more often controlled by the plant rather than the animal. There are many different kinds of phytotelmata that have varying degrees of control over the biotic and abiotic activity within the aquatic microhabitat, but the most basic kind of influence that they all exert is some kind of modulation of the chemical composition of the fluid they contain (Kitching 2000). The discreteness of these phytotelmata is one reason they have often been employed in community ecology and food web research (Kitching 2000). Phytotelmata can be seen as discrete ecological islands with aquatic inhabitants that typically cannot persist solely in the surrounding terrestrial environment. Organisms with biphasic life histories with aquatic larvae and terrestrial adults, including insects and amphibians and some crustaceans, are completely confined to the phytotelm during the larval phase and disperse as adults, possibly selecting a new phytotelm for oviposition based on site quality. As long as the larvae live in the phytotelm, though, they must contend with the particular conditions of that environment.

Much work has been done describing the community-level composition of various phytotelmata and other container microhabitats, but less is known about the direct influence that plant traits have on their symbionts. These influences can take the form of plant secondary chemistry, morphological defense, manipulation of plant microclimate at the level of the leaf, and indirect effects via third parties such as endophytic fungi and bacteria.

Here, I examine the roles that *Nepenthes* pitcher plants play in influencing their interactions with arthropods, both in terms of prey capture and especially with regard to the community of symbionts living within the specialized phytotelm system. In this dissertation, I: (1) assess the effect that variability in pitcher coloration has on the plants' interactions with insect inquilines, prey, and herbivores, and more broadly explore the possible adaptive underpinnings of this trait in an evolutionary context; (2) investigate whether interspecific differences in pitcher traits can lead to ecological filtering of their microbiomes despite sharing a common external environment and similar starting conditions; and (3) examine the eukaryotic and bacterial communities of wild pitchers within a single species along an elevational gradient in order to assess the relative effects of external environmental conditions and intraspecific variation of plant-regulated traits on different taxa.

Chapter 1: Keeping an eye on coloration: Ecological correlates of the evolution of pitcher traits in the genus *Nepenthes* (Caryophyllales)

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Running title: Pitcher evolution in *Nepenthes*

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Abstract

Nepenthes is a genus of carnivorous pitcher plants with high intra- and interspecific morphological diversity. Many species produce dimorphic pitchers, and the relative production rate of the two morphs varies interspecifically. Despite their likely ecological importance to the plants, little is known about the selective context under which various pitcher traits have evolved. This is especially true of color-related traits, which have not been examined in a phylogenetic context. Using field observations of one polymorphic species (*N. gracilis*) and phylogenetic comparative analysis of 85 species across the genus, we investigate correlations between color polymorphism and ecological factors including altitude, light environment, and herbivory. In *N. gracilis*, color does not correlate to amount of prey-capture, but red pitchers experience less herbivory. Throughout the genus, color polymorphism with redder lower pitchers appears to be evolutionarily favored. We found a lack of phylogenetic signal for most traits, either suggesting that most traits are labile or reflecting the uncertainty regarding the underlying tree topology. This work highlights ecological correlates of the vast phenotypic diversity of this group of tropical plants. We point to a need for future work examining herbivores of *Nepenthes* and experimental investigations on color polymorphism.

Key Words: altitude - carnivorous plants - coloration - comparative methods - herbivory - intraspecific diversity - *Nepenthes* L. - pitcher plants - plant-animal interactions

Introduction:

Competition for resources can lead to the divergence of a clade into multiple niches and the evolution of novel morphological features. This can be seen in many plant radiations such as the bromeliads, where the species that came to occupy water- and nutrient-limited habitats evolved tightly pressed leaf tanks capable of collecting water and nutrient-rich debris (Benzing & Renfrow, 1974; Givnish et al., 2011). In addition to resource limitation and/or competition, plants must also routinely respond to a suite of interspecific interactions. For instance, animal pollinators have a prominent role in shaping floral evolution (e.g. Muchhala & Potts, 2007; Kay & Sargent, 2009; Alcantara & Lohmann, 2011; van der Niet & Johnson, 2012; Anderson et al., 2014; Boberg et al., 2014; Muchhala, Johnsen, & Smith, 2014; Lagomarsino et al., 2016). Thus plant morphological evolution can have multiple, interacting biotic and abiotic drivers. However, disentangling the effects of these various drivers is difficult and has not been achieved in many groups.

Pitcher plants are one such group characterized by an adaptation that is subject to multiple interacting drivers. Their pitchers are modified leaves used to capture and digest animal prey—they are nitrogen-acquiring organs analogous to bromeliad tanks, but are also like flowers in their potential to coevolve with animal visitors. Pitcher plants are thus a useful system to investigate the roles of abiotic and biotic effects on the diversification of an adaptive trait. Here we examine the carnivorous plant genus *Nepenthes* L., the most diverse (>140 species: Cheek & Jebb, 2014) and widespread family of carnivorous pitcher plants. Its core distribution spans most of Southeast Asia, with a few outlying species in Madagascar to the west and New Caledonia to the east, and ranges in altitude from 0 up to 3520 m asl (McPherson, Robinson, & Fleischmann, 2009). The pitchers of different species are used to prey on insects, but additionally may be involved in interactions spanning from commensalism to parasitism and mutualism (e.g. Beaver, 1979; Adlassnig, Peroutka, & Lendl, 2011; Bazile et al., 2012; Thornham et al., 2012; Scharmann et al., 2013). Pitchers are morphologically complex, exhibiting an array of traits that are under selection by both biotic and abiotic factors that are difficult to tease apart.

Recent work has elucidated the functional significance of pitcher traits including the thickness of the peristome, presence of a waxy layer, viscosity of the fluid, and digestive gland structure in relation to prey trapping efficiency (Bonhomme et al., 2011; Renner & Specht, 2011; Bauer et al., 2012). Although it has not yet been well documented whether interspecific differences can be explained by niche-partitioning (Chin, Chung, & Clarke, 2014; but see Peng & Clarke, 2015; Gaume et al., 2016), the few known specialist trappers point to the importance of animal visitors to pitcher phenotypic evolution; this includes traits such as the parabolic structure in *N. hemsleyana* Macfarl. that functions as an echolocation guide for its mutualist bat (Schöner et al., 2015; Schöner et al., 2017). Despite growing knowledge of the significance of different trapping features to the genus, less than 10% of all species have been the subject of ecological studies detailing their specific prey capture strategies (Clarke and Moran, 2011), and the functional significance of many pitcher traits has yet to be explored.

One potentially important, yet understudied set of traits in pitcher plants are those related to intraspecific polymorphism. Many species produce two distinct pitcher morphologies ("morphs") throughout the lifespan of an individual plant: lower pitchers ("lowers"), which grow gravitropically from plants in the rosette phase and possess winged fringes of tissue ("wings"); and upper pitchers ("uppers"), which grow from plants in the climbing phase, twine onto surrounding vegetation via their tendrils, and possess a more streamlined form lacking wings (Jebb and Cheek, 1997; Figure 1.1). There is some evidence of prey-partitioning between pitcher morphs (Moran, 1996; Rembold et al., 2010; Gaume et al., 2016) and differences in symbiont communities of different pitcher morphs (Clarke, 1997a), but little is known about the evolution or functional significance of pitcher dimorphism.

In addition to shape, many species also have pitchers that vary from red to green, often with discrete color differences between the lower and upper morphs (Figure 1.1—we hereafter refer to the occurrence of discrete color differences between pitcher morphs within a plant as "color polymorphism"). Despite being a conspicuous feature of *Nepenthes*, pitcher coloration is poorly understood. A few studies have examined the role of red pigmentation as a visual signal in carnivorous plants (Schaefer & Ruxton, 2008; Bennett & Ellison, 2009; Foot, Rice, & Millet, 2014; Jürgens et al., 2015; El-Sayed, Byers, &

Suckling, 2016), and a number have hypothesized that the contrast of red against a green background of foliage could be attractive, although many insects lack red perception (Briscoe and Chittka, 2001). Red was not found to be a prey attractant in studies with sundews (Foot, Rice, & Millet, 2014; Jürgens et al., 2015; El-Sayed, Byers, & Suckling, 2016), but results have been conflicting in pitcher plants (Schaefer & Ruxton, 2008; Bennett & Ellison, 2009).

Figure 1.1. (A-D) Photographs showing polymorphism in *Nepenthes gracilis*: (A) green lower pitcher, (B) red lower pitcher, (C) green upper pitcher, and (D) red upper pitcher. An (E) upper and (F) lower pitcher of *N. rafflesiana* showing a second example of pitcher dimorphism. Note the difference in tendrils (black arrows) between morphs, which twine in uppers and grow gravitropically in lowers, and note the wings (white arrows) in lower pitchers, which are lacking or reduced in uppers. (G) A lower pitcher of *N. hamata*, indicating the peristome with tall ribs (blue arrow) (H) Diagram of a generalized *Nepenthes* plant with key morphological features labelled. Diagram of plants with (I,J) similar coloration between morphs, (K) redder lower pitchers, and (L) redder upper pitchers. Photo credits: A and C-G, K. Gilbert; B, S. Johnson-Freyd. Illustration credit: H-L, Abraham Cone.

As red pigmentation in *Nepenthes* is due to anthocyanins (Kováčik, Klejdus, & Repčáková, 2012), which are costly to produce (Gould, 2004), the existence of intraspecific color polymorphism in pitcher plants is particularly puzzling. More generally speaking, the role of plant anthocyanins as visual signals in flowers and fruits is well understood, but the function of anthocyanins in leaves is less resolved.

Multiple competing, though not necessarily mutually exclusive, hypotheses have been proposed for the role of anthocyanins in leaves (Gould, 2004), the majority of which can be divided into two camps: those that argue that anthocyanins primarily serve a physiological role, vs. those that posit that they are visual signals and primarily a result of coevolving with herbivores (Archetti & Brown, 2004; Archetti et al., 2009). Some potential physiological functions of anthocyanins involve protecting leaves from excess light, including UV shielding and free radical scavenging (Feild, Lee, & Holbrook, 2001; Hoch, Zeldin, & McCown, 2001; Neill & Gould, 2003), and facilitating nutrient resorption in the context of deciduous color-changing leaves (Hughes, Singsaus, & McCown, 2003). Additionally, abaxial anthocyanins in understory plants have been proposed to improve photosynthetic efficiency in the "back-scatter hypothesis", though there is some experimental evidence against this (Hughes, Vogelman, & Smith, 2008). Considering the coevolution hypothesis, anthocyanins may serve as either direct (Schaefer, Rentzsch, & Breuer, 2008; Tellez, Rojas, & Van Bael, 2016) or indirect (Page & Towers, 2002; Archetti & Brown, 2004; Karageorgou & Manetas, 2006; Schaefer & Rolshausen, 2006; Lev-Yadun & Gould, 2008; Archetti et al., 2009) defense against herbivores and pathogens.

Here, we seek to better understand the functional significance and diversification of dimorphic traits in pitchers using two complementary approaches: (1) a field study of the polymorphic species *N. gracilis* Korth., exploring the functional significance of intraspecific variation in pitcher traits and (2) a comparative phylogenetic analysis of species across the genus exploring trait evolution more broadly. This approach should allow us to identify broad patterns across the genus that can be verified in more detail within a particular species.

Our study of *N. gracilis* tests the following hypotheses: 1) red pigmentation promotes prey capture and/or symbiont colonization; 2) red pigmentation increases with increasing light intensity; 3) red-pigmented pitchers show fewer signs of herbivory. Our comparative phylogenetic study first tests for phylogenetic signal in pitcher traits (Felsenstein, 1985). We then use stochastic character mapping to determine if particular color states are evolutionarily favored. Finally, we test for the correlation of pitcher traits with each other and with environmental traits, including habitat type and altitude. Moran et

al. (2013) found precipitation to be a key factor behind the distribution of the traits they examined (peristome width, wax presence, and presence of viscoelastic fluid). Furthermore, previous studies hypothesize that the decreasing availability of ants with altitude (Hölldobler & Wilson, 1990) increases selective pressure for evolving specialized dietary strategies (Clarke et al., 2009), which could impact many pitcher traits including coloration and dimorphism. As both precipitation and ant abundance covary with altitude, so we analyze the role of altitude as a primary abiotic driver of trait evolution. In addition to altitude, we explore habitat and growth habit as proxies for abiotic drivers of coloration evolution.

Methods:

1.**Intraspecific variation in *N. gracilis***

a. **Field sites**

Singapore (1.5° N) is aseasonal, with an average annual rainfall of 2,340 mm, an average minimum diurnal temperature of 25° C, an average maximum diurnal temperature of 37° C, and relative humidity levels generally above 90% in the morning and down to 60% later in the day. The highest point in Singapore is 165 m asl (Bukit Timah Hill). The natural areas utilized in this study include Kent Ridge Park (1°17'13.00"N, 103°47'10.91"E) and MacRitchie Reservoir Park (1°20'34.99"N, 103°49'47.96"E). Kent Ridge Park is a secondary "adinandra belukar" forest, dominated by simpoh air (*Dillenia suffruticosa* Martelli) trees. Adinandra belukar type vegetation is characterized by acidic soils (3.3 – 3.9 pH) and low nitrogen and phosphorous (Chan et al., 1997). The MacRitchie Reservoir Park pitcher plants examined grow on the coast of an artificial water reservoir supported by *Ploiarium alternifolium* Melch. and simpoh air trees. *Nepenthes gracilis* is abundant throughout natural areas in Singapore. We chose this species for its abundance and high level of intraspecific variability. We specifically worked in microhabitats where *N. gracilis* grew isolated from its local congeners (*N. ampullaria* Jack and *N. rafflesiana* Jack).

b. **Assessment of insect accumulation rates in different pitcher variants**

In mid-July 2014, pitchers were sampled from two separate areas within Kent Ridge Park separated by about 0.3 km and at one site in MacRitchie Reservoir, which is about 8 km from Kent Ridge. As we could not know how long each pitcher had been open prior to our survey, we needed to "reset" all of the pitchers in our study sites to be able to compare arthropod colonization rates across pitchers given equal time. We first emptied each pitcher, marked it with a small tag attached to the base of the lamina distal from the pitcher, and then returned to collect its entire fluid contents one month after emptying. Arthropods contained in the pitchers were filtered out from the fluid and stored in 100% ethanol prior to being counted and identified according to higher level classification (e.g. order or family depending on the taxon) under a dissecting scope.taxa under a dissecting scope. We recorded the following characteristics from each sampled pitcher: pitcher morph (upper or lower), pitcher color (red or green), pitcher condition (healthy or damaged/senescent), the length and width of the pitcher, fluid volume, its distance from the ground, a rank of "connectedness" (degree to which pitchers formed physical connections with surrounding plants via twining, scored from 1-3, with 1 being no connection to other vegetation and 3 being fully twined and well-connected), and the node on which the pitcher occurred.

To determine whether counts of insect prey and symbionts differed significantly between pitchers of differing traits, we performed Poisson regressions using the 'glmer' function in the 'lme4' package (Bates et al., 2014) in R 3.3.2 (RCoreTeam, 2013). We collected from multiple pitchers per plant, so we set plant as a random effect, as well as collection site. We included all examined traits (pitcher color, pitcher morph, connectedness, pitcher size, and distance from the ground) as fixed effects in one model in order to account for any correlations among traits. To avoid the potential confounding effects of senescence or increased herbivory, pitchers that deteriorated in condition over the one month period after emptying were excluded from the analysis We tested for statistically significant differences in numbers of ants, culicid larvae (mosquitoes), non-culicid larvae (all low-abundance dipteran taxa), mites, and flying prey items between pitchers that differed in all of the aforementioned measured pitcher characteristics.

c. **Assessment of relationship between pitcher color and canopy coverage**

In January 2014, for 8 arbitrarily-selected locations within Kent Ridge Park, we laid out plots of approximately 1.5 m in diameter and then exhaustively tallied all of the pitchers within the plots. Based on morph and color, pitchers were assigned to one of four categories: red lower, red upper, green lower, and green upper. We estimated the canopy coverage by photographing the canopy above each plot (pointing upwards from the level of the pitchers at the center of the plot) using a digital camera (Canon PowerShot ELPH 170IS) and calculating the total area of shade-free space in each image by counting white cells using 625 $pixel^2$ per cell grids in ImageJ (Rasband, 2012). We tested for a correlation between canopy coverage and the proportion of red pitchers per plot using a linear regression.

d. **Assessment of relationship between pitcher color and herbivory**

In late January to early February 2016, we tallied pitchers within 8 plots in Kent Ridge Park as described above. To test for a relationship between red pigmentation and herbivory in *N. gracilis*, we scored each pitcher within a plot for pitcher type (the four categories of color and morph described above) and for whether or not the pitcher exhibited signs of herbivory or pathogen attack. Pitchers were scored as having signs of attack based on the presence of localized spots of discolored, senescent, or missing tissue anywhere on the pitcher body (this was treated as a binary character, so any pitchers lacking such signs were scored as "not attacked"). We performed a logistic regression using the 'glmer' function in the 'lme4' package (Bates et al., 2014) in R 3.3.2 (RCoreTeam, 2013) to test for a relationship between pitcher color and signs of attack, including plot as a random effect. We also performed a logistic regression in the same way on the subset of lower pitchers to examine the effect of color while controlling for morph. To test for a relationship between pitcher morph and signs of attack while controlling for color, we performed a logistic regression on the subset of green pitchers. The number of red upper pitchers (n=1) was too small to meaningfully compare red uppers and lowers or red and green uppers.

2. Comparative analysis of interspecific variation in Nepenthes

a. Sequence mining and phylogenetic inference

Previous molecular studies of the genus have utilized different markers: the peptide transferase single copy nuclear gene (*PTR1*: Meimberg & Heubl, 2006), the plastid *trnK* intron (Meimberg et al., 2001, 2006; Merckx et al., 2015) and the nuclear ribosomal transcribed spacers (*nrITS1-5.8S-nrITS2*, (Alamsyah & Ito, 2013; Schwallier et al., 2016). Only those studies using *PTR1* and *trnK* shared voucher specimens, so these were the two markers we chose for phylogenetic inference to ensure the taxonomic identity of the specimens was consistent between sequences, especially considering that the risk of misidentified sequences is a caveat inherent to the use of sequences obtained from a database. While currently available sequence data have proven insufficient to conclusively resolve the phylogeny of *Nepenthes* (Meimberg et al., 2001, 2006; Alamsyah & Ito, 2013), they nevertheless provide a working hypothesis with which to begin looking for patterns. Sequences were downloaded from Genbank, resulting in 87 sequences for the ~2500 bp *trnK* plastid gene region, and 40 sequences for the ~1605 bp *PTR1* nuclear gene. We did not use sequences for the pseudogenized copy of the *trnK* gene (Meimberg et al., 2006). Our outgroups were *Triphyophyllum peltatum* and *Ancistrocladus abbreviatus*, which both have *trnK* sequences (Meimberg et al., 2001). Specimen information and sequences used are summarized in Supplemental Table 1.1. Sequences for each of the genes were aligned separately using MUSCLE (Edgar, 2004) in the Geneious 7.0 platform. To remove ambiguously aligned regions, Gblocks 091 with relaxed parameters (Castresana, 2000; Talavera & Castresana, 2007) was applied to the *trnK* alignment. Best-fitting models for DNA substitution for each marker were selected according to the corrected Akaike information criterion (AICc) in jModeltest ver. 0.118 (Posada, 2008). These resulted in GTR+G for *trnK* and GTR+I for *PTR1*.

An ultrametric tree was inferred using Bayesian MCMC in the program BEAST v1.8.3 (Drummond et al., 2012). A Yule tree prior model and a strict clock were applied (as no definitive fossils of Nepenthaceae are known, no fossil calibration points were used), and two independent chains were run

for 10 million generations. Convergence was inspected in Tracer v.1.5 and a 10% burn-in was applied to each chain to obtain the final tree.

b. Character matrix

A character matrix was gathered from species descriptions in McPherson, Robinson, & Fleischmann (2009), which includes accounts of 125 species and incompletely diagnosed taxa. Using a single source has the advantage of greater consistency in the scoring of characters, in particular those related to color. Scoring of such traits may be subjective and vary between accounts; furthermore, original species descriptions do not always describe color variation in depth or provide color photographs. Another problem with this source is that the information on color variation within species is based on qualitative descriptions as opposed to quantitative descriptions of the proportions of color variants within pitcher morphs. Some species have variable coloration, and without data on the proportions of color variants, both morphs may be described as "variable", which may mask finer details (i.e. whether the two morphs have different probabilities of being red); however, this still allows us to examine broad patterns. We note that this field guide is not a peer-reviewed source, so wherever possible we have also cross-checked this information against the Jebb and Cheek (1997) *Nepenthes* monograph. We have also included some additional data (peristome width/slope and viscosity) from Bauer et al. (2012) for further comparison. Finally, our data are constrained to colors that are found in the visible spectrum. Certain pitcher plant species are known to be strongly reflective and/ or absorbing in the UV as well as long wavelength (e.g. Joel et al., 1985; Moran et al., 1999), and the UV in particular may be important in signals involving insects. However, since only a few pitcher plant species have been assessed for their spectral qualities outside the visible, we were unable to include a wider range of wavelengths in our analysis.

In our scoring for color polymorphism, we scored species either as "redder lower", "redder upper", or "similar coloration". All of these scores deal specifically with levels of red pigmentation. If a species produces mostly solid-colored pitchers and the lower pitchers are generally red (to the human eye)

and the upper pitchers are generally green (to the human eye), then it was scored as “redder lower”—the reverse was scored as “redder upper”. For species with patterning (blotches, spots, stripes, or mottles of red/dark pigmentation on the outer pitcher wall), the pitcher morph with denser pigment patterning was considered to be “redder”. Darker colored pigmentation was assumed to be the result of increased expression of anthocyanin, so a morph with solid or patterned “black”, “purple”, or “brown” color was considered to be redder than a morph with solid or patterned “red”, “orange”, or “pink” color. In cases of variation within a pitcher morph, the most commonly observed coloration was used for the comparison. Species with pitcher morphs that are deemed to be generally equivalent for all of the above-described properties were scored as “similar coloration”. Species where both pitcher morphs exhibit color variation and both pitcher morphs are described as “equally variable” were also scored as ‘1’ for “similar coloration”.

“Lid contrast” and “peristome contrast” refer to whether the lid/peristome differs in coloration from the pitcher body; e.g., a green pitcher body with a red lid/peristome or a red pitcher body with a green lid/peristome. The underside of the pitcher lid is generally lighter in color than the outer wall of the pitcher body, so this did not factor into the scoring of this set of characters. However in terms of increased pigment relative to the body, a pitcher was scored as having “lid contrast” based on either the entire lid, the upper surface of the lid, or the under surface of the lid—wherever the strongly contrasting red or green coloration is expressed. The contrast scores for lids/peristomes were based primarily on solid colors and any spots or stripes were not considered. Peristome striping was scored as a binary trait, where the trait was scored as present whenever any expression of the trait is reported in a given species. All patterning traits were scored independently for each pitcher morph.

We scored presence/absence of pitcher dimorphism and a related yet distinct trait we refer to as “reduced lower pitcher production” or “reduced lowers”. These species still produce both morphs, except that they only produce lowers in young plants and then switch to solely producing uppers, as opposed to other species that continue to produce both when mature. Both pitcher dimorphism and reduced lowers were scored as binary.

We scored each of three growth habits (terrestrial, epiphytic, lithophytic) and each of nine habitats (lowland dipterocarp forest, peat swamp, heath forest, montane forest, scrub, cliff, mangrove, seasonal grassland, and degraded—which includes all anthropogenically-modified environments) as binary traits, denoting the presence or absence of a species in that habit/habitat.

Species designations follow the taxonomy of McPherson, Robinson, & Fleischmann (2009). Given this, the Meimberg et al. (2001) accession named as *N. anamensis* was scored according to the McPherson, Robinson, & Fleischmann (2009) account of *N. smilesii*. *Nepenthes xiphioides* and *N. pectinata* were both scored identically to *N. gymnamphora*. As what Meimberg et al. (2001) designates as *N. pilosa* is likely *N. chaniana* (Clarke, Lee, and McPherson, 2006), we have relabeled their "*N. pilosa*" sequence as "*N. chaniana* 3" ("*N. chaniana*" and "*N. chaniana* 2" are from Merckx et al., 2015 data).

c. Phylogenetic tests

All statistical analyses were conducted in R 3.3.2 (R Core Team 2013). We tested for phylogenetic signal in continuous traits with Blomberg's K (Blomberg, Garland, & Ives, 2003) and Pagel's lambda (Pagel, 1999) using the 'phylosig' function in the 'phytools' package (Revell, 2012), and in binary traits with Fritz and Purvis's D (Fritz & Purvis, 2010) using the 'phylo.d' function in the 'caper' package in R (Orme, 2013). To find the number of transitions between states for color polymorphism we used the 'countSimmap' function using the 'phytools' package in R; this method is a form of stochastic character mapping and has the advantage of accounting for uncertainty in the underlying topology (Bollback, 2006). To test for the influence of altitude on pitcher traits, we performed a phylogenetic generalized least squares (pgls; for continuous traits) using the 'pgls' function in the 'caper' package and utilized the 'brunch' function (for discrete traits) in the 'caper' package in R. We used pgls to examine correlations between morphological traits. To test for correlated evolution between color polymorphism and patterned pitchers, between reduced lowers and color traits, and between various traits and habitat/growth habit, we used a binary PGLMM (phylogenetic generalized linear mixed model) using the ape package (Paradis et al. 2016).

Results:

Intraspecific variation in *Nepenthes gracilis*

At Kent Ridge Park and our site in MacRitchie Reservoir, we collected all the fluid and associated organisms from 83 pitchers of *Nepenthes gracilis* (31 individual plants, Supplemental Table 1.2). We counted total of 822 pitchers in Kent Ridge Park during our January 2014 survey of *N. gracilis* in relation to canopy coverage, and a total of 605 pitchers during our January 2016 survey of *N. gracilis* in relation to herbivory.

Our data show no significant differences in counts of prey (ants, mites, and flying prey) or symbionts (culicids and other larvae) explained by pitcher color, morph, connectedness, distance from the ground, or size (Poisson regression, $p>0.05$ in all cases, Supplemental Table 1.3), except that pitcher size is positively correlated with number of ants ($p<0.05$, Supplemental Table 1.3).

From our January 2014 survey, we found that lower pitchers were disproportionately more likely to be red-pigmented than upper pitchers (chi-squared test, $chi^2= 148.3$, $p<0.001$; Supplemental Table 1.4), showing that *N. gracilis* has "redder lower" color polymorphism. We also found a significant positive correlation between the proportion of red pitchers in a site and canopy cover ($r^2=0.79$, $p<0.01$, Figure 1.2). From our January 2016 survey, we found that red pitchers were disproportionately less likely to show signs of herbivore or pathogen attack in the field (logistic regression, $p=0.002$). This result was similar when accounting for pitcher morph by only comparing red and green lowers (logistic regression, $p=0.002$). However there was no difference in the likelihood of attack due to pitcher morph, either in the full dataset (logistic regression, $p=0.72$) or between the green subset of uppers and lowers (logistic regression, $p=0.47$).

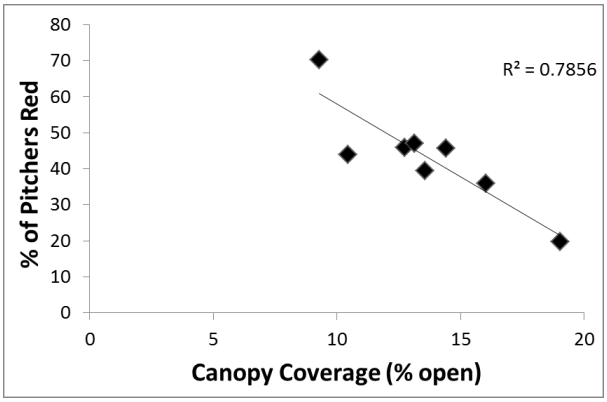

Figure 1.2. A linear regression between canopy coverage and percentage of red pitchers for our January 2014 field observations in Kent Ridge Park, Singapore shows a negative correlation between red pigmentation and light environment. Each point represents a single patch. p=0.003.

Phylogenetic inference

The phylogeny we constructed using the *trnK* and *PTR1* genes (Figures 3-4) is similar to the phylogenies published by Meimberg et al. (2001) and Meimberg and Heubl (2006). The first split within *Nepenthes* separates a clade consisting of *Nepenthes khasiana* and *N. madagascariensis* + *N. masoalensis* from the remaining species, which are then split into a clade consisting of *N. pervillei* + *N. distillatoria* and the rest of *Nepenthes*. These two smaller clades include the "outlying" species from the Western limits of the genus' range (India, Madagascar, the Seychelles, and Sri Lanka), which have appeared in a similar position in all of the phylogenies published thus far (often referred to as "basal" species in previous studies, e.g. Meimberg et al. 2001, 2006; Alamsyah & Ito, 2013). The branch lengths become much shorter and less well-resolved for the numerous species from the Southeast Asian center of distribution. Within this large Southeast Asian clade, a clade consisting mostly of Papuan species is sister to the remaining species. As with previous studies, however, several nodes are poorly resolved, particularly within the aforementioned large Southeast Asian clade containing species from Sundaland, the Philippines, and western Wallacea.

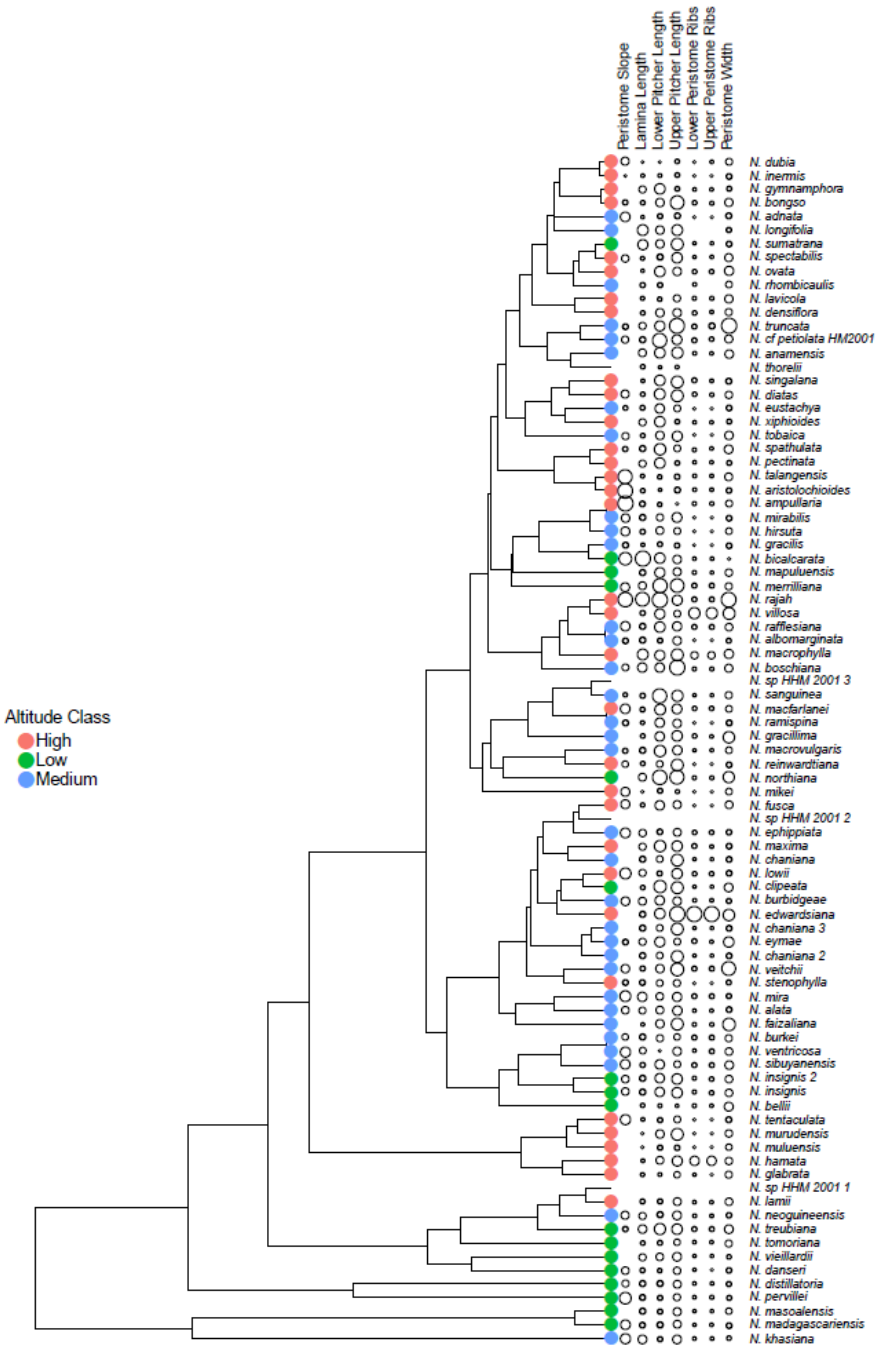

Figure 1.3. Phylogeny displaying topology for the *trnK+PTR1* tree mapped with quantitative morphological traits from McPherson et al. (2009) and Bauer et al. (2012). Each taxon is placed into an altitude class based on its recorded maximum occurring altitude (McPherson et al. 2009): low (0-1000 m asl), medium (1001-2000 m asl), and high (2001-3520 m asl). The size of the circle icon corresponds to the relative magnitude of that trait for the given taxon.

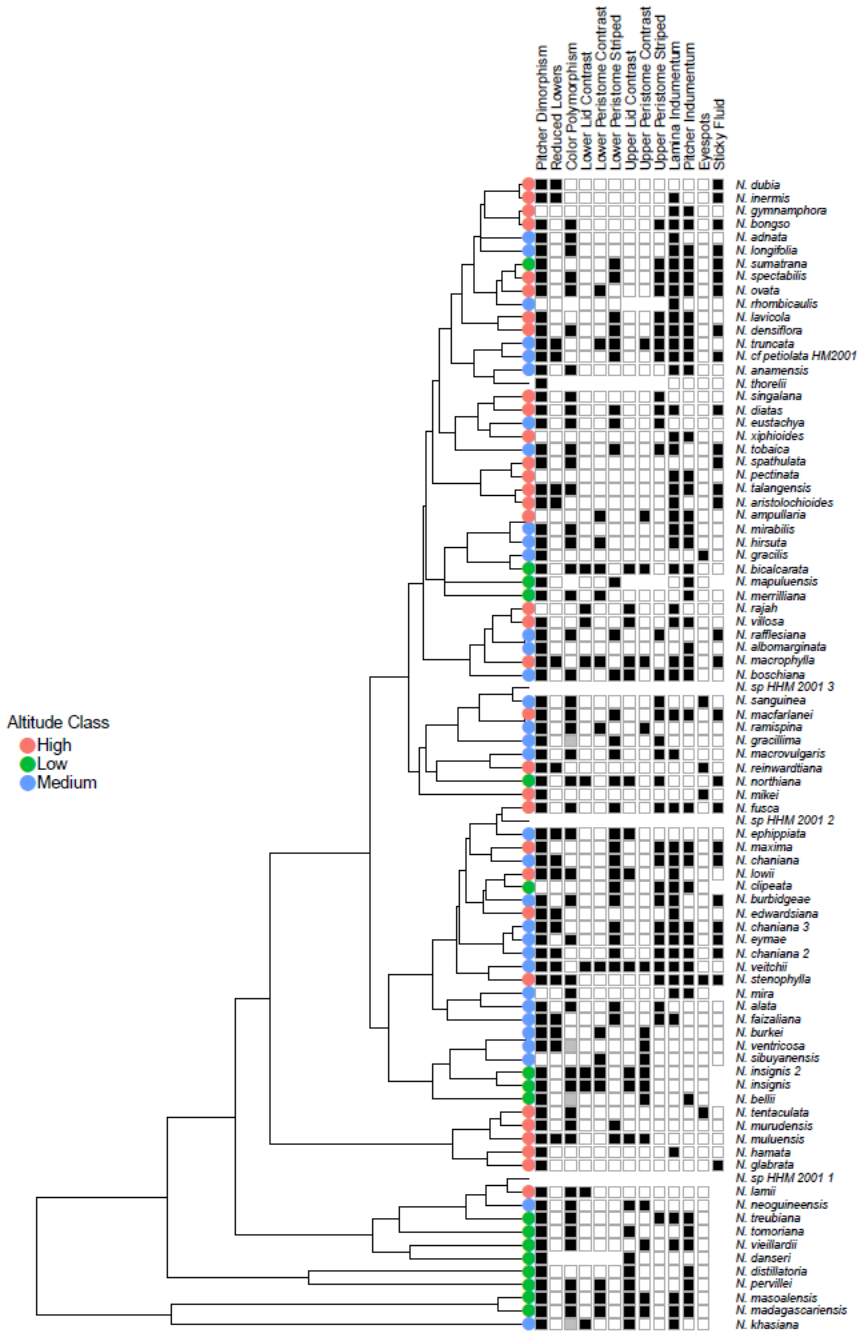

Figure 1.4. Phylogeny displaying topology for the *trnK+PTR1* tree mapped with qualitative morphological traits from McPherson et al. (2009) data. Each taxon is placed into an altitude class based on its recorded maximum occurring altitude (McPherson et al. 2009): low (0-1000 m asl), medium (1001-2000 m asl), and high (2001-3520 m asl). Where information is available, the presence of the trait is represented by a black square and the absence of the trait with an open square. Color polymorphism is the exception as the sole non-binary qualitative trait. Here the three states are similar coloration between morphs (open square), redder lower pitchers (black square), and redder upper pitchers (grey square).

Phylogenetic signal

None of the quantitative traits we examined exhibit significant phylogenetic signal, neither with Pagel's lambda nor with Blomberg's K (Table 1.1). None of the binary traits we examined exhibit significant phylogenetic signal (Table 1.2), except for lower peristome stripes (probability random distribution= 0.003). The lack of significant signal in the majority of these traits may suggest evolutionary lability in *Nepenthes* pitcher evolution or may equally plausibly be attributed to the lack of topological resolution inferred from the currently available genetic data.

Table 1.1. Phylogenetic signal in continuous (quantitative) traits using Pagel's lambda (Pagel 1999) and Blomberg's K statistic (Blomberg et al. 2013), p-values are in parentheses. Lamina length, upper/lower pitcher length, and upper/lower peristome rib heights are values from McPherson et al. (2009). Peristome width (peristome width values corrected for pitcher length) and peristome slope (the length of the inward sloping portion of the peristome) are values taken from Bauer et al. (2012) for comparison. Values significant at the Bonferroni-corrected alpha value of 0.00625 are indicated by an asterisk. Significant values indicate a trait with phylogenetic signal.

Trait	lambda	K
Lamina Length	0 (p = 1)	0.11 (p = 0.4)
Lower Pitcher Length	0.22 (p = 0.31)	0.16 (p = 0.02)
Upper Pitcher Length	0 (p = 1)	0.09 (p = 0.48)
Lower Peristome Rib Height	0 (p = 1)	0.13 (p = 0.38)
Upper Peristome Rib Height	0 (p = 1)	0.13 (p = 0.33)
Peristome Width	0 (p = 1)	0.18 (p = 0.02)
Peristome Slope	0 (p = 1)	0.06 (p = 0.81)

Table 1.2. Phylogenetic signal in binary traits using Fritz and Purvis's D statistic. Given is the estimated D statistic for each trait (Fritz and Purvis 2010) as well as the probability that the trait is randomly distributed in the phylogeny (for a true random distribution D not significantly different from 1), and the probability that the trait is distributed according to a Brownian pattern (D not significantly different from 0). The extreme values for the D statistic are -2.4 for clumped and 1.9 for overdispersed. The scoring of these binary traits derived from McPherson et al. (2009) is described in the methods. "Similar Coloration", "Redder Lowers", and "Redder Uppers" are all elements of color polymorphism converted to binary. Values significant at the Bonferroni-corrected alpha value of 0.0035 are indicated by an asterisk.

Trait	Estimated D	prob_random	prob_brownian
Pitcher Dimorphism	1.13	0.567	0.056
Reduced Lower Pitchers	0.22	0.007	0.333
Similar Coloration	1.14	0.674	0.003*
Redder Lowers	0.93	0.401	0.008
Redder Uppers	0.53	0.251	0.383
Lower Lid Contrast	0.57	0.144	0.165
Lower Peristome Contrast	0.68	0.137	0.046
Lower Peristome Stripes	0.13	0.003*	0.393
Upper Lid Contrast	0.20	0.005	0.32
Upper Peristome Contrast	0.46	0.041	0.126
Upper Peristome Stripes	0.53	0.042	0.077
Lamina Indumentum	0.78	0.199	0.009
Pitcher Indumentum	0.85	0.277	0.009
Eyespots	0.74	0.243	0.106

State switches in color polymorphism

Our analysis of state switches in the color polymorphism trait yielded "redder lowers" as the state with the longest evolutionary residence time, followed by "similar coloration", and the shortest time for "redder uppers" (proportion of time spent in state 0.52, 0.31, and 0.17. respectively). Switches happening between "redder lowers" and "similar coloration" are more numerous than any of the switches involving "redder uppers". Switches away from "redder uppers" are more numerous than switches to "redder uppers". Switches between "redder lowers" and "redder uppers" are more numerous than those between "similar coloration" and "redder uppers". Overall, together with the likelihood that "redder lowers" is

ancestral, these trends imply that “redder lowers” is the default state and “redder uppers” is evolutionarily disfavored relative to the other two states. (Figure 1.5)

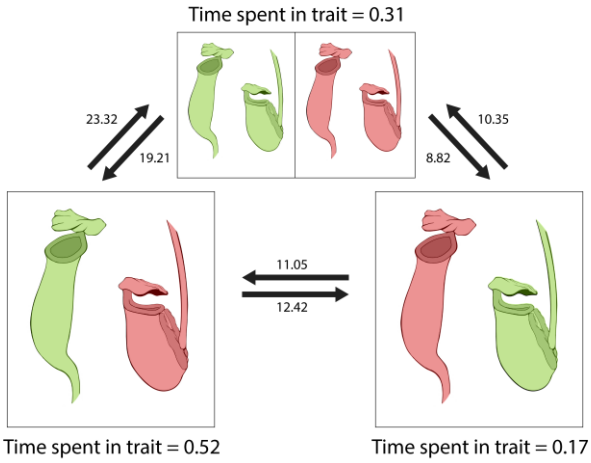

Figure 1.5. Evolutionary pathways of color polymorphism. Illustrated are the three states of color polymorphism we scored (from top, counterclockwise): similar coloration between pitcher morphs, lower pitchers more red-pigmented, and upper pitchers more red-pigmented. Arrows show direction of state change. Numbers above arrows represent the frequency of that transition in our character-mapped phylogeny. Note that the majority of transitions initiate from the “Redder Lowers” state. Illustration credit: Abraham Cone.

Correlations with altitude and between morphological traits

None of the quantitative or binary traits we examined exhibit a significant relationship with altitude (pgls, Table 1.3). Lamina length increases with lower pitcher length (pgls, $p<0.001$, Table 1.3) and lower peristome rib height increases with upper peristome rib height (pgls, $p<0.0001$, Table 1.3).

Table 1.3: Correlations among quantitative traits using phylogenetic generalized least squares. Pearson's correlation coefficients are in bold, p-values are in parentheses. An asterisk indicates statistical significance at a Bonferroni-corrected alpha level of 0.0017.

	Min Altitude	Max Altitude	Altitudinal Range	Lamina Length	Lower Pitcher Length	Upper Pitcher Length	Lower Peristome Rib Height
Min Altitude	**1**	-	-	-	-	-	-
Max Altitude	**0.53** (2.04E-7*)	**1**	-	-	-	-	-
Altitudinal Range	**-0.47** (8.40E-6*)	**0.41** (1.00E-4*)	**1**	-	-	-	-
Lamina Length	**-0.25** (0.0224)	**-0.25** (0.0245)	**0.04** (0.7133)	**1**	-	-	-
Lower Pitcher Length	**-0.12** (0.2860)	**-0.19** (0.0884)	**-0.08** (0.4938)	**0.37** (5.27E-4*)	**1**	-	-
Upper Pitcher Length	**-0.03** (0.7656)	**-0.28** (0.0109)	**-0.30** (0.0062)	**0.19** (0.0789)	**0.27** (0.0152)	**1**	-
Lower Peristome Rib Height	**0.29** (0.0081)	**0.33** (0.0028)	**0.04** (0.7544)	**-0.13** (0.2325)	**0.26** (0.0207)	**0.03** (0.8004)	**1**
Upper Peristome Rib Height	**0.26** (0.0204)	**0.30** (0.0070)	**0.07** (0.5102)	**-0.11** (0.3344)	**0.12** (0.2532)	**0.08** (0.4632)	**0.95** (0.0000*)

Discussion:

Species of *Nepenthes* represent a plant radiation with high morphological and ecological diversity in their pitchers. We took two approaches to evaluating the ecological drivers of dimorphism- and color-related morphological traits in *Nepenthes*: a field study of the polymorphic species *N. gracilis* and comparative phylogenetic analysis across the genus. Our field studies of *N gracilis* showed the potential importance of light environment and herbivore pressure to color polymorphism, with lower pitchers disproportionately more likely to be red-pigmented than upper pitchers (Supplemental Table 1.4), and the proportion of red pitchers in a site significantly correlated with canopy cover (Figure 1.2). Our comparative analysis further showed that redder lower pitchers may be evolutionarily favored across the genus. We found little evidence supporting that altitude, growth habit, or habitat are key drivers of the traits we examined (Supplemental Tables 7). We further discuss our results below.

Pitcher dimorphism

We found no evidence that dimorphism is correlated to altitude (Supplemental Tables 7), contrary to our expectation that dimorphism would be lost with the decreasing availability of ants at higher altitudes. We also examined situations in which the production of lower pitchers is reduced. We expected this trait to increase with altitude, but we again found no significant relationship (Supplemental Tables 7). However, we found that the evolution of reduced lower pitchers is positively correlated with the evolution of epiphytism (Supplemental Tables 7), possibly reflecting that upper pitchers are better suited to an arboreal environment than lowers. Interestingly, only one of the species in our dataset is a strict epiphyte—the rest grow terrestrially as well—so reduced lowers may be a means of entering an epiphytic niche rather than a consequence of becoming epiphytic.

We found no difference in rate of prey capture between upper and lower pitchers of *N. gracilis* in our observations of this species, and no evidence for partitioning of crawling and flying insects between morphs (Supplemental Table 1.3). This is consistent with the results of Gaume et al. (2016), a study examining prey capture in seven sympatric Bornean taxa with morphological differences, where *N. gracilis* showed far less difference in prey composition of upper and lower pitchers than the other species examined. This shows that while dimorphism may have a pronounced ecological role in some species, this pattern is not universal throughout the genus.

Color polymorphism

We tested three hypotheses for the function of red pitchers in *N. gracilis*: (1) red coloration acts as a visual signal to prey and symbionts, (2) red pigmentation protects pitchers from excess solar radiation, and (3) red pigmentation is related to defense.

We found no support for our first two hypotheses: red and green pitchers did not differ in their prey capture rates in *N. gracilis* (Supplemental Table 1.3), and red pitchers were significantly less common in areas with greater sun exposure (Figure 1.2). The lack of difference in prey capture between red and green pitchers makes sense from the perspective of insect vision: ants, the main prey items, lack

an ability to perceive red (Briscoe and Chittka, 2001). Our finding on sun exposure is the opposite from what we would predict if the anthocyanins in red pitchers function primarily to protect against UV. This does not necessarily rule out all possible physiological functions (e.g., protection against sun flecks; Gould et al., 1995), but pitchers are also less photosynthetically active than the laminae (Pavlovič, Masarovičová, & Hudák, 2007; Pavlovič et al., 2009; Adamec, 2010a,b), further diminishing the likelihood of a photosynthetic function. However, our observations were consistent with our third hypothesis, that red pigmentation is related in some way to defense.

Defense is likely prioritized more as plants become more nutrient-stressed or energy-limited (Gianoli, 2015). In Moran & Moran (1998), it was shown that red coloration in *N. rafflesiana* can be induced by nutrient stress. In our study, red pitchers were more likely to occur in the shade, and showed significantly fewer signs of herbivory than green pitchers (logistic regression, $p<0.01$). Thus pigmentation could play a defensive role in *N. gracilis,* possibly an indication that red pitchers are more chemically defended (Menzies et al., 2016), or less nutritionally valuable, and/or that the coloration defends against herbivores via crypsis (Fadzly et al., 2009; Klooster, Clark, & Culley, 2009; Fadzly & Burns, 2010; Niu et al., 2014; Fadzly et al., 2016), which is plausible considering that lowers often grow in reddish-brown leaf litter while uppers tend to grow embedded in green foliage (K. Gilbert, pers. obs.). The greater likelihood for lowers to be red compared to uppers also supports the defense hypothesis, as a climbing habit reduces herbivore pressure (Gianoli, 2015). The results of our phylogenetic analyses suggest that the selection for redder lowers we see in *N. gracilis* may be generalizable to the genus as a whole. Not only is the number of clades with redder lowers much greater than those with redder uppers, but the "redder uppers" state has the lowest residence time in our state switch analysis (Figure 1.5), suggesting that pigmented upper pitchers are generally not evolutionarily favored.

Another line of evidence for the putative role of herbivory in shaping color polymorphism is that the evolution of reduced lowers is associated with the loss of color polymorphism and a shift away from "redder lowers" (Supplemental Table 6). A lessened investment in producing lowers could conceivably lead to a lessened investment in pigmenting them. Alternatively, as species with reduced lowers have a

tendency towards epiphytism, both morphs may experience more similar environmental conditions than usual, leading to similar coloration. It is notable that epiphytes completely avoid the relatively higher herbivore pressure of terrestrial areas (Gianoli, 2015), so the defensive role of pigmentation would be relaxed. When both pitcher morphs are red in epiphytes, it could be due to a stronger signaling role (see *N. macrophylla* in Moran et al., 2012).

The evolution of contrasting color patterns within pitchers

In addition to examining color polymorphism, we explored the evolution of interspecific diversity in contrasting color patterns, which include a striped peristome, contrast between the color of peristome and that of the pitcher body, and contrast between the color of the pitcher lid and pitcher body. This kind of patterning seems likely to play a role in signaling to visually-oriented animals that can distinguish between red and green, and a contrasting pattern has already been shown to be important in signaling to vertebrate visitors in coprophagous species (*N. lowii*, *N. rajah*, and *N. macrophylla*: Moran et al., 2012). More generally, pitcher contrast may be important to anthophilous insects as well (*N. rafflesiana*; Moran, Booth, & Charles, 1999). We found more origins of peristome and lid contrast in upper pitchers than in lower pitchers (Figure 1.4), possibly because upper pitchers tend to be in higher light environments that could make such patterns more effective in signaling.

Correlations between altitude, habit, and habitat and morphological evolution

The potential ecological drivers we explored in our comparative analysis include altitude, growth habit, and habitat. None of the quantitative traits we examined significantly correlate to altitude in our phylogeny (Table 1.3). However, the trend of increasing lower and upper peristome rib heights in relation to maximum altitude is compelling given the wetness-dependent function of the peristome (Bohn & Federle, 2004; Bauer & Federle, 2009), which is favored in climates with greater precipitation (Moran et al., 2013; Schwallier et al., 2016). As precipitation increases with altitude, the trend of peristome rib height increasing with altitude also makes sense. Altitude, growth habit, and habitat are all proxies for

multiple abiotic factors, so our inability to find significant results for most our morphological traits using these environmental traits could mean that abiotic factors are generally less important to pitcher evolution than biotic factors, or that our metrics do not accurately capture enough relevant environmental variables.

Conclusions:

Although much remains to be learned about functional diversity of *Nepenthes* pitchers in relation to diet and prey capture, even less emphasis has been placed on the adaptations used by the plant to deter its own enemies. Our analysis of *Nepenthes* pitcher coloration indicates that herbivory may play a role in maintaining pitcher color polymorphism, and should be explored further experimentally. Herbivory is an understudied subject in *Nepenthes*, with few publications directly addressing herbivores that attack *Nepenthes* (Clarke, 1997b; Merbach et al., 2007; Bauer, Rembold, & Grafe 2016). More generally, the role of anthocyanins as an herbivore defense remains unsettled (Menzies et al., 2016), so an improved understanding of the influence of herbivores on pitchers' complex pigmentation strategies may yield novel insights into the broader use of red coloration in leaves—and such questions require polymorphic species as models (Gould et al., 2000; Menzies et al., 2016). The unique nutritional challenges of carnivorous plants in general (Givnish et al., 1984) adds weight to the importance of herbivore defense in their ecology; and the biphasic life history of climbing *Nepenthes* emphasizes how environmental context interacts with the potential defensive role of anthocyanins, as evidenced by the prevalence of redder lowers. It is our hope that this study will serve to both review the current state of knowledge of *Nepenthes* diversity and stimulate future phylogenetic explorations of this unique plant group.

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Chapter 1 References:

Adamec L. **2010a**. Ecophysiological look at organ respiration in carnivorous plants: a review. *Cell Respiration and Cell Survival: Processes, Types and Effects. New York: Nova Science Publishers, Inc*: 225–235.

Adamec L. **2010b**. Dark respiration of leaves and traps of terrestrial carnivorous plants: are there greater energetic costs in traps? *Central European Journal of Biology* **5**: 121–124.

Adlassnig W, Peroutka M, Lendl T. **2011**. Traps of carnivorous pitcher plants as a habitat: composition of the fluid, biodiversity and mutualistic activities. *Annals of Botany* **107**: 181–94.

Alamsyah F, Ito M. **2013**. Phylogenetic Analysis of Nepenthaceae, Based on Internal Transcribed Spacer Nuclear Ribosomal DNA Sequences. *APG: Acta Phytotaxonomica et Geobotanica* **64**: 113–126.

Alcantara S, Lohmann LG. **2011**. Contrasting phylogenetic signals and evolutionary rates in floral traits of Neotropical lianas. *Biological Journal of the Linnean Society* **102**: 378–390.

Anderson B, Ros P, Wiese T, Ellis A. **2014**. Intraspecific divergence and convergence of floral tube length in specialized pollination interactions. *Proceedings of the Royal Society of London B: Biological Sciences* **281**: 20141420.

Archetti M, Brown SP. **2004**. The coevolution theory of autumn colours. *Proceedings. Biological sciences / The Royal Society* **271**: 1219–23.

Archetti M, Döring TF, Hagen SB, Hughes NM, Leather SR, Lee DW, Lev-Yadun S, Manetas Y, Ougham HJ, Schaberg PG, *et al.* 2009. Unravelling the evolution of autumn colours: an interdisciplinary approach. *Trends in ecology & evolution* **24**: 166–73.

Bauer U, Clemente CJ, Renner T, Federle W. **2012**. Form follows function: Morphological diversification and alternative trapping strategies in carnivorous *Nepenthes* pitcher plants. *Journal of evolutionary biology* **25**: 90–102.

Bauer U, Federle W. **2009**. The insect-trapping rim of *Nepenthes* pitchers: Surface structure and function. *Plant Signaling & Behavior* **4**: 1019–1023.

Bauer U, Rembold K, Grafe TU. **2016**. Carnivorous *Nepenthes* pitcher plants are a rich food source for a diverse vertebrate community. *Journal of Natural History* **50**: 483–495.

Bates D, Mächler M, Bolker B, Walker S. **2014**. Fitting linear mixed-effects models using lme4. *arXiv preprint arXiv:1406.5823*.

Bazile V, Moran JA, Le Moguédec G, Marshall DJ, Gaume L. **2012**. A carnivorous plant fed by its ant symbiont: a unique multi-faceted nutritional mutualism. *PloS one* **7**: e36179.

Beaver R. **1979**. Biological studies of the fauna of pitcher plants [*Nepenthes*] in west Malaysia. *Annales de la Société Entomologique de France*.3–17.

Bennett KF, Ellison AM. **2009**. Nectar, not colour, may lure insects to their death. *Biology Letters*: rsbl20090161.

Benzing D, Renfrow A. **1974**. The mineral nutrition of Bromeliaceae. *Botanical Gazette*: 281–288.

Blomberg SP, Garland T, Ives AR. **2003**. Testing for phylogenetic signal in comparative data: Behavioral traits are more labile. *Evolution; International Journal of Organic Evolution* **57**: 717–45.

Boberg E, Alexandersson R, Jonsson M, Maad J, Ågren J, Nilsson LA. **2014**. Pollinator shifts and the evolution of spur length in the moth-pollinated orchid *Platanthera bifolia*. *Annals of Botany* **113**: 267–275.

Bohn HF, Federle W. **2004**. Insect aquaplaning: *Nepenthes* pitcher plants capture prey with the peristome, a fully wettable water-lubricated anisotropic surface. *Proceedings of the National Academy of Sciences of the United States of America* **101**: 14138–14143.

Bollback JP. **2006**. SIMMAP: stochastic character mapping of discrete traits on phylogenies. *BMC Bioinformatics* **7**: 88.

Bonhomme V, Pelloux-Prayer H, Jousselin E, Forterre Y, Labat J-J, Gaume L. **2011**. Slippery or sticky? Functional diversity in the trapping strategy of *Nepenthes* carnivorous plants. *The New Phytologist* **191**: 545–54.

Briscoe AD, Chittka L. **2001**. The evolution of color vision in insects. *Annual Review of Entomology* **46**: 471–510.

Castresana J. **2000**. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution* **17**: 540–52.

Chan KL, Chen LMJ, Choo JPS, Ling KT, Ming LT, Ng PKL, Tan HTW, Kiat TW, Lum WC. **1997**. *Guide to the Carnivorous Plants of Singapore* (Tan, Hugh T.W., Ed.). Singapore Science Centre.

Cheek M, Jebb M. **2014**. Expansion of the *Nepenthes alata* group (Nepenthaceae), Philippines, and descriptions of three new species. *Blumea-Biodiversity, Evolution and Biogeography of Plants* **59**: 144–154.

Chin L, Chung AY, Clarke C. **2014**. Interspecific variation in prey capture behavior by co-occurring *Nepenthes* pitcher plants: evidence for resource partitioning or sampling-scheme artifacts? *Plant Signaling & Behavior* **9**: e27930.

Clarke C. **1997a**. The effects of pitcher dimorphism on the metazoan community of the carnivorous plant *Nepenthes bicalcarata* Hook. f. *The Malayan Nature Journal (Malaysia)*.

Clarke C. **1997b**. *Nepenthes* of Borneo. *Kota Kinabalu, Sabah: Natural History Publications (Borneo) xi, 207p-col. illus.. ISBN* **1248185562**.

Clarke C, Lee CC, and McPherson S. 2006. *Nepenthes chaniana* (Nepenthaceae), a new species from north-western Borneo. *Sabah Parks Nature Journal*, **7:** 53-66.

Clarke CM, Bauer U, Lee CC, Tuen AA, Rembold K, Moran JA. **2009**. Tree shrew lavatories: A novel nitrogen sequestration strategy in a tropical pitcher plant. *Biology Letters* **5**: 632–635.

Clarke C, Moran J. **2011**. Incorporating ecological context: a revised protocol for the preservation of *Nepenthes* pitcher plant specimens (Nepenthaceae). *Blumea-Biodiversity, Evolution and Biogeography of Plants* **56**: 225–228.
Drummond AJ, Suchard MA, Xie D, Rambaut A. **2012**. Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution* **29**: 1969–1973.

Edgar RC. **2004**. MUSCLE: A multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* **5**: 1.

El-Sayed AM, Byers JA, Suckling DM. **2016**. Pollinator-prey conflicts in carnivorous plants: when flower and trap properties mean life or death. *Scientific Reports* **6**: 21065.

Fadzly N, Jack C, Schaefer HM, Burns K. **2009**. Ontogenetic colour changes in an insular tree species: signalling to extinct browsing birds? *New Phytologist* **184**: 495–501.

Fadzly N, Burns K. **2010**. Hiding from the ghost of herbivory past: evidence for crypsis in an insular tree species. *International journal of plant sciences* **171**: 828–833.

Fadzly N, Zuharah WF, Mansor A, Zakaria R. **2016**. Cryptic coloration of *Macaranga bancana* seedlings: A unique strategy for a pioneer species. *Plant signaling & behavior* **11**: e1197466.

Feild TS, Lee DW, Holbrook NM. **2001**. Why leaves turn red in autumn. The role of anthocyanins in senescing leaves of red-osier dogwood. *Plant Physiology* **127**: 566–74.

Felsenstein J. **1985**. Phylogenies and the comparative method. *American Naturalist*: 1–15.

Foot G, Rice SP, Millett J. **2014**. Red trap colour of the carnivorous plant *Drosera rotundifolia* does not serve a prey attraction or camouflage function. *Biology Letters* **10**: 20131024.

Fritz SA, Purvis A. **2010**. Selectivity in mammalian extinction risk and threat types: a new measure of phylogenetic signal strength in binary traits. *Conservation Biology* **24**: 1042–1051.

Gaume L, Bazile V, Huguin M, Bonhomme V. **2016**. Different pitcher shapes and trapping syndromes explain resource partitioning in *Nepenthes* species. *Ecology and Evolution*.

Gianoli E. **2015**. The behavioural ecology of climbing plants. *AoB Plants* **7**: plv013.

Givnish TJ, Burkhardt EL, Happel RE, Weintraub JD. **1984**. Carnivory in the bromeliad *Brocchinia reducta*, with a cost/benefit model for the general restriction of carnivorous plants to sunny, moist, nutrient-poor habitats. *American Naturalist*: 479–497.

Givnish TJ, Barfuss MH, Van Ee B, Riina R, Schulte K, Horres R, Gonsiska PA, Jabaily RS, Crayn DM, Smith JAC, others. **2011**. Phylogeny, adaptive radiation, and historical biogeography in Bromeliaceae: insights from an eight-locus plastid phylogeny. *American Journal of Botany* **98**: 872–895.

Gould KS, Kuhn DN, Lee DW, Oberbauer SF, others. **1995**. Why leaves are sometimes red. *Nature* **378**: 241–242.

Gould KS, Markham KR, Smith RH, Goris JJ. **2000**. Functional role of anthocyanins in the leaves of *Quintinia serrata* A. Cunn. *Journal of Experimental Botany* **51**: 1107–1115.

Gould KS. **2004**. Nature's Swiss army knife: the diverse protective roles of anthocyanins in leaves. *BioMed Research International* **2004**: 314–320.

Hoch WA, Zeldin EL, McCown BH. **2001**. Physiological significance of anthocyanins during autumnal leaf senescence. *Tree Physiology* **21**: 1–8.

Jebb M, Cheek M. **1997**. A skeletal revision of *Nepenthes* (Nepenthaceae). *Blumea-Biodiversity, Evolution and Biogeography of Plants* **42**: 1–106.

Joel, DM, Juniper, BE, Dafni, A. 1985. Ultraviolet patterns in the traps of carnivorous plants. *New Phytologist* 101: 585-593.

Juniper BE, Robins RJ, Joel DM. **1989**. *The Carnivorous Plants*. London, etc.: Academic Press.

Jürgens A, Witt T, Sciligo A, El-Sayed AM. **2015**. The effect of trap colour and trap-flower distance on prey and pollinator capture in carnivorous *Drosera* species. *Functional Ecology* **29**: 1026–1037.

Hölldobler B, Wilson EO. **1990**. *The Ants*. Harvard University Press.

Karageorgou P, Manetas Y. **2006**. The importance of being red when young: anthocyanins and the protection of young leaves of *Quercus coccifera* from insect herbivory and excess light. *Tree Physiology* **26**: 613–621.

Kay KM, Sargent RD. **2009**. The role of animal pollination in plant speciation: Integrating ecology, geography, and genetics. *Annual Review of Ecology, Evolution, and Systematics* **40**: 637–656.

Klooster MR, Clark DL, Culley TM. **2009**. Cryptic bracts facilitate herbivore avoidance in the mycoheterotrophic plant *Monotropsis odorata* (Ericaceae). *American Journal of Botany* **96**: 2197–2205.

Kováčik J, Klejdus B, Repčáková K. **2012**. Phenolic metabolites in carnivorous plants: Inter-specific comparison and physiological studies. *Plant Physiology and Biochemistry : PPB / Société Française de Physiologie Végétale* **52**: 21–7.

Lagomarsino LP, Condamine FL, Antonelli A, Mulch A, Davis CC. **2016**. The abiotic and biotic drivers of rapid diversification in Andean bellflowers (Campanulaceae). *New Phytologist* **210**: 1430–1442.

Lev-Yadun S, Gould KS. **2008**. Role of anthocyanins in plant defence. *Anthocyanins*. Springer, 22–28.

McPherson S, Robinson A, Fleischmann A. **2009**. *Pitcher plants of the Old World*. Redfern Natural History Productions Dorset.

Meimberg H, Heubl G. **2006**. Introduction of a nuclear marker for phylogenetic analysis of Nepenthaceae. *Plant Biology (Stuttgart, Germany)* **8**: 831–40.

Meimberg H, Thalhammer S, Brachmann A, Heubl G. **2006**. Comparative analysis of a translocated copy of the *trnK* intron in carnivorous family Nepenthaceae. *Molecular Phylogenetics and Evolution* **39**: 478–490.

Meimberg H, Wistuba A, Dittrich P, Heubl G. **2001**. Molecular phylogeny of Nepenthaceae based on cladistic analysis of plastid *trnK* intron sequence data. *Plant Biology* **3**: 164–175.

Menzies IJ, Youard LW, Lord JM, Carpenter KL, Klink JW, Perry NB, Schaefer HM, Gould KS. **2016**. Leaf colour polymorphisms: a balance between plant defence and photosynthesis. *Journal of Ecology* **104**: 104–113.

Merbach MA, Zizka G, Fiala B, Merbach D, Booth WE, Maschwitz U. **2007**. Why a carnivorous plant cooperates with an ant—Selective defense against pitcher-destroying weevils in the myrmecophytic pitcher plant *Nepenthes bicalcarata* Hook. f. *Ecotropica* **13**: 45–56.

Merckx VS, Hendriks KP, Beentjes KK, Mennes CB, Becking LE, Peijnenburg KT, Afendy A, Arumugam N, de Boer H, Biun A, others. **2015**. Evolution of endemism on a young tropical mountain. *Nature* **524**: 347–350.

Moran JA. **1996**. Pitcher dimorphism, prey composition and the mechanisms of prey attraction in the pitcher plant *Nepenthes rafflesiana* in Borneo. *Journal of Ecology*: 515–525.

Moran JA, Moran AJ. **1998**. Foliar reflectance and vector analysis reveal nutrient stress in prey-deprived pitcher plants (*Nepenthes rafflesiana*). *International Journal of Plant Sciences* **159**: 996–1001.

Moran JA, Booth WE, Charles JK. **1999**. Aspects of pitcher morphology and spectral characteristics of six Bornean *Nepenthes* pitcher plant species: implications for prey capture. *Annals of Botany* **83**: 521–528.

Moran JA, Clarke C, Greenwood M, Chin L. **2012**. Tuning of color contrast signals to visual sensitivity maxima of tree shrews by three Bornean highland *Nepenthes* species. *Plant Signaling & Behavior* **7**: 1267–1270.

Moran JA, Gray LK, Clarke C, Chin L. **2013**. Capture mechanism in Palaeotropical pitcher plants (Nepenthaceae) is constrained by climate. *Annals of Botany*: mct195.

Muchhala N, Johnsen S, Smith SD. **2014**. Competition for hummingbird pollination shapes flower color variation in Andean Solanaceae. *Evolution* **68**: 2275–2286.

Muchhala N, Potts MD. **2007**. Character displacement among bat-pollinated flowers of the genus *Burmeistera*: Analysis of mechanism, process and pattern. *Proceedings of the Biological Sciences of the Royal Society* **274**: 2731–7.

Neill SO, Gould KS. **2003**. Anthocyanins in leaves: light attenuators or antioxidants? *Functional Plant Biology* **30**: 865–873.

Niu Y, Chen G, Peng DL, Song B, Yang Y, Li ZM, Sun H. **2014**. Grey leaves in an alpine plant: a cryptic colouration to avoid attack? *New Phytologist* **203**: 953–963.

Van der Niet T, Johnson SD. **2012**. Phylogenetic evidence for pollinator-driven diversification of angiosperms. *Trends in Ecology & Evolution* **27**: 353–361.

Orme D. **2013**. The caper package: comparative analysis of phylogenetics and evolution in R. *R package version* **5**.

Page JE, Towers GHN. **2002**. Anthocyanins protect light-sensitive thiarubrine phototoxins. *Planta* **215**: 478–84.

Pagel M. **1999**. Inferring the historical patterns of biological evolution. *Nature* **401**: 877–84.

Paradis E, Blomberg S, Bolker B, Claude J, Cuong HS, Desper R, Didier G, Durand B, Dutheil J, Gascuel O. **2016**. Package "ape." *Analyses of phylogenetics and evolution, version*: 2–4.

Pavlovič A, Singerová L, Demko V, Hudák J. **2009**. Feeding enhances photosynthetic efficiency in the carnivorous pitcher plant *Nepenthes talangensis*. *Annals of Botany*: mcp121.

Pavlovič A, Masarovičová E, Hudák J. **2007**. Carnivorous syndrome in Asian pitcher plants of the genus *Nepenthes*. *Annals of Botany* **100**: 527–536.

Peng HS, Clarke C. **2015**. Prey capture patterns in *Nepenthes* species and natural hybrids - Are the pitchers of hybrids as effective at trapping prey as those of their parents? *Carnivorous Plant Newsletter* **44**: 62–79.

Posada D. **2008**. jModelTest: phylogenetic model averaging. *Molecular biology and evolution* **25**: 1253–1256.

R Core Team. **2013**. R: A Language and Environment for Statistical Computing. **55**: 275–286.

Rasband W. **2012**. ImageJ: Image processing and analysis in Java. *Astrophysics Source Code Library*.

Rembold K, Fischer E, Wetzel MA, Barthlott W. **2010**. Prey composition of the pitcher plant *Nepenthes madagascariensis*. *Journal of Tropical Ecology* **26**: 365–372.

Renner T, Specht CD. **2011**. A sticky situation: assessing adaptations for plant carnivory in the Caryophyllales by means of stochastic character mapping. *International Journal of Plant Sciences* **172**: 889–901.

Revell LJ. **2012**. phytools: an R package for phylogenetic comparative biology (and other things). *Methods in Ecology and Evolution* **3**: 217–223.

Schaefer HM, Rentzsch M, Breuer M. **2008**. Anthocyanins reduce fungal growth in fruits. *Natural Product Communications* **3(8)**: 1267–1272.

Schaefer HM, Rolshausen G. **2006**. Plants on red alert: do insects pay attention? *BioEssays : News and reviews in molecular, cellular and developmental biology* **28**: 65–71.

Schaefer HM, Ruxton GD. **2008**. Fatal attraction: carnivorous plants roll out the red carpet to lure insects. *Biology Letters* **4**: 153–5.

Scharmann M, Thornham DG, Grafe TU, Federle W. **2013**. A novel type of nutritional ant-plant interaction: ant partners of carnivorous pitcher plants prevent nutrient export by dipteran pitcher infauna. *PloS one* **8**: e63556.

Schwallier R, Raes N, Boer HJ, Vos RA, Vugt RR, Gravendeel B. **2016**. Phylogenetic analysis of niche divergence reveals distinct evolutionary histories and climate change implications for tropical

carnivorous pitcher plants. *Diversity and Distributions* **22**: 97–110.

Schöner MG, Schöner CR, Simon R, Grafe TU, Puechmaille SJ, Ji LL, Kerth G. **2015**. Bats are acoustically attracted to mutualistic carnivorous plants. *Current Biology* **25**: 1911–1916.

Schöner MG, Schöner CR, Kerth G, Suhaini SNBP, Grafe TU. **2017**. Handle with care: enlarged pads improve the ability of Hardwicke's woolly bat, *Kerivoula hardwickii* (Chiroptera: Vespertilionidae), to roost in a carnivorous pitcher plant. *Biological Journal of the Linnean Society*: blx098.

Talavera G, Castresana J. **2007**. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Systematic Biology* **56**: 564–577.

Tellez P, Rojas E, Van Bael S. **2016**. Red coloration in young tropical leaves associated with reduced fungal pathogen damage. *Biotropica* **48**: 150–153.

Thornham DG, Smith JM, Ulmar Grafe T, Federle W. **2012**. Setting the trap: cleaning behaviour of *Camponotus schmitzi* ants increases long-term capture efficiency of their pitcher plant host, *Nepenthes bicalcarata*. *Functional Ecology* **26**: 11–19.

Chapter 2: Tropical pitcher plant species in the genus *Nepenthes* as ecological filters

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Abstract

Tropical pitcher plants in the genus *Nepenthes* are carnivorous plants with modified leaves (pitchers) containing rainwater and digestive fluid for trapping and digesting prey; in natural populations, this pitcher fluid hosts a diverse community of aquatic arthropods and microbes. With over 140 described species, the genus *Nepenthes* exhibits vast interspecific diversity in pitcher morphology and physiology. For instance, pitchers can actively control the pH of their fluid, and some species produce viscoelastic fluid. This study investigated the extent to which *Nepenthes* species differentially regulate pitcher traits under common conditions, and the effects of interspecific trait differences on microbial community assembly. Sixteen species of *Nepenthes* representing wide phylogenetic diversity within the genus, were reared in a common garden experiment in the controlled environment of a glasshouse and treated with commonly-sourced pH 6.5 water. After a two-week acclimation period, we sampled fluids from the pitchers and used 16S and 18S metabarcoding to analyze the bacterial and eukaryotic communities. Different species differentially altered fluid pH, viscosity, and color, and these had strong effects on the community structure of their microbiota. Since pitcher physiology influences the abiotic conditions of the pitcher fluid, these trait differences, and pH in particular, can mediate their interactions with symbionts and affect the resultant community composition of the pitcher microbiome, allowing species of *Nepenthes* to act as ecological filters that can cultivate distinct microbial communities despite similar external conditions.

Introduction

Living organisms can act as ecological filters that alter the ability of other species to establish and persist in defined local environments. The term "ecological filter" has predominantly been used in plant community ecology (e.g. George and Bazzaz 1999); however, it can also be applied to any system in which some key ecological factor influences the assembly of the local community, regardless of which taxon comprises that community (e.g. plants: Itô and Hino 2007; insects: Tiitsaar et al. 2013; vertebrates: Muñoz et al. 2013; microbes: Stagaman et al. 2017), and its use is not always strictly limited to biotic filtering agents (e.g. woody debris as an ecological filter: Krueger and Peterson 2009; vertebrate carcasses as an ecological filter: Bump et al. 2009; landscape heterogeneity as an ecological filter: Duflot et al. 2014).

Many studies treat biotic and abiotic filters as conceptually separate, distinguishing between abiotic (often termed "environmental") and biotic filters (Kraft et al. 2014; Ovaskainen et al. 2017). However, such a distinction may not always be a useful simplification. Abiotic factors do not only affect organisms; the reverse can also occur through a process that has been called "ecological niche construction" (Lewontin 1983; Odling-Smee 1988). At the largest scale, anthropogenic climate change and the Great Oxygenation Event are examples of this. The distinction between biotic and abiotic influence also becomes blurred in small, contained environments such as inside termite mounds or phytotelmata (plant-held waters). In such environments, the products of the inhabitants' metabolism, such as exhaled CO_2, can accumulate and directly impact the entire community (Turner 2000); and furthermore, the species identity of those inhabitants matter, as opposed to the effect being a function of the mere presence of respiring organisms (Turner 2002). Biotic-abiotic interactions may have an even larger impact when the contained environment is within a living organism; in this case, the host's "extended phenotype" (Dawkins 1978) can directly influence the entire inhabitant community and define the bulk of the abiotic conditions experienced—especially if the host has evolved to regulate those conditions in specific ways.

Because of this, the ecological filter concept can be of great utility to the study of host-microbiome interactions. A general concept of ecological filters that recognizes the integrated nature of abiotic and biotic factors can be useful in inquiring the nature of microbial community assembly: to what extent can closely related host species produce characteristically distinguishable microbiota given shared external environmental conditions?

A suitable system for this question can be found in the phytotelmata of tropical pitcher plants (*Nepenthes* L.: Nepenthaceae: Caryophyllales). These carnivorous plants have modified leaves known as pitchers that contain a pool of plant secretions and rainwater used for the capture and digestion of arthropod prey (Juniper et al. 1989). Additionally, however, this digestive fluid serves as a habitat for a diverse community of symbiotic dipteran larvae, mites, bacteria, fungi, and protists (Beaver 1979; Kitching 1987; Fashing 2002; Bittleston et al. 2016). The genus, which evolved on islands throughout the Sundaland shelf of Southeast Asia, contains over 140 described species, and pitchers of different species exhibit a high level of morphological diversity, varying widely in shape, size, and coloration (McPherson et al. 2009; Gilbert et al. 2018). Physiological traits related to the abiotic conditions of pitcher fluid also can vary among species (Adlassnig et al. 2011). Interspecific trait differences in pitchers may mediate their interactions with symbionts and affect the resultant community composition of the microbiome. Multiple studies have shown that different plant species (Vorholt 2012; Vacher et al. 2016) or even genotypes within species (Wagner et al. 2016) can have distinguishable microbiota when placed in a common environment. Due to the nature of the digestive fluid, however, pitcher pools could be considered a "harsher" environment than other kinds of leaf surfaces, and thus potentially be able to exert greater selective control over the microbiota contained within.

One possible means of selective control is via the biochemistry of the fluid. Pitchers secrete a variety of digestive enzymes including proteases, chitinases, glucanases, glucosidases, nucleases, esterases, peroxidases, phosphatases, and ureases (Renner and Specht 2013; Ravee et al. 2018). The aspartic proteases nepenthesin I and nepenthesin II are characteristic of *Nepenthes* (Athauda et al. 2004; Buch et al. 2015). Additionally, pitchers secrete compounds with anti-bacterial (Buch et al. 2012) and

anti-fungal properties (Eilenberg et al. 2010) that could directly constrain which taxa can colonize and establish in these environments. A number of other fluid traits may also act as ecological filters. For instance, pitchers alter the viscosity of their fluid: some species produce highly viscoelastic fluid, well adapted to increase prey retention (Gaume and Forterre 2007; Bonhomme et al. 2011; Moran et al. 2013; Bazile et al. 2015). Viscosity could potentially differentially affect bacterial taxa, as has been seen in animal gut environments (Goddard and Spiller 1996; McDonald et al. 2001; Umu et al. 2015) and motor oil (Amund and Adebiyi 1991); the effect of viscosity on flagellar motility (Schneider and Doetsch 1974) may well favor the growth of certain taxa and thus may have broad effects on a community level.

Pitchers can also actively regulate their pH levels, with likely species differences in this trait (Moran et al. 2010). Individual bacteria species are typically constrained to specific pH ranges (Rabotnova 1963) and given the strong effect of pH in structuring microbial communities in a wide variety of other contexts including roots (Dennis et al. 2009; Hartmann et al. 2009; Rascovan et al. 2016), soils (Fierer and Jackson 2006), and guts (Beasley et al. 2015), it is highly likely pH can act as a filter for pitchers. Indeed, prior studies of *Nepenthes* microbiota indicate that pH can be a major factor in shaping communities (Kanokratana et al. 2016; Bittleston et al. 2018) and field studies also point to interspecific differences in fluid pH (Clarke 1997; Bittleston 2018).

The goal of the present study is to determine the extent to which *Nepenthes* species differentially regulate pitcher traits given common rearing conditions, and the effect that such interspecific trait differences have on community assembly of the microbiota. This study takes advantage of the highly controlled environmental conditions within a horticultural glasshouse. We used plants reared in a *Nepenthes* nursery at Singapore's "HortPark". This nursery contained a large collection of *Nepenthes* species with geographic origins across the distribution of the genus; plants were propagated in a common garden setting within a climate-controlled glasshouse. An earlier study (Kanokratana et al. 2016) similarly utilized a common garden setup to explore ecological filtering in 7 local *Nepenthes* species found in Thailand. Our study differed from this one in several respects: our comparison included 16 species collected throughout the range of *Nepenthes*, each represented by fluids from 2-3 pitchers growing on 2-3

different individual plants; our plants were kept fully indoors rather than outdoors in a semi-natural setting where they might be exposed to a large uncharacterized potential arthropod pool and variable external conditions. We filled pitchers with water from a common source and standardized pH in order to maximize the similarity of the starting conditions, thereby increasing confidence that any resulting differences in fluid properties and microbiome communities could be attributed primarily to plant physiological traits. We used 16S and 18S metabarcoding to characterize the bacterial and eukaryotic communities within our experimental plants. Although artificial in nature, this study provides novel data on the *Nepenthes* microbiome that can be compared with results from previous analyses and applied to the task of understanding wild community assembly as well as developing a general understanding of the power of trait-based ecological filtering.

Materials and Methods

Rearing conditions

The present study used plants cultivated for horticultural purposes in a dedicated *Nepenthes* glasshouse at the HortPark nursery in Singapore. The 16 species of *Nepenthes* were originally imported to HortPark in 2014 as micropropagated clones from Borneo Exotics (Pvt) Ltd., a nursery located in Sri Lanka. The plants were grown together in the HortPark glasshouse under common conditions and thus were all roughly the same age at the time that their pitchers were sampled. The plants were potted in *Sphagnum* medium and foliar-fed using Gaviota 63 fertilizer. The glasshouse environment was regulated to mimic conditions representative of the natural habitat of high-altitude *Nepenthes* species: the temperature was kept at 16°C and the humidity at 80% relative humidity, maintained via an automatic misting system. The sealed indoor environment largely precluded entry by arthropods; however, fungus gnats (Sciaridae) could be found living in the *Sphagnum* medium. The water source used for misting and watering the plants was tap water that was filtered and mixed with enough hydrochloric acid to reach a target pH of 6.5. This pH-altered water was added to the pitchers *ad libitum* such that they remained one-third full of

fluid. Water addition was ceased for two weeks prior to sampling in order to allow time for pitchers to adjust fluid properties.

Sampling design

Sampling took place in July 2016. We aimed to sample from species that were represented by at least 2-3 individual plants and where possible, we sampled from 2 or 3 pitchers per individual (see Table 2.1 for full list of sampled plant species with successful extractions). We chose healthy pitchers of comparable size and age (noted by nodal position on the plant); however, the exact ages and time of opening were not known. For individual plants with multiple pitcher samples, we noted the ages of the pitchers relative to each other. We collected the entire fluid contents into sterile Falcon tubes using sterile Pasteur pipettes, wearing gloves as a further safeguard against contamination. Fluid pH was measured by placing a small drop of remnant fluid from the pipette directly onto a Millipore ColorpHast (0-14) indicator strip. Indicator strips provide a resolution of 1 pH unit, which is somewhat course (e.g. a sample with a reading of pH 3 on a strip may actually fall somewhere between 3 and 4 if using a finer resolution), however this scale was fairly suitable for our study as the range of pH values was wide (recall that pH is on a logarithmic scale). To preserve the DNA, we added 1 mL of Cetyl trimethylammonium bromide (CTAB) solution (recipe following Bittleston et al. 2016) for every 1 mL of pitcher fluid. The volume and color (e.g. clear or brightly yellow/green/pink-colored) of the fluid sample was also noted prior to addition of CTAB. Fluid samples that had the property of forming unbroken strands between the pitcher and pipette tip during collection were considered to be viscoelastic (hereafter "viscous" for brevity). After DNA was extracted from the sample, solid particles (prey contents) were filtered out of the fluid using sterile gauze and the contents were examined under a dissecting microscope, identified, and counted.

Table 2.1: Summary of *Nepenthes* species sampled for this study.

Name	Region of Origin	Total no. of plants	Mean no. pitchers sampled per plant ±standard deviation (sd)	Mean± sd pH	Mean ± sd Fluid Volume (mL)	Mean ± sd Pitcher Length (mm)
Nepenthes boschiana	Borneo	2	3 ± 0	3.9± 1.2	5.93± 2.51	136.83± 9.67
Nepenthes copelandii	Philippines	2	2 ± 0	3.8± 1.5	7.6± 1.02	153.41±33.08
Nepenthes dubia	Sumatra	3	1.67 ± 0.58	1.8± 0.8	0.48± 0.39	40.94±3.94
Nepenthes eymae	Sulawesi	2	1.50 ± 0.71	3.7± 1.2	1.9± 0.26	78.93±10.46
Nepenthes fusca	Borneo	3	2.33 ± 0.58	4.9± 0.4	1.98±0.30	139.66±12.79
Nepenthes hamata	Sulawesi	2	1 ± 0	3± 1.4	1.76±2.45	86.66±30.56
Nepenthes inermis	Sumatra	3	2 ± 0	2.2± 0.4	0.66±0.27	58.48±27.18
Nepenthes jacquelineae	Sumatra	4	1.25 ± 0.50	2± 1.0	1.57±0.40	45.08±25.86
Nepenthes khasiana	India	2	1.50 ± 0.71	3.3± 0.6	1.33±1.04	102.93±10.98
Nepenthes maxima	Sulawesi & New Guinea	4	1.75 ± 0.50	3± 0.8	4.29±1.32	100.29±32.99
Nepenthes ramispina	Malayan Peninsula	2	2 ± 0	2.3± 0.5	1.4±0.47	109.71±18.03
Nepenthes sanguinea	Malayan Peninsula	3	1.67 ± 0.58	2± 0.7	1.38±0.16	107.44±33.98
Nepenthes singalana	Sumatra	3	1.33 ± 0.58	2.3± 2.5	1.76±0.28	110.86±31.85
Nepenthes tentaculata	Borneo & Sulawesi	3	1.67 ± 0.58	4.2± 0.4	1.04±0.62	41.01±7.01
Nepenthes truncata	Philippines	4	1.25 ± 0.50	3± 1.4	1.78±0.12	137.73±21.31
***N. ventricosa* "Bill Bailey" (*N. ventricosa* x *singalana*)**	Unnatural Hybrid, *N. ventricosa*= Philippines	6	2 ± 0.63	2.3± 0.9	1.86±2.05	117.99±20.22

Sequencing

We used a metabarcoding approach to sequence the 16S and 18S rRNA genes in the fluid to represent the entire prokaryotic and eukaryotic communities in the pitcher fluid. DNA was extracted using a bead-beating and phenol-chlorofform extraction method after concentrating the cells with a centrifuge. The 16S and 18S rRNA gene regions were sequenced via Illumina Amplicon sequencing. Sequences were

assembled and assigned to operational taxonomic units (OTUs) using the QIIME pipeline and Harvard's Odyssey computer cluster (following Bittleston et al. 2018). We used the Greengenes and SILVA database for 16S and 18S sequences, respectively, for taxonomic classification of OTUs, with a cutoff of 97% sequence identity. In some cases, further taxonomic assignment was determined using NCBI BLAST. Neighbor-joining phylogenies were constructed for all bacterial (16S) and eukaryotic (18S) OTUs. 16S OTUs classified as chloroplast and mitochondrial sequences, and 18S OTUs classified as Embryophyte (land plant) sequences were removed from downstream analyses of community similarity to avoid inclusion of possible contaminants from host plant cells. Additionally, we repeated certain analyses for eukaryotes after removing all OTUs classified as fungus gnats (Insecta: Diptera: Sciaridae), in order to probe whether trends in community similarity were sensitive to the high levels of prey DNA in some samples.

Statistical analyses

All analyses were conducted in R version 3.5.0. In addition to testing for correlations between traits, we tested for phylogenetic signal in traits using phylogenetic data from Gilbert et al. (2018), the Pagel (1999) lambda and Blomberg et al. (2003) K statistic for continuous traits, and the Fritz and Purvis (2010) D statistic for qualitative traits. We used the function 'betadisper', paired with 'permutest' to calculate the homogeneity of variance in community similarity across species. Some samples were excluded in order to achieve homogeneity of variance for analysis of community similarity. We excluded *N. dubia* and *N. hamata* from our analysis of community similarity, each of which had only a single sample from one pitcher that was successfully extracted. For eukaryotes, *N. jacquelineae* also had only a single successful extraction and was also excluded from ordinations and downstream analyses. Lastly, we excluded the hybrid taxon *N. ventricosa* x "Bill Bailey" for both bacterial and eukaryotic community similarity analyses, due to its violation of homogeneity of variance in order to limit the comparison of community structure to well recognized species rather than commercially produced hybrids."

Since it was not possible to extract DNA successfully from all of the samples, we also performed logistic regressions (generalized linear models, binomial family with "logit" link) to assess potential biases in extraction success as a consequence of pitcher properties.

Using the 'vegan' package, we assessed community-level similarity the using non-metric multidimensional scaling (NMDS) ordination method and the unweighted Unifrac distance metric. We assessed the significance of clustering by pitcher traits (species, pH, fluid color, and viscoelasticity) using the 'adonis' function in the 'vegan' package (Oksanen et al. 2013), which conducts a specialized PERMANOVA test. For quantitative traits such as pH, we also performed a Mantel test. We further conducted canonical correspondence analysis (CCA), a form of constrained ordination, in order to more directly test the correlation between community similarity and pH. We calculated alpha diversity according to the Shannon Index using the function 'diversity' in the 'vegan' package.

In order to examine patterns of differential abundance of individual OTUs in relation to fluid properties we performed ANCOM (Analysis of Composition of Microbiomes, Mandal et al. 2015), a test designed to examine taxon abundance while accounting for the fact that metagenomics studies yield relative abundance data as opposed to absolute abundance; one advantage of this test is that it can reveal changes in differential abundance of rare OTUs that otherwise do not affect community-level properties. For ANCOM tests, we the full set of successfully extracted samples, only included OTUs with sequence counts above 100, and corrected for multiple testing (FDR) at a significance level of 0.05.

Results

Fluid properties and community composition

The mean pH differed significantly between species (Kruskal-Wallis, χ^2= 42.98, p<<0.001, Figure 2.1A). Some species (*N. dubia*, *N. jacquelineae*, *N. sanguinea*, *N. inermis*, and *N. ramispina*) had a low mean pH (~2) and a narrow range; others, such as *N. singalana* and *N. ventricosa* x "Bill Bailey" also had a low mean pH, but with at least one high pH outlier each. Most of the remaining species had a more moderate mean pH (~3-4) and a wide range of values, while *N. fusca* had a relatively high mean pH (4.86) and

narrow range. *N. fusca*'s pH is significantly different from that of *N. dubia*, *N. inermis*, *N. jacquelineae*, *N. ramispina*, *N. sanguinea*, *N. singalana*, *N. tentaculata*, and *N. ventricosa* x "Bill Bailey" (posthoc Dunn Test with Benjamini-Hochberg correction, $p<0.05$ for all pairs). We found no phylogenetic signal in mean pH, minimum pH, maximum pH, or pH range (Pagel's lambda and Blomberg's K, $p>0.10$ in all cases; Supplementary Figure 2.1) for the species tested.

The different species also varied in their ability to produce viscous fluids: *N. sanguinea*, *N. ramispina*, *N. ventricosa* x "Bill Bailey", *N. eymae*, *N. tentaculata*, and *N. fusca* did not display the ability to do so (Figure 2.1A). A few species produce colored fluid in some pitchers, including *N. singalana*, *N. ventricosa* x "Bill Bailey", *N. hamata*, *N. maxima, N. khasiana*, *N. eymae*, *N. copelandii*, and *N. boschiana* (Figure 2.1A). We observed colored fluid in some unopened or newly opened pitchers from different (unsampled) individuals in the HortPark glasshouse, including members of our study species (data not shown), thus suggesting that the fluid coloration is largely plant-produced rather than a function of external inputs from the environment. Colored fluids were either reddish/pinkish/orange or yellowish/greenish (interestingly, we observed that initially yellowish/greenish samples instantly turned reddish/pinkish/orange upon the addition of CTAB, hinting at a common chemical nature of all colored fluid samples in our study).

The species did not significantly differ in prey capture (visible fungus gnat abundance, Kruskal-Wallis test, $p>0.05$). Regarding correlations between traits, viscous pitcher samples tended to be more acidic (Kruskal-Wallis test, $\chi^2=6.20$, $p=0.012$), and pitchers with visible prey tended to have a lower pH (Kruskal-Wallis test, $\chi^2=5.03$, $p=0.025$). Colored fluid samples did not differ from clear fluid samples in pH, viscosity, or prey capture (Kruskal-Wallis test, $p>0.05$ in all cases); however, a disproportionately higher number of clear fluid samples were without visible prey (logistic regression, $p=0.003$). We found no phylogenetic signal in presence of colored fluid (Fritz and Purvis's $D= -0.14$, $p=0.08$) or viscous fluid (Fritz and Purvis's $D= 0.74$, $p=0.32$; Supplementary Figure 2.1).

To assess potential biases in extraction success as a consequence of pitcher properties, we tested for correlations between extraction success and fluid properties. A greater number of visible gnats

increased the likelihood of extraction success for 16S (logistic regression, $p=0.02$), but this did not correlate significantly with the presence of prey for 18S (logistic regression, $p=0.21$). Viscous samples were less likely to provide successful extractions both for 16S and 18S (logistic regressions, $p=0.02$ and $p=0.01$, respectively). Finally, decreasing pH decreased the likelihood of extraction success overall both for 16S and 18S (logistic regressions, $p=0.023$ and $p=0.012$, respectively); however, there was no significant effect of pH on extraction success for either 16S or 18S when controlling for viscosity (logistic regressions, $p>0.05$ in all cases).

The bacterial communities found in our samples consist of several phyla, predominantly Proteobacteria (Alpha-, Beta-, and Gammaproteobacteria) and Bacteroidetes. Within Alphaproteobacteria, the order Rhodospirillales, containing the families Acetobacteraceae and Rhodospirillaceae, was particularly dominant in some samples (Figure 2.1B). The eukaryotic communities found in our samples consist of a diverse assemblage of Metazoa, Fungi, Amoebozoa, Archaeplastida, Stramenopiles, Alveolata, Rhizaria, and Discoba (Figure 2.1C).

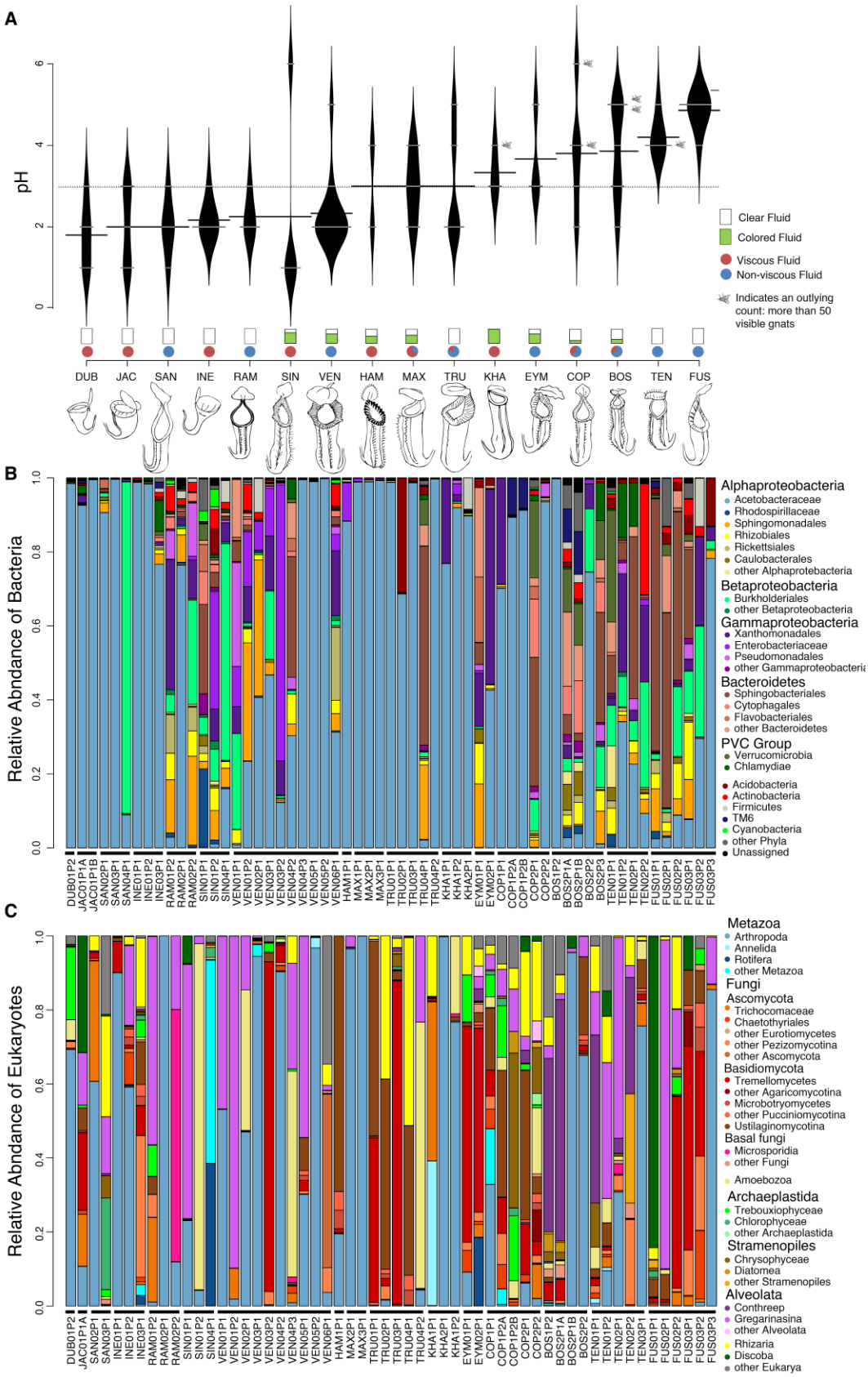
A
pH
0
2
4
6
Clear Fluid
Colored Fluid
Viscous Fluid
Non-viscous Fluid
Indicates an outlying count: more than 50 visible gnats
DUB JAC SAN INE RAM SIN VEN HAM MAX TRU KHA EYM COP BOS TEN FUS
B
Relative Abndance of Bacteria
0.0
0.2
0.4
0.6
0.8
1.0
Alphaproteobacteria
Acetobacteraceae
Rhodospirillaceae
Sphingomonadales
Rhizobiales
Rickettsiales
Caulobacterales
other Alphaproteobacteria
Betaproteobacteria
Burkholderiales
other Betaproteobacteria
Gammaproteobacteria
Xanthomonadales
Enterobacteriaceae
Pseudomonadales
other Gammaproteobacteria
Bacteroidetes
Sphingobacteriales
Cytophagales
Flavobacteriales
other Bacteroidetes
PVC Group
Verrucomicrobia
Chlamydiae
Acidobacteria
Actinobacteria
Firmicutes
TM6
Cyanobacteria
other Phyla
Unassigned
DUB01P2 JAC01P1A JAC01P1B SAN02P1 SAN03P1 SAN04P1 INE01P1 INE01P2 INE03P1 RAM01P2 RAM02P1 RAM02P2 SIN01P1 SIN01P2 SIN04P1 VEN01P1 VEN01P2 VEN02P1 VEN03P1 VEN03P2 VEN04P2 VEN04P3 VEN05P1 VEN05P2 VEN06P1 HAM1P1 MAX1P1 MAX2P1 MAX3P1 TRU01P1 TRU02P1 TRU03P1 TRU04P1 TRU04P2 KHA1P1 KHA1P2 KHA2P1 EYM01P1 EYM02P1 COP1P1 COP1P2A COP1P2B COP2P1 COP2P2 BOS1P2 BOS2P1A BOS2P1B BOS2P2 BOS2P3 TEN01P1 TEN01P2 TEN02P1 TEN02P2 FUS01P1 FUS02P1 FUS02P2 FUS03P1 FUS03P2 FUS03P3
C
Relative Abndance of Eukaryotes
0.0
0.2
0.4
0.6
0.8
1.0
Metazoa
Arthropoda
Annelida
Rotifera
other Metazoa
Fungi
Ascomycota
Trichocomaceae
Chaetothyriales
other Eurotiomycetes
other Pezizomycotina
other Ascomycota
Basidiomycota
Tremellomycetes
other Agaricomycotina
Microbotryomycetes
other Pucciniomycotina
Ustilaginomycotina
Basal fungi
Microsporidia
other Fungi
Amoebozoa
Archaeplastida
Trebouxiophyceae
Chlorophyceae
other Archaeplastida
Stramenopiles
Chrysophyceae
Diatomea
other Stramenopiles
Alveolata
Conthreep
Gregarinasina
other Alveolata
Rhizaria
Discoba
other Eukarya
DUB01P2 JAC01P1A SAN02P1 SAN03P1 INE01P1 INE01P2 INE03P1 RAM01P2 RAM02P1 RAM02P2 SIN01P1 SIN01P2 SIN04P1 VEN01P1 VEN01P2 VEN02P1 VEN03P1 VEN03P2 VEN04P2 VEN04P3 VEN05P1 VEN05P2 VEN06P1 HAM1P1 MAX2P1 MAX3P1 TRU01P1 TRU02P1 TRU03P1 TRU04P1 TRU04P2 KHA1P1 KHA2P1 KHA1P2 EYM01P1 EYM02P1 COP1P1 COP1P2A COP1P2B COP2P1 COP2P2 BOS1P2 BOS2P1A BOS2P1B BOS2P2 TEN01P1 TEN01P2 TEN02P1 TEN02P2 TEN03P1 FUS01P1 FUS02P1 FUS02P2 FUS03P1 FUS03P2 FUS03P3

Figure 2.1: Illustration of fluid properties and community composition of experimental pitchers. (A) Beanplot representing pH levels of each species. The width of short white lines represents the number of samples at each value; long black lines represent means. Small barplots represent portion of pitchers per species that exhibited colored fluid. Circles represent portion of pitchers per species that produced viscous fluid. Fly icons represent individual pitcher samples that contained more than 50 visible gnats. Illustrations of pitchers from each species by LSB; illustrations are not to scale and are for representative purposes only. Species are organized by mean pH. Species codes: DUB = *N. dubia*, JAC = *N. jacquelineae*, SAN = *N. sanguinea*, INE = *N. inermis*, RAM = *N. ramispina*, SIN = *N. singalana*, VEN = *N. ventricosa* x "Bill Bailey", HAM = *N. hamata*, MAX = *N. maxima*, TRU = *N. truncate*, KHA = *N. khasiana*, EYM = *N. eymae*, COP = *N. copelandii*, BOS = *N. boschiana*, TEN = *N. tentaculata*, FUS = *N. fusca* (B) Stacked barplot representing relative abundances of bacterial taxa. (C) Stacked barplot representing relative abundances of eukaryotic taxa.

Influence of pitcher traits on community similarity and alpha diversity

Nepenthes species

Both bacteria (PERMANOVA, R^2= 0.39, p<0.001) and eukaryotes (PERMANOVA, R^2= 0.32, p=0.004) show significant clustering by host species; however, a pairwise Adonis test shows no significant differences between individual species pairs (with Benjamini-Hochberg correction, p>0.05 for all pairs; Figure 2.2). Regarding alpha diversity, bacterial communities differ in the mean and range of sample Shannon index by species; these means appear to be significantly different (Kruskal-Wallis test, χ^2= 29.637, p<0.001), but individual species pairs are not significantly different under a Benjamini-Hochberg corrected post hoc Dunn test (p>0.05 for all pairs). For eukaryotes, there were no significant differences in alpha diversity across species (Kruskal-Wallis test, χ^2= 18.38, p=0.07). We found no differences in the resulting trends when we repeated these analyses with fungus gnat OTUs removed.

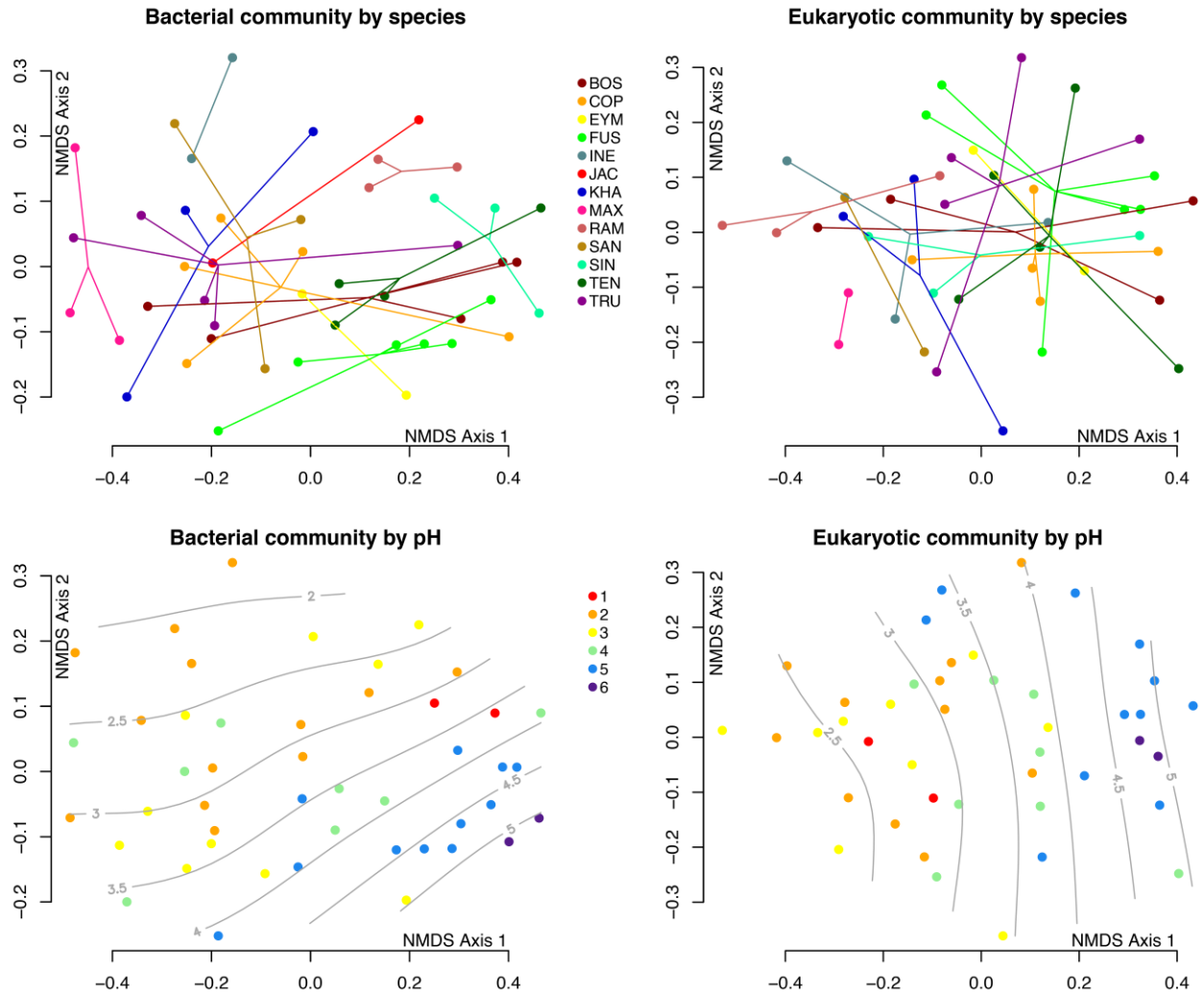

Figure 2.2: Non-metric multidimentional scaling (NMDS) plots representing community similarity (UniFrac distances) of bacteria (A,C) and eukaryotes (B,D) by host plant species identity (A,B) and by pH (C,D). Each point represents a sample and distance between points represents degree of similarity in community composition. Lines in A and B connect points belonging to the same species. Grey contour lines in C and D are smooth surfaces calculated based on variation in pH using the 'ordisurf' function in the 'vegan' package of R.

Fluid pH

Pitcher fluid pH was significantly correlated with community similarity for both bacteria (Mantel test, r= 0.23, $p<0.001$) and eukaryotes (Mantel test, r= 0.32, $p<0.001$). The CCA test also revealed that community similarity changed as a function of changing pH levels, and this was also highly significant for both bacteria ($\chi^2=0.62$, $p<0.001$) and eukaryotes ($\chi^2=0.62$, $p<0.001$). Alpha diversity increases with increasing pH for both bacteria (linear regression, $R^2=0.27$, $p<<0.001$) and eukaryotes (linear regression,

R^2=0.39, p<<0.001). When we repeated these analyses for eukaryotes with fungus gnat OTUs removed, we found no differences in the resulting trends.

Fluid color

There was significant clustering by fluid color for eukaryotes (PERMANOVA, R^2= 0.04, p=0.03) but not bacteria (PERMANOVA, R^2= 0.025, p=0.23). However, when we repeated the analysis for eukaryotes with all OTUs assigned as fungus gnats removed, the significant difference for eukaryotes disappeared (Adonis R^2= 0.0374, p=0.07). Alpha diversity was not significantly different between colored and clear fluid samples for bacteria (Kruskal-Wallis test, χ^2 = 0.56, p= 0.46). For eukaryotes, alpha diversity tended to be lower in colored fluid samples (Kruskal-Wallis test, χ^2 = 6.13, p= 0.013), however fluid color had no significant relationship with alpha diversity once fungus gnats are removed (Kruskal-Wallis test, χ^2 = 3.10, p= 0.078).

Viscosity

There was no significant clustering by fluid viscosity for either bacterial (PERMANOVA, R^2= 0.024, p=0.26) or eukaryotic community similarity (PERMANOVA, R^2= 0.028, p=0.22). However, viscous samples had lower alpha diversity for both bacteria (Kruskal-Wallis test, χ^2 = 4.43, p= 0.035) and eukaryotes (Kruskal-Wallis test, χ^2 = 4.26, p= 0.039), though the effect was modest, and if fungus gnats are removed from the eukaryotic OTU table, viscosity had no effect on eukaryotic alpha diversity (Kruskal-Wallis test, χ^2 = 2.88, p= 0.089).

Influence of pitcher traits on individual OTUs

Fluid pH

According to the ANCOM test, the abundances of 25 bacterial and 17 eukaryotic OTUs were significantly differentially abundant across the different pH levels of the pitcher fluids. Bacteria exhibited variation in individual OTU response to pH level, with most OTUs increasing in abundance with increasing pH (all

OTUs within Bacteroidetes, Cyanobacteria, and Verrucomicrobia), and others decreasing with increasing pH (Figure 2.3). The minority of OTUs that decreased with increasing pH include members of Rhodospirillales, both *Acidocella* and an unassigned Rhodospirillaceae. A third OTU in Rhodospirillales (unassigned Acetobacteriaceae) exhibits the opposite pattern from its confamilial *Acidocella*. Most other OTUs in Alphaproteobacteria generally increased with increasing pH, except *Sphingomonas*, which generally decreased with increasing pH. In Betaproteobacteria, one OTU (*Dechloromonas*) decreased with increasing pH, while the other one (unassigned Comamondaceae) increased with increasing pH. All significant eukaryotic OTUs decreased with increasing pH, and all are assigned as fungus gnats (Diptera: Sciaridae: Bradysia).

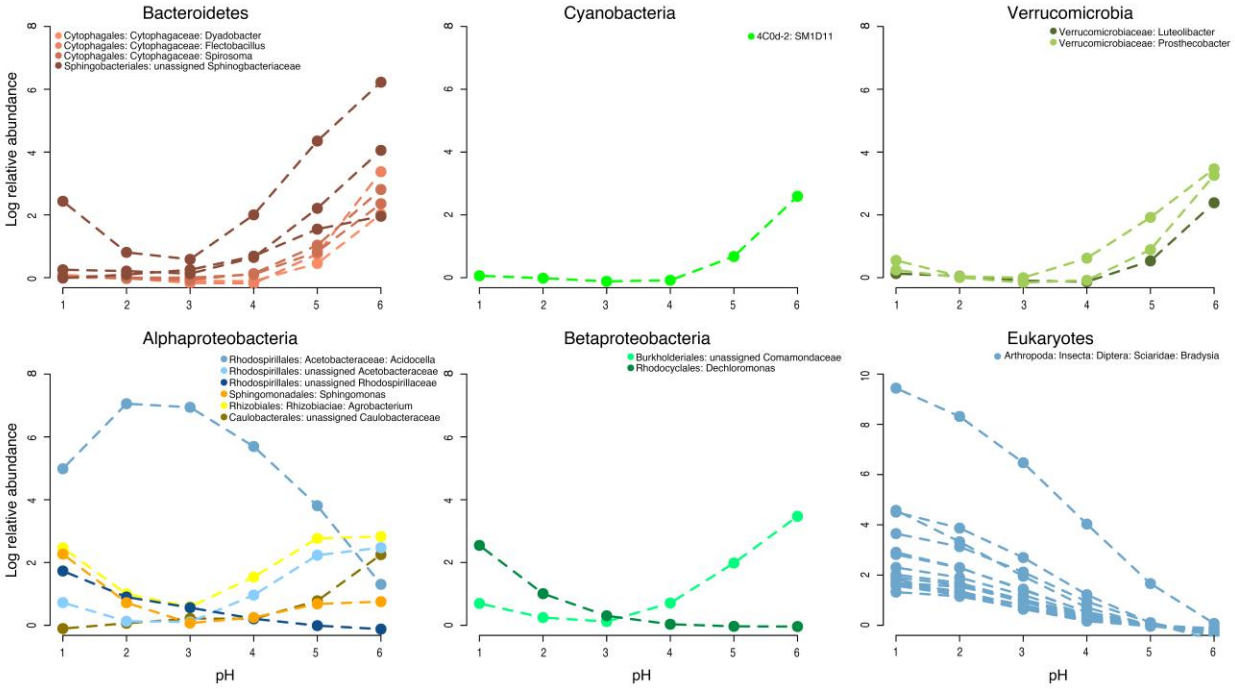

Figure 2.3: Results of Analysis of composition of microbiomes (ANCOM) test, showing OTUs that are differentially abundant across pH levels. The points and dotted lines are smooth splines generated to summarize the individual trends in change of mean log relative abundance for each OTU across pH levels.

Fluid color

The ANCOM test reveals 23 eukaryotic OTUs differentially abundant by fluid color: 5 OTUs in Fungi and 18 OTUs in Metazoa (Figure 2.4). The metazoan OTUs were more abundant in colored fluid (most of

which are absent from clear fluid); conversely the fungal OTUs are more abundant in clear fluid (completely absent from colored fluid). Only a single bacterial OTU was significantly differentially abundant by fluid color, an unassigned Enterobacteriaceae, and it was more abundant in colored fluid than clear fluid.

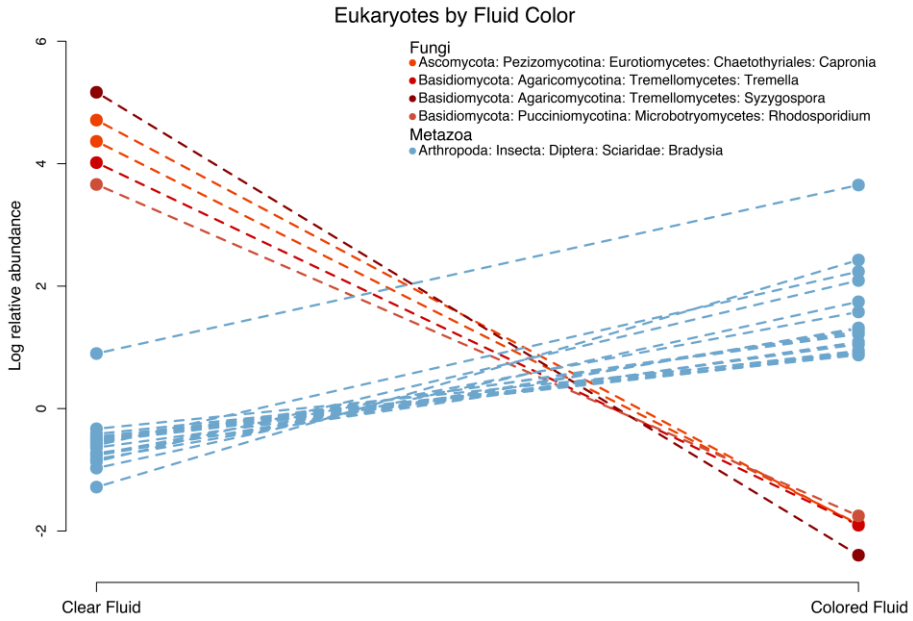

Figure 2.4: Results of Analysis of composition of microbiomes (ANCOM) test, showing OTUs that are differentially abundant between the two categories of fluid color (clear and colored). The points and dotted lines are regression lines showing the slope of the change in mean log relative abundance for each OTU between categories.

Viscosity

The ANCOM test shows that only a single bacterial OTU was significantly differentially abundant by fluid viscosity, *Cryocola* (Actinobacteria), and it was more abundant in viscous fluid than non-viscous fluid. Only a single eukaryotic OTU had a significant ANCOM result by viscosity, *Chlamydomonas* (Archaeplastida: Chlorophyceae), and it was more abundant in viscous fluid than non-viscous fluid.

Discussion

We investigated how *Nepenthes* pitchers might act as ecological filters, especially via their manipulation of the abiotic properties of their fluid. In order for *Nepenthes* species to affect the ability of microbes to establish and persist in their pitchers, they must be able to alter their fluid properties. We demonstrated that the various species in our experiment do in fact alter their fluid properties, including pH levels and the production of viscous and/or colored fluid. The pitchers in our study were all filled with the same pH 6.5 water at the beginning of the experiment and yet after about two weeks of acclimation, they ended with a pH range spanning from ~ 1-6, and clear differences in fluid viscosity and color. We designate the differences in fluid properties as both biotic (fluid viscosity, color) and abiotic factors (pH); however, the designation is not clear cut. The pH level, like viscosity or fluid color, is largely a function of pitcher physiology, and accordingly shows interspecific variation.

We first tested how *Nepenthes* species identity shapes variation in the community compositions of organisms housed within pitchers. At a first approximation, the magnitude of the effect of host species identity on community composition is similar to that of pH; however, a post hoc test reveals that with the number of species involved, the differences between any species pair taken in isolation lacks statistical significance. To further probe the role of species identity as a force in shaping microbial assembly, we performed the ANCOM test for the presence of significantly differentially abundant OTUs within *N. ramispina* (a species with a visibly separated cluster in the ordination for bacteria, Figure 2.2) versus all other species. While *N. ramispina* did contain OTUs that were unique or differentially abundant with respect to all other species pooled, these were OTUs that did not differ across the other species that share a similar pH mean and range (low and narrow, Figure 2.1A). Hence these OTUs were likely associated with *N. ramispina*'s particular pH regulatory properties rather than *N. ramispina* itself. The fact that species do not contain characteristic and significantly different OTUs when compared with the pitchers of other species within a group of physiologically similar species further suggests that trait variation rather than species identity *per se* is the factor that acts as an ecological filter, at least in this study. This supports

pH as the primary factor of importance among the traits we measured for community assembly within *Nepenthes* pitchers.

The influence of pH on both bacteria and eukaryotes is strong, both in terms of community similarity and in terms of the dynamics of individual OTUs (as seen in ANCOM results, Figure 2.3). Most bacterial OTUs are less abundant in highly acidic fluid, and the overall alpha diversity is lower as well. This speaks to the harshness of low pH conditions, where only a few specialized acidophiles are able to thrive, such as the Acetobacteraceae. Interestingly, species in the Acetobacteraceae, especially of the genus *Acidocella*, appear to be common associates of *Nepenthes*, not only in this study, but in wild samples as well (supplemental discussion).

For eukaryotes, all OTUs with significant differential abundance at different pH levels were found to decrease with increasing pH. All of these OTUs were assigned as fungus gnats (Insecta: Diptera: Sciaridae) by BLAST. The high numbers of fungus gnat sequences at low pH levels may be because prey capture induces a decrease in pH, or they may indicate that pitchers belonging to high-acidity species also tend to be more successful at prey capture, assuming no bias in fungus gnat occurrence throughout the glasshouse. The former is likely given that prey capture indeed induces fluid acidification (Lloyd 1942; Saganová et al. 2018). Despite this, pH differences can still be largely explained by species differences, as even with prey induction, it has been demonstrated that not all species are capable of achieving the same levels of acidity (Saganová et al. 2018). Also, the samples with the greatest number of visible gnats had relatively moderate to high pH (pH 4-6, Figure 2.1). However, it is still possible that the more acidic pitchers in fact caught more prey than the less acidic pitchers, but acidic pitchers were also better at digestion, thus leaving less physical evidence of their prey capture success. Future studies examining how prey abundances in pitchers correlate to 18S rRNA sequence counts could help to clarify this (Bittleston et al. 2016). The effect of pH on eukaryotes is not exclusively a result of prey capture, however, as pH still has a significant effect on eukaryotic community similarity after removing fungus gnats from the OTU table. This means that microbial eukaryotes living symbiotically in the fluid such as fungi, algae,

and amoebae experience physiological challenges in acidic conditions similar to the bacteria, or appear to be similarly affected due to their interactions with the bacteria themselves.

It was surprising that viscosity, a biotic factor and definitively plant-regulated trait, had only a weak relationship to community structure, with no significant difference in community similarity for either bacteria or eukaryotes between species with different fluid viscosities. The only effect we noted was that viscous pitchers had lower alpha diversity for both bacteria and eukaryotes. This might suggest that viscous fluid presents a harsher environment for inquilines, similar to how low pH environments lead to reduced diversity. Extractions from viscous samples were also more likely to fail than those from non-viscous samples. Even though extraction success generally increases as pH rises, there was no effect of pH on extraction success within either category of viscous or non-viscous pitchers. Thus, despite the fact that species with viscous fluid were generally more acidic, viscosity appears to have more of an impact on DNA extraction success than pH. However, without qPCR data directly measuring numbers of ribosomal RNA genes, it is not possible to ascertain whether extraction failure can be attributed to reduced microbial abundance or to some form of bias in the extraction process. In any case, viscosity might have a larger impact on individual OTUs than on community composition, as our ANCOM results revealed OTUs with significant differential abundances between viscous and non-viscous fluids.

For eukaryotes, but not for bacteria, colorful pitcher fluids had significantly lower alpha diversity than clear fluids, suggesting fluid color to be a more important factor for eukaryotic communities. However, the effects of fluid color on eukaryotic community similarity and alpha diversity are not robust, and disappear when fungus gnats are removed from the dataset. Like viscosity, fluid color appears to be more important at the individual OTU level than it is at the community level. Fluid color could be an indication of the production of droserone and 5-O methyl droserone. Past studies have shown that the presence of these compounds results in reddish (Eilenberg et al. 2010; Raj et al. 2011) or yellowish (Baby et al. 2017) fluid coloration. Droserone and 5-O methyl droserone are anti-fungal agents induced by prey capture, specifically in response to chitin (Eilenberg et al. 2010; Raj et al. 2011; Baby et al. 2017). This could explain the higher abundance of fungus gnat DNA in colored samples relative to clear fluid

samples, accompanied by a decrease in the relative abundance of certain fungal OTUs (Figure 2.4). Without confirmation by GCMS this explanation remains somewhat speculative, but the pattern is suggestive. Pitcher fluid coloration is not a well-documented trait in the literature; to our knowledge, plant-produced colored fluid has only been reported for *N. khasiana* (Eilenberg et al. 2010; Raj et al. 2011; Baby et al. 2017). Our observation of colored fluid in more species is novel, and future work should investigate this potentially ecologically meaningful trait, both in the field and in cultivation.

In this study, it was not possible to assess the functional significance of differentially abundant microbial OTUs, but these could be probed by future transcriptomic or proteomic work. The observation of certain OTUs frequently occurring in *Nepenthes* pitchers in both natural and artificial situations could indicate that these particular associations are ecologically significant, so the common *Nepenthes* symbionts like *Acidocella* found here merit further research from a functional perspective. Pitchers may also modify other abiotic features of the fluid such as dissolved oxygen levels or temperature, so additional fluid properties should be examined in future work as well.

Our research supports the hypothesis that *Nepenthes* pitcher plants regulate abiotic factors, potentially as a means of maintaining species-specific microbial associations. This is important in considering the possibility of community codiversification (Ley et al. 2008; Sanders et al. 2014). From the perspective of the host, as long as an abiotic factor is under host control, it functionally becomes an extended phenotype with the same potential for evolution in response to interspecies interactions as any other biotic phenotype. However, from the perspective of the microbial symbionts, evolution in response to host conditions becomes much less tight. Microbes that respond to a purely biotic factor, such as secondary metabolites, can be considered to be necessarily linked to the evolution of the host, as the exact biochemical compounds involved are unlikely to be found in other environmental contexts. On the other hand, when microbes respond to an abiotic factor, such as fluid pH, those microbes may have been pre-adapted to live in a wide range of environments that incidentally fit that factor, such as other small aquatic environments. So even if the abiotic factor is a product of host evolution in one context, the symbionts may not have evolved in response to the host. Thus, the evolutionary implications of biotic and abiotic

filters are quite different from the perspective of the symbiont, despite having similar implications from the perspective of the host.

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Chapter 2 References:

Adlassnig W, Peroutka M, Lendl T. **2011**. Traps of carnivorous pitcher plants as a habitat: composition of the fluid, biodiversity and mutualistic activities. *Annals of Botany* **107**: 181–94.

Amund O, Adebiyi A. **1991**. Effect of viscosity on the biodegradability of automotive lubricating oils. *Tribology International* **24**: 235–237.

Athauda SBP, Matsumoto K, Rajapakshe S, Kuribayashi M, Kojima M, Kubomura-Yoshida N, Iwamatsu A, Shibata C, Inoue H, Takahashi K. **2004**. Enzymic and structural characterization of nepenthesin, a unique member of a novel subfamily of aspartic proteinases. *The Biochemical Journal* **381**: 295–306.

Baby S, Johnson AJ, Zachariah EJ, Hussain AA. **2017**. *Nepenthes* pitchers are CO_2-enriched cavities, emit CO_2 to attract preys. *Scientific Reports* **7**.

Bazile V, Le Moguédec G, Marshall DJ, Gaume L. **2015**. Fluid physico-chemical properties influence capture and diet in *Nepenthes* pitcher plants. *Annals of Botany* **115**: 705–716.

Beasley DE, Koltz AM, Lambert JE, Fierer N, Dunn RR. **2015**. The evolution of stomach acidity and its relevance to the human microbiome. *PloS one* **10**: e0134116.

Beaver R. **1979**. Biological studies of the fauna of pitcher plants [*Nepenthes*] in west Malaysia. *Annales de la Société Entomologique de France*.3–17.

Bittleston LS. **2018**. Commensals of *Nepenthes* Pitchers, in *Carnivorous Plants: Physiology, Ecology, and Evolution*. Ellison, Aaron M and Adamec, Lubomír ed. Oxford University Press, 314–332.

Bittleston LS, Baker C, Strominger LB, Pringle A, Pierce NE. **2016**. Metabarcoding as a tool for investigating arthropod diversity in *Nepenthes* pitcher plants. *Austral Ecology* **41**: 120–132.

Bittleston LS, Wolock CJ, Yahya BE, Chan XY, Chan KG, Pierce NE, Pringle A. **2018**. Convergence between the microcosms of Southeast Asian and North American pitcher plants. *eLife* **7**.

Blomberg SP, Garland T, Ives AR. **2003**. Testing for phylogenetic signal in comparative data: behavioral traits are more labile. *Evolution; International Journal of Organic Evolution* **57**: 717–45.

Bonhomme V, Pelloux-Prayer H, Jousselin E, Forterre Y, Labat J-J, Gaume L. **2011**. Slippery or sticky? Functional diversity in the trapping strategy of *Nepenthes* carnivorous plants. *The New Phytologist* **191**: 545–54.

Buch F, Kaman WE, Bikker FJ, Yilamujiang A, Mithöfer A. **2015**. Nepenthesin protease activity indicates digestive fluid dynamics in carnivorous *Nepenthes* plants. *PloS one* **10**: e0118853.

Buch F, Rott M, Rottloff S, Paetz C, Hilke I, Raessler M, Mithöfer A. **2012**. Secreted pitfall-trap fluid of carnivorous *Nepenthes* plants is unsuitable for microbial growth. *Annals of Botany*: mcs287.

Bump JK, Webster CR, Vucetich JA, Peterson RO, Shields JM, Powers MD. **2009**. Ungulate carcasses perforate ecological filters and create biogeochemical hotspots in forest herbaceous layers allowing trees a competitive advantage. *Ecosystems* **12**: 996–1007.

Clarke C. **1997**. *Nepenthes* of Borneo. *Kota Kinabalu, Sabah: Natural History Publications (Borneo) xi, 207p-col. illus.. ISBN* **1248185562**.

Dawkins R. **1978**. Replicator Selection and the Extended Phenotype 3. *Zeitschrift für Tierpsychologie* **47**: 61–76.

Dennis PG, Hirsch PR, Smith SJ, Taylor RG, Valsami-Jones E, Miller AJ. **2009**. Linking rhizoplane pH and bacterial density at the microhabitat scale. *Journal of Microbiological Methods* **76**: 101–4.

Duflot R, Georges R, Ernoult A, Aviron S, Burel F. **2014**. Landscape heterogeneity as an ecological filter of species traits. *Acta Oecologica* **56**: 19–26.

Eilenberg H, Pnini-Cohen S, Rahamim Y, Sionov E, Segal E, Carmeli S, Zilberstein A. **2010**. Induced production of antifungal naphthoquinones in the pitchers of the carnivorous plant *Nepenthes khasiana*. *Journal of Experimental Botany* **61**: 911–22.

Fashing NJ. **2002**. *Nepenthacarus*, a new genus of Histiostomatidae (Acari: Astigmata) inhabiting the pitchers of *Nepenthes* mirabilis (Lour.) Druce in far north Queensland, Australia. *Australian Journal of Entomology* **41**: 7–17.

Fierer N, Jackson RB. **2006**. The diversity and biogeography of soil bacterial communities. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 626–631.

Fritz SA, Purvis A. **2010**. Selectivity in mammalian extinction risk and threat types: a new measure of phylogenetic signal strength in binary traits. *Conservation Biology* **24**: 1042–1051.

Gaume L, Forterre Y. **2007**. A viscoelastic deadly fluid in carnivorous pitcher plants. *PloS one* **2**: e1185.

George LO, Bazzaz F. **1999**. The fern understory as an ecological filter: emergence and establishment of canopy-tree seedlings. *Ecology* **80**: 833–845.

Gilbert KJ, Nitta JH, Talavera G, Pierce NE. **2018**. Keeping an eye on coloration: ecological correlates of the evolution of pitcher traits in the genus *Nepenthes* (Caryophyllales). *Biological Journal of the Linnean Society* **123**: 321–327.

Goddard A, Spiller R. **1996**. The effect of omeprazole on gastric juice viscosity, pH and bacterial counts. *Alimentary Pharmacology & Therapeutics* **10**: 105–109.

Hartmann A, Schmid M, van Tuinen D, Berg G. **2009**. Plant-driven selection of microbes. *Plant and Soil* **321**: 235–257.

Itô H, Hino T. **2007**. Dwarf bamboo as an ecological filter for forest regeneration. *Ecological Research* **22**: 706–711.

Juniper BE, Robins RJ, Joel DM. **1989**. *The Carnivorous Plants*. London, etc.: Academic Press.

Kanokratana P, Mhuanthong W, Laothanachareon T, Tangphatsornruang S, Eurwilaichitr L, Kruetreepradit T, Mayes S, Champreda V. **2016**. Comparative Study of Bacterial Communities in

Nepenthes. *Microbial Ecology* **72**: 381–393.

Kitching R. **1987**. A preliminary account of the metazoan food webs in phytotelmata from Sulawesi. *Malay. Nat. J* **41**: 1–12.

Kraft NJ, Adler PB, Godoy O, James EC, Fuller S, Levine JM. **2014**. Community assembly, coexistence and the environmental filtering metaphor. *Functional Ecology*.

Krueger LM, Peterson CJ. **2009**. Effects of woody debris and ferns on herb-layer vegetation and deer herbivory in a Pennsylvania forest blowdown. *Ecoscience* **16**: 461–469.

Lewontin RC. 1983. Gene, Organism and Environment. In: *Evolution from Molecules to Men*. Bendall, DS, editor. Cambridge: Cambridge University Press.

Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, Schlegel ML, Tucker TA, Schrenzel MD, Knight R, others. **2008**. Evolution of mammals and their gut microbes. *Science* **320**: 1647–1651.

Lloyd F 1942. *The Carnivorous Plants.* Waltham, MA: Chronica Botanica Company

Mandal S, Van Treuren W, White RA, Eggesbø M, Knight R, Peddada SD. **2015**. Analysis of composition of microbiomes: a novel method for studying microbial composition. *Microbial Ecology in Health and Disease* **26**: 27663.

McDonald D, Pethick D, Mullan B, Hampson D. **2001**. Increasing viscosity of the intestinal contents alters small intestinal structure and intestinal growth, and stimulates proliferation of enterotoxigenic *Escherichia coli* in newly-weaned pigs. *British Journal of Nutrition* **86**: 487–498.

McPherson S, Robinson A, Fleischmann A. **2009**. *Pitcher plants of the Old World*. Redfern Natural History Productions Dorset.

Moran JA, Gray LK, Clarke C, Chin L. **2013**. Capture mechanism in Palaeotropical pitcher plants (Nepenthaceae) is constrained by climate. *Annals of Botany*: mct195.

Moran JA, Hawkins BJ, Gowen BE, Robbins SL. **2010**. Ion fluxes across the pitcher walls of three Bornean *Nepenthes* pitcher plant species: flux rates and gland distribution patterns reflect nitrogen sequestration strategies. *Journal of Experimental Botany*: erq004.

Muñoz JC, Aerts R, Thijs KW, Stevenson PR, Muys B, Sekercioglu CH. **2013**. Contribution of woody habitat islands to the conservation of birds and their potential ecosystem services in an extensive Colombian rangeland. *Agriculture, Ecosystems & Environment* **173**: 13–19.

Odling-Smee FJ. 1988. Niche constructing phenotypes. In: *The role of Behavior in Evolution*. Plotkin HC, editor. Cambridge: MIT Press. p 73–132.

Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'hara R, Simpson GL, Solymos P, Stevens MHH, Wagner H, others. **2013**. Package "vegan." *Community Ecology Package, version* **2**.

Ovaskainen O, Tikhonov G, Norberg A, Guillaume Blanchet F, Duan L, Dunson D, Roslin T, Abrego N. **2017**. How to make more out of community data? A conceptual framework and its implementation as models and software. *Ecology Letters* **20**: 561–576.

Pagel M. **1999**. Inferring the historical patterns of biological evolution. *Nature* **401**: 877–84.

Rabotnova IL. **1963**. *The importance of physical-chemical factors (pH and rH2) for the life activity of microorganisms*. Ft. Belvoir Defense Technical Information Center, Army Biological Labs, Frederick, MD, Translation of German edition of Russian book, Jena, 226p

Raj G, Kurup R, Hussain AA, Baby S. **2011**. Distribution of naphthoquinones, plumbagin, droserone, and 5-O-methyl droserone in chitin-induced and uninduced *Nepenthes khasiana*: molecular events in prey capture. *Journal of Experimental Botany* **62**: 5429–5436.

Rascovan N, Carbonetto B, Perrig D, Díaz M, Canciani W, Abalo M, Alloati J, González-Anta G, Vazquez MP. **2016**. Integrated analysis of root microbiomes of soybean and wheat from agricultural fields. *Scientific Reports* **6**: 28084.

Ravee R, Salleh FM, Goh H-H. **2018**. Discovery of digestive enzymes in carnivorous plants with focus on proteases. *PeerJ* **6**: e4914.

Renner T, Specht CD. **2013**. Inside the trap: gland morphologies, digestive enzymes, and the evolution of plant carnivory in the Caryophyllales. *Current Opinion in Plant Biology* **16**: 436–42.

Saganová M, Bokor B, Stolárik T, Pavlovi\vc A. **2018**. Regulation of enzyme activities in carnivorous pitcher plants of the genus *Nepenthes*. *Planta*: 1–14.

Sanders JG, Powell S, Kronauer DJ, Vasconcelos HL, Frederickson ME, Pierce NE. **2014**. Stability and phylogenetic correlation in gut microbiota: lessons from ants and apes. *Molecular Ecology* **23**: 1268–1283.

Schneider WR, Doetsch RN. **1974**. Effect of viscosity on bacterial motility. *Journal of Bacteriology* **117**: 696–701.

Stagaman K, Burns AR, Guillemin K, Bohannan BJ. **2017**. The role of adaptive immunity as an ecological filter on the gut microbiota in zebrafish. *The ISME Journal* **11**: 1630.

Tiitsaar A, Kaasik A, Teder T. **2013**. The effects of seasonally variable dragonfly predation on butterfly assemblages. *Ecology* **94**: 200–7.

Turner J. **2000**. *The Extended Organism. The Physiology of Animal-Built Structures*. Cambridge: Harvard University Press.

Turner JS. **2002**. A superorganism's fuzzy boundaries. *Natural History* **111**: 62–69.

Umu ÖC, Frank JA, Fangel JU, Oostindjer M, Da Silva CS, Bolhuis EJ, Bosch G, Willats WG, Pope PB, Diep DB. **2015**. Resistant starch diet induces change in the swine microbiome and a predominance of beneficial bacterial populations. *Microbiome* **3**: 16.

Vacher C, Hampe A, Porté AJ, Sauer U, Compant S, Morris CE. **2016**. The phyllosphere: microbial jungle at the plant-climate interface. *Annual Review of Ecology, Evolution, and Systematics* **47**: 1–24.

Vorholt JA. **2012**. Microbial life in the phyllosphere. *Nature Reviews. Microbiology* **10**: 828–40.

Wagner MR, Lundberg DS, Tijana G, Tringe SG, Dangl JL, Mitchell-Olds T. **2016**. Host genotype and age shape the leaf and root microbiomes of a wild perennial plant. *Nature Communications* **7**: 12151.

Chapter 3: Eukaryotic and bacterial communities in *Nepenthes* phytotelmata along an elevational gradient

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Abstract

The genus *Nepenthes* shows great diversity in altitudinal distribution, spanning from sea level to over 3500 meters above sea level in the highest-altitude species. Altitude has been an important factor in the field of community ecology—one that integrates several abiotic features and leads to strong repeatable patterns of community assembly, such as the mid-altitude diversity peak seen in disparate taxa in numerous studies. We focus on one species from the Philippines, *N. mindanaoensis*, which has a relatively large altitudinal span for *Nepenthes* (~400-1600 m a.s.l.) to investigate whether altitude plays a role in structuring the community of microbes and inquilines found in the fluid of its pitchers. Two outcomes are possible: either altitude plays a role in community structure as seen in many classic studies, or alternatively, no structuring by altitude occurs, possibly due to internal homeostatic features of the pitchers being more important. We show differences in patterns of community assembly for bacteria and eukaryotes, despite their living together in the same aquatic microhabitats. Community similarity of eukaryotes, but not bacteria, are significantly influenced by altitude. On the other hand, pitcher dimorphism has an effect on eukaryotes but not bacteria, while variation in pH levels strongly influences both taxa. Additionally, we show that arthropod abundance in this system follows the classical trend of decreasing with elevation, and point to some differences in the patterns of abundance for living inquilines insects as opposed to insect prey, in relation to intraspecific plant trait variation.

Introduction

Since its inception, a major direction within the field of ecology has been to uncover large-scale patterns of biodiversity; such macroecological research ultimately seeks to find mechanisms or fundamental rules governing organisms and ecosystems that can explain the patterns we see. Much like the latitudinal gradient in species diversity, the elevational diversity gradient is a well-examined phenomenon in macroecology. Mountains, especially tropical mountains, can exhibit levels of climactic variation across their ranges that are comparable to the climactic changes that occur at much larger distances over the globe moving from equator to poles. Because mountains present spatially compact environmental gradients, they have been very useful for exploring broad patterns across taxa. Similar patterns have been found across diverse taxa over decades: whether monotonic or hump-shaped, plants, mammals, birds, amphibians, reptiles, insects, and other invertebrates have all generally exhibited decreasing diversity and abundance with increasing elevation (Rahbek 1995). While the history of elevational gradient studies can be traced back to the time of Linnaeus, bacteria and other microbes have only been the focus of such studies relatively recently, largely within the past decade (Bryant et al. 2008). The extent to which microbes follow the same macroecological trends established in plants and animals (macro-organisms or "macrobes") remains an unresolved question. One major line of thought on microbial macroecology has roots in the Baas-Becking hypothesis: "everything is everywhere, and the environment selects", suggesting that bacteria are not dispersal-limited and thus are not subject to the broad-scale spatial structuring seen in macrobes (O'Malley 2008; Shen et al. 2013; Yuan et al. 2014; Wang et al. 2015; Debnath et al. 2016; Zhao et al. 2018).

Microbes have not been as extensively studied in the context of elevational gradients, but the past decade has seen an increasing number of studies examining elevational patterns of bacteria in soil (Bryant et al. 2008; Fierer et al. 2011; Shen et al. 2013; Zhang et al. 2013; Yuan et al. 2014; Wang et al. 2015; Debnath et al. 2016; Nottingham et al. 2018; Zhao et al. 2018) and to a lesser extent, aquatic habitats (Wang et al. 2011, 2012; Zeng et al. 2016; Li et al. 2017). Similar studies have also been conducted for fungi (Bahram et al. 2012; Coince et al. 2014; Liu et al. 2015; Yang et al. 2016, 2017; Ni et al. 2018;

Qian et al. 2018; Schön et al. 2018) and to a lesser extent, protists (Soininen et al. 2011; Grossmann et al. 2016; Teittinen et al. 2016; Boenigk et al. 2018). Although some studies have found trends of decreasing richness with elevation, the pattern has been inconsistent. Of the growing number of elevational studies of microbes, only a handful have sought to directly compare diversity patterns of microbes and macro-organisms coinhabiting the same transect. The results of these studies have also been inconsistent. Bryant et al. (2008) found that both microbes (Acidobacteria) and macrobes (angiosperms) decrease in richness with elevation, though the decrease was monotonic in microbes and unimodal in macrobes. Fierer et al. (2011) showed generally decreasing richness patterns for macro-organisms (bats, birds, and trees) but no pattern for microbes (soil bacteria). Wang et al. (2011) found monotonic increase with elevation for bacteria, monotonic decrease with elevation for diatoms, and a hump-shaped pattern for macroinvertebrates. Shen et al. (2013) found that plant richness decreases with elevation while eukaryotic microbial richness has no significant pattern with elevation. Nottingham et al. (2018) found concordance between plant and soil microbe (both bacterial and fungal) diversity patterns: generally decreasing with elevation (depending on the soil horizon used for either microbial taxon).

There are some potential limitations to the interpretability of such comparative studies, however. One is the question of whether the environmental conditions microbes experience makes for an ecologically relevant comparison to plants or animals, even if they are sampled from the same site. For example, in terrestrial systems, it can be argued that the environment experienced by soil microbes is completely different from the environment experienced by free-roaming animals or even plants, as the soil microbes are much more insulated from the aboveground climate. Rather than air temperature, the immediate conditions belowground biota experience are factors such as soil horizon and pH, biotic and abiotic factors that can vary at a much finer scale than the aboveground environmental conditions (Vos et al. 2013). This may even be true for microbes on aboveground surfaces as they are still in direct contact with chemical gradients that may have much more negligible effects on macrobes. Aquatic environments are often better mixed than terrestrial systems, which might aid in addressing this issue. However, the question of the scale of interactions remains, and it might be difficult to define the bounds of a single

community or to subsample extensively enough within a single water body (e.g. a lake, river, stream) to capture a complete picture of that community with confidence. The small, specialized ecosystems contained in phytotelmata provide another way to address this issue. Phytotelmata are water-bodies held within plant tissues, including water-filled tree holes, bromeliad tanks, and the modified leaves of carnivorous pitcher plants (Kitching 2001). This class of small ecosystems has proven exceptionally useful to the field of community ecology as they are discrete communities where it is relatively straightforward to identify the participating members. Though small (typically much less than a liter in volume), phytotelmata often encompass a diverse set of taxa, which can include bacteria, archaea, algae, protozoans, rotifers, annelids, mites, crustaceans, insects, and amphibians (Kitching 2001). Thus, they serve as convenient systems where multiple taxa can be compared simultaneously. Metabarcoding makes it possible to get a much fuller view of the entire taxonomic composition of phytotelmata than would be possible in larger ecosystems.

Tropical pitcher plants (*Nepenthes*: Nepenthaceae: Caryophyllales) are a genus of carnivorous plant with modified leaves; these leaves or "pitchers" act as a pitfall trap, with slippery surfaces and a pool of fluid at the bottom for trapping and digesting prey (Juniper et al. 1989). This digestive fluid doubles as a phytotelm habitat (Adlassnig et al. 2011). With over 140 described species (Cheek and Jebb 2013), there is high interspecific diversity in pitcher form and function, but there can also be considerable intra-specific variation within a typical species (McPherson et al. 2009). For instance, most species exhibit pitcher dimorphism, producing different pitcher morphs depending on the growth phase (mature *Nepenthes* are lianas): one morph produced from the terrestrial rosette phase (known as "lower pitchers") and another distinct morph produced from the climbing phase (known as "upper pitchers"). Intraspecific color polymorphism is also common, with pitchers varying from green to heavily red-pigmented. The plants actively regulate the pH levels of their pitcher fluid (Moran et al. 2011) and individuals vary with respect to this trait both within species (Hua and Li 2005; Bittleston 2018) and between species (Moran et al. 2010; Bittleston 2018). *Nepenthes* phytotelm communities typically include bacteria, fungi, algae, protozoans, mites, aquatic insect larvae (Kitching 2001; Adlassnig et al. 2011; Bittleston 2018), and rarely

anuran tadpoles (Lim and Ng 1991; Malkmus and Dehling 2008; Das and Haas 2010). Previous metabarcoding studies of pitcher plants have revealed that these communities are specialized and distinct from the surrounding environment (Bittleston et al. 2018), which can partly be attributed to the specific conditions within the plants such as the acidic pH levels of the fluids (Kanokratana et al. 2016; Bittleston et al. 2018).

In this study, we examine phytotelm communities of *Nepenthes mindanaoensis* along a 400-1200 m a.s.l. elevational gradient on Mt. Hamiguitan, Mindano, the Philippines. We collected the entire fluid contents and used a metabarcoding approach to sequence 16S and 18S rDNA genes to capture the community-level diversity of bacteria and eukaryotes, respectively. The eukaryotes can be loosely classified into two types: the inquiline species whose larvae are typically aquatic and complete their development living and feeding inside the pitcher fluids, and the prey species that are either attracted to, or fall into the pitchers where they are digested by the plants. We identified and counted physical specimens of arthropods representing both the inquiline larvae and the partially digested prey remains in the pitcher. Our major goal was to determine whether pitcher phytotelm communities are at all structured by elevation, or whether plant-regulated factors such as pH and morphology have a greater effect that may swamp out the effect of elevation, which is largely generated by external climactic factors (i.e. temperature and precipitation).

We assessed the pitcher contents along several axes: we compared the microbes as well as the macrobes found in these common environments; we also compared the inquiline (living) versus prey (dead) components in the pitchers. We aimed to determine whether there are concordant responses to elevation between microbes and macrobes, as they both are found in the exact same microenvironment, or whether they have differential responses due to differential relative importance of elevation and plant-regulated factors to the different groups. Yet another interesting component of the pitcher system is the presence of insect prey. One could infer that the pitchers act as passive pitfall traps and can thus give an accurate picture into the patterns of diversity for the entire insect fauna in the region, again allowing for a multi-taxon comparison within the class Insecta. Alternatively, prey composition may not reflect overall

patterns of insect diversity if pitchers target non-random prey spectra (Chin et al. 2014), perhaps via visual signaling. It is interesting to compare and contrast patterns of abundance and richness for pitcher prey and inquilines, as the former are not part of the living community and thus may not be regulated by environmental conditions in the same way.

Methods

Site

Mount Hamiguitan (N 06°43'1.81", E 126°10'24.35") is part of a mountain range located in the southernmost peninsula of eastern Mindanao, the southernmost major island of the Philippines (an archipelago of ~7000 islands). Much like the broader Southeast Asian biogeographic region of Malesia, to which it belongs, the Philippines is a hotbed of biodiversity. Due to its unique geological history, the Philippines boasts impressive levels of endemism. Unlike the neighboring islands of Sundaland, the majority of Philippine islands were never connected to mainland Southeast Asia; Mindanao, the Visayas, and Luzon are true oceanic islands (Hall 2002). Sea level changes due to Pleistocene glaciation cycles led to a complex history of connection and separation among the islands. The Philippines overall has high levels of endemism, with an estimated 45% of vertebrate species, 50% of plant species, and 70% of insect species being endemic (Pelser et al. 2011). Additionally, the flora and fauna of the individual islands are often highly endemic to that particular island; Mindanao is no exception. Further still, some species are endemic to just Mt. Hamiguitan itself, including 5 species of *Nepenthes* (Gronemeyer et al. 2016; Amoroso et al. 2017). This site-level endemism on the mountain is one of the criteria that led to the designation of the Mt. Hamiguitan Range Wildlife Sanctuary as an UNESCO World Heritage Site. The site ranges from 75 to 1637 m a.s.l. and has several habitat types, including dipterocarp forest, montane forest, mossy forest, and the unusual pygmy mossy forest or "bonsai forest" at the highest elevation. Following the general pattern observed in elevational gradients on tropical mountains, there is a pronounced gradient in both temperature and precipitation along the transect. We determined the site's climactic properties using data from WorldClim (Fick and Hijmans 2017), analyzed using the 'st'

package in R (RCoreTeam 2013) (resolution of 5 minutes for max temperature and 2.5 minutes for all other variables).

Sample collection

Sampling was conducted 14-17 July 2016. We worked along a transect going from ~400 m a.s.l. up to ~1200 m a.s.l. (a ground distance of about 4.2 km, Figure 3.1). Individuals of *Nepenthes mindanaoensis* were quite abundant throughout this transect, allowing for fairly systematic sampling, however there is less representation from ~600 to 800 m a.s.l. due to more challenging terrain conditions. We collected from a total of 33 pitchers of *N. mindanaoensis*; we selected only a single healthy mature pitcher per individual plant and chose plants that were several paces apart in order to avoid sampling from multiple plants sharing a root system. We poured the entirety of the fluid contents from each pitcher directly into separate sterile 50 mL Falcon tubes, sealing the capped tubes with parafilm to safeguard against spillage or contamination prior to the addition of preservative. We added preservative within 24 hours of collection of a sample, adding 1 mL of Cetyl trimethylammonium bromide (CTAB) solution for every 1 mL of pitcher fluid (recipe following Bittleston et al. 2018). Prior to the addition of CTAB, we measured the pH of the sample with ColorPhast pH strips using a tiny portion of the sample dropped onto the strip with a sterile Pasteur pipette. We also recorded the volume of the fluid sample. We measured the length (distance from the base of the pitcher to the insertion of its lid) and width (the diameter of the widest section of the pitcher) of each pitcher *in-situ* using digital calipers. We also recorded pitcher morph (upper or lower) and color (primarily green or red-pigmented) for each sample. We estimated "canopy openness" for each pitcher by photographing the sky from pitcher's-eye-view using a digital camera (Canon PowerShot ELPH 170IS) and calculating the area not covered by vegetation using ImageJ (Rasband 2012); this metric does not only cover leaf area of canopy trees, but also all herbaceous layers shading that pitcher. We obtained GPS coordinates and elevation of each sampled pitcher using a Garmin handheld GPS unit.

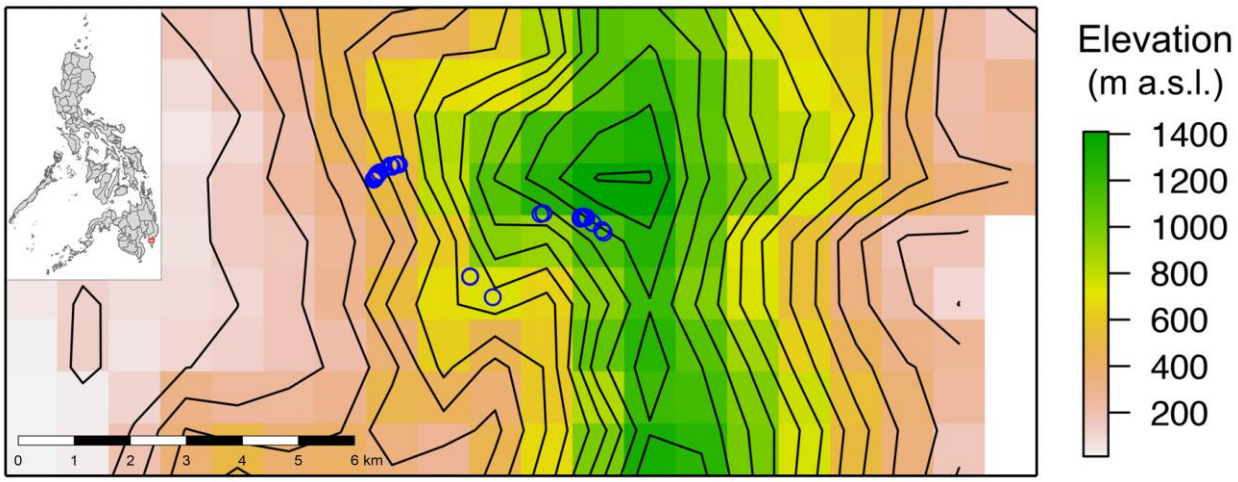

Figure 3.1. Sampling area for the study, blue circles indicate the locations of the sampled pitchers on Mount Hamiguitan. Location of the area within the Philippines is indicated by a red box in the inset.

Arthropod identification and analysis

After DNA extraction, we filtered out arthropod bodies and debris from samples using fine gauze (<0.5 mm pore size) and separated out taxa under a dissecting microscope. We photographed specimens using an automontage system and then stored them in 100% ethanol. We counted total arthropod numbers from each pitcher. For arthropods classed as insect prey, these numbers were based on a combination of head capsule counts and wing counts, in the case of wings we counted 2 morphologically similar wings as 1 individual. Arthropod counts were categorized as culicid (mosquito) larvae, ceratopogonid (midge) larvae, brachyceran (a suborder of Diptera) larvae, mites (Acari), ants (Formicidae), and other insect prey. Ants and mosquitoes were identified down to species where possible or morphospecies, using General and Alpert (2012) for ants, and Rattanarithikul (1982) for keying culicids to genus. Order-level designation of insect prey was based on wing venation patterns or overall gestalt of head capsules.

Extraction and sequencing

We used a metabarcoding approach to sequence the 16S and 18S genes in the fluid to represent the entire prokaryotic and eukaryotic communities in the pitcher fluid. Samples were sent for extraction within a

week after the end of the sampling period. DNA was extracted using a bead-beating and phenol-chloroflorm extraction method after concentrating the cells with a centrifuge. The 16S and 18S rRNA gene regions were sequenced via Illumina Amplicon sequencing. Sequences were assembled and assigned to operational taxonomic units (OTUs) using the QIIME pipeline and Harvard's Odyssey computer cluster (following Bittleston et al. 2018). We used the Greengenes and SILVA database for 16S and 18S sequences, respectively, for taxonomic classification of OTUs, with a cutoff of 97% sequence identity. In some cases, further taxonomic assignment was determined using NCBI BLAST. Neighbor-joining phylogenies were constructed for all bacterial (16S) and eukaryotic (18S) OTUs. 16S OTUs classified as chloroplast and mitochondrial sequences, and 18S OTUs classified as Embryophyte (land plant) sequences were removed from downstream analyses of community similarity to avoid inclusion of possible contaminants from host plant cells.

Statistical analysis

All analyses were conducted in R version 3.5.0. We used the function 'betadisper', together with 'permutest' in order to calculate and compare levels of betadiversity (turnover from pitcher to pitcher) between low (<700 m a.s.l.) and high (>700 m a.s.l.) elevation for bacteria and eukaryotes.
We first conducted a PCA of all recorded sample traits (elevation, physical ground distance between plants along the transect, fluid pH, canopy openness, fluid volume, pitcher length, pitcher width, pitcher morph, and pitcher color) in order to determine the axes of variation and assess correlation between traits. Based on this assessment, we determined ground distance is on an identical axis with elevation, and thus did not include ground distance as a variable in our analyses. We chose to focus on pitcher length as the sole pitcher dimension as opposed to also including pitcher width in tests. Pitcher length is positively correlated with width, but unlike pitcher length, width is also correlated with fluid volume.

We analyzed community-level similarity with the 'vegan' package, using the non-metric multidimensional scaling (NMDS) ordination method and the unweighted Unifrac distance metric. We assessed the significance of clustering by categorical variables (pitcher morph and color) using the

'adonis' function in the 'vegan' package, which performs a PERMANOVA test. For quantitative traits (elevation, pH, canopy openness, fluid volume, pitcher length) we performed Mantel tests. We calculated alpha diversity according to the Shannon Index using the function 'diversity' in the 'vegan' package.

In order to examine patterns of differential abundance of individual OTUs in relation to fluid properties we performed ANCOM, short for Analysis of Composition of Microbiomes (Mandal et al. 2015), a test designed to examine taxon abundance while accounting for the fact that metagenomics studies yield relative abundance data as opposed to absolute abundance; one advantage of this test is that it can reveal changes in differential abundance of rare OTUs that otherwise do not affect community-level properties. For ANCOM tests, we used the full set of successfully extracted samples, only included OTUs with sequence counts above 100, and corrected for multiple testing (FDR) at a significance level of 0.05. For the ANCOM tests, pH was binned into three categories: low (≤3.0), mid (3.0-4.5), and high (≥5.0). Elevation was binned into three categories: low (400-600 m a.s.l.), mid (600-900 m a.s.l.), and high (>900 m a.s.l.). Canopy openness was binned into three categories, which roughly correspond to our preliminary qualitative assessments of canopy openness in the field: closed (~0-20% open), semi-open (~20-40% open), and open (~40-100% open).

In order to assess the correlation between arthropod abundance and elevation, we conducted Poisson regressions using the 'glm' function in the 'lme4' package in R. We conducted a separate regression for the abundance of culicids (mosquito larvae), ceratopogonids (midge larvae), brachyceran larvae (dipteran inquiline families other than mosquitoes and midges), mites, ants, and other insect prey separately and applied a Bonferroni correction in assessing significance for the family-wise set of six arthropod groups. The same method was used to assess correlation between elevation and richness for the family-wise set of three arthropod groups: ant morphospecies, culicid morphospecies, and non-ant prey insect orders. For assessing the correlation between arthropod abundance/richness and the other examined factors (pH, canopy openness, fluid volume, pitcher length, pitcher morph, and pitcher color), we included all these factors together with elevation into a single generalized linear model (Poisson regression), in order to account for correlations between factors. We tested each taxon separately, and

again applied a Bonferroni correction for assessing significance of the family-wise set of six arthropod groups for abundance, and to the family-wise set of three arthropod groups for richness.

Results

Climate

In any given month, the mean temperature for the lowest elevation region of Mt. Hamiguitan is 26-27ºC compared to 21-22ºC at the highest elevation. For monthly minimum temperature, the range is from 21-22ºC at the lowest elevation down to 16-18ºC at the highest elevation. For maximum temperature, the range is 30-30.5ºC at low elevation and 27.5-29ºC at high elevation. Precipitation patterns are more variable throughout the year than temperature patterns, but conditions are wetter at high elevation than low elevation for most months; this is true from October to May where total monthly precipitation is 11-22mm at the lowest elevation and 16-26mm at the highest elevation. For the months of June to September (monsoon season), conditions tend to be wetter at low elevation: 18-36mm at the lowest elevation compared to 17-24mm at the highest elevation.

Correlation among factors

The seven continuously varying factors we recorded (elevation, ground distance between pitchers, canopy openness, pH, fluid volume, and pitcher length and width) capture considerable variation among our sampled pitchers (Figure 3.2), with 68% of the total variation explained in the PCA plot. Elevation and ground distance strongly covary. While there is a correlation between length and width, width correlates with fluid volume whereas length does not. Few upper pitchers were found at high elevation.

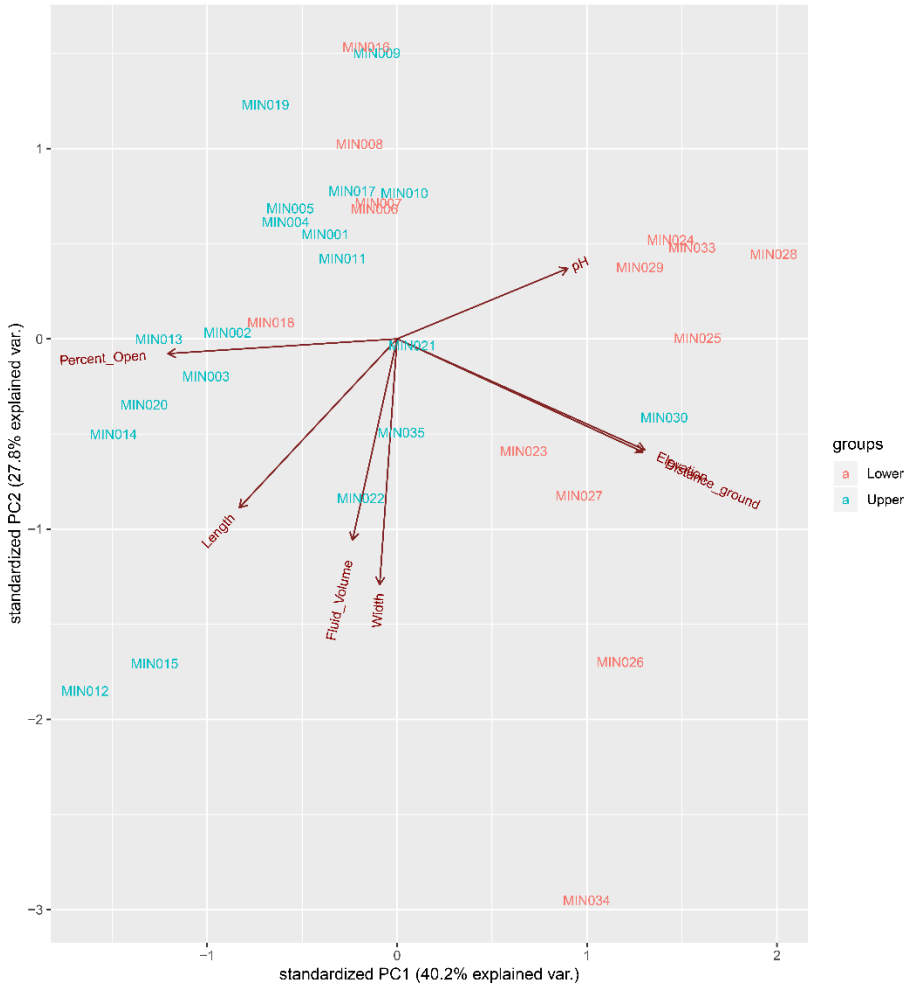

Figure 3.2. Principle coordinates analysis (PCA) plot showing axes of variation for all sampled pitchers: elevation (m a.s.l.), ground distance (m), pH level, the amount of open sky above from the perspective of the pitcher ("Percent_Open", i.e. canopy openness), the length and width of the pitcher (mm), and the volume of the fluid sample (mL). Points are labelled with the sample ID and colored by pitcher morph: "lowers" in red and "uppers" in blue.

Taxonomic composition

The bacterial taxonomic composition is dominated by Proteobacteria, particularly Rhodospirillales in Alphaproteobacteria, Burkholderiales in Betaproteobacteria, and Enterobacteriales in Gammaproteobacteria; Actinobacteria, Bacteroidetes, and Firmicutes are also common across samples, with less relative abundance (Figure 3.3). The eukaryotic communities consist of many taxa, including Metazoa, Alveolata, Stramenopiles (especially Chrysophyceae), Rhizaria, Cyptophyceae (especially *Goniomonas*), Discoba (primarily euglinids), Fungi, and Amoebozoa. Within Metazoa, Insecta is

dominant, followed by Arachnida (specifically mites, Acari). Other arthropods and nematodes appear far less frequently and with lower abundance. We observed frog eggs (from an unidentified rhacophorid) in one of the pitchers we sampled, and the 18S data was able to capture this. (Figure 3.3)

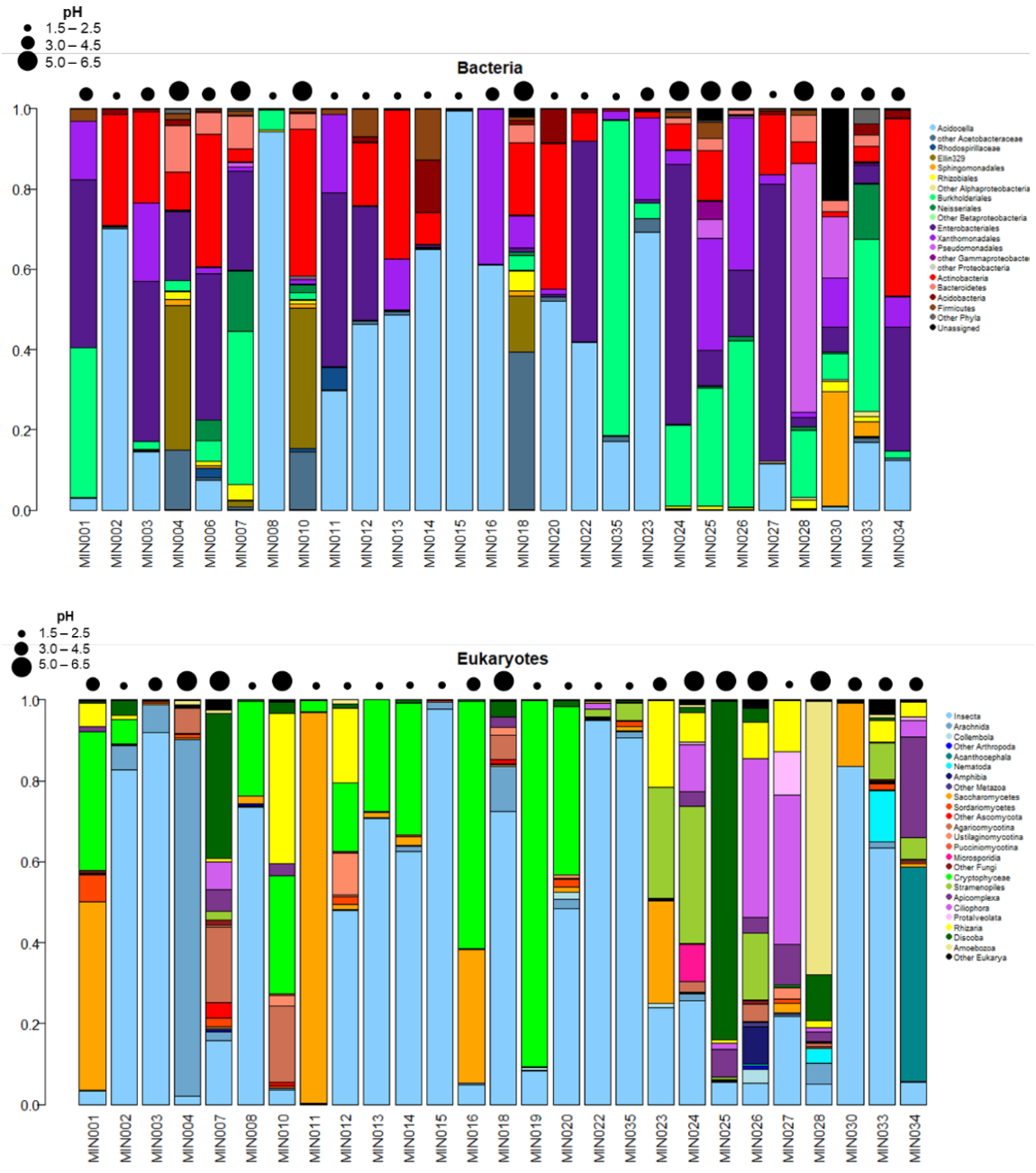

Figure 3.3. Stacked barplots showing relative abundances of bacterial taxa and eukaryotic taxa as determined by 16S and 18S metabarcoding, respectively. Samples arranged by increasing elevation. The fluid pH of the samples is indicated by proportionally sized circles.

Community similarity

For bacteria, pH is the only factor significantly structuring community similarity (Mantel r= 0.64, p= 0.001). While not significant at the Bonferroni-corrected alpha level of 0.007, bacteria appear to be somewhat structured by elevation as well, though to a lesser degree than pH (Mantel r= 0.22, p= 0.009). For eukaryotes, both elevation (Mantel r= 0.40, p= 0.002) and pH (Mantel r= 0.31, p= 0.004) have a significant effect on community similarity, with elevation having a somewhat stronger effect. However, when Metazoa is excluded from the OTU table, elevation is the only factor with a significant effect (Mantel r= 0.35, p= 0.001). In this case, pH shows a possible slight effect though not significant (Mantel r= 0.31, p= 0.015). (Table 3.1)

Table 3.1. Results of Mantel ([1]) or PERMANOVA ([2]) tests of community similarity for Bacteria, Eukaryotes, and Eukaryotes without Metazoa. The seven factors (elevation, pH, percent open, fluid volume, pitcher length, pitcher morph, pitcher color) constitute separate tests on the same ordination for each of the respective three taxa, so the set of tests for each taxon is accordingly considered a family-wise set to account for multiple testing. "Coefficient" refers either to Mantel r or PERMANOVA R^2 depending on the test. *Indicates significance at Bonferroni-corrected alpha level of 0.007.

	Bacteria		Eukaryotes		Eukaryotes without Metazoa	
Factor	**Coefficient**	**p-value**	**Coefficient**	**p-value**	**Coefficient**	**p-value**
Elevation[1]	0.220	0.009	0.400	0.002*	0.351	0.001*
pH[1]	0.640	0.001*	0.310	0.004*	0.300	0.015
Percent Open[1]	0.020	0.350	0.070	0.130	0.020	0.399
Fluid Volume[1]	0.060	0.280	0.060	0.280	0.023	0.397
Pitcher Length[1]	0.002	0.468	-0.050	0.700	-0.095	0.803
Pitcher Morph[2]	0.060	0.020	0.080	0.016	0.066	0.216
Pitcher Color[2]	0.030	0.910	0.039	0.572	0.050	0.586

Bacteria show no significant difference in between-pitcher beta diversity between low (average distance to median= 0.52) and high (average distance to median= 0.51) elevation (permutation test for homogeneity of dispersions, F= 0.27, p= 0.59). Eukaryotes show no significant difference in between-pitcher beta diversity between low (average distance to median= 0.462) and high (average distance to median= 0.457) elevation (permutation test for homogeneity of dispersions, F= 0.04, p= 0.85). Without Metazoa, eukaryotes still show no significant difference in between-pitcher beta diversity between low

(average distance to median= 0.441) and high (average distance to median= 0.447) elevation (permutation test for homogeneity of dispersions, F= 0.04, p= 0.85)

Alpha diversity

Bacterial alpha diversity does not significantly correlate with elevation (Table 3.2, glm, t value= 1.096, p= 0.287). The only factor that significantly correlates with bacterial alpha diversity is pH, with greater alpha diversity at higher pH (glm, t value= 4.259, p<0.001). Eukaryotic alpha diversity does not significantly correlate with elevation (Table 3.3, glm, t value= 0.187, p= 0.854). Neither does it significantly correlate with pH (glm, t value= 1.46, p= 0.161). The only factor that significantly correlates with eukaryotic alpha diversity is pitcher morph, with greater alpha diversity in lower pitchers (glm, t value= -3.434, p=0.003). When Metazoa is removed from the eukaryotic OTU table, the only factor that significantly correlates with alpha diversity is pH (Table 3.4, glm, t value= 2.1, p= 0.049), more closely resembling the results for bacteria Bacterial and eukaryotic alpha diversity do not clearly correlate with one another (linear model, R^2= 0.11, p= 0.09) unless metazoans are removed from the eukaryotic OTU table (linear model, R^2= 0.28, p= 0.006), in which case non-metazoan eukaryotic alpha diversity positively correlates with bacterial alpha diversity.

Table 3.2. Results of generalized linear model test of factors correlating with bacterial alpha diversity (Shannon Index). All factors included in one model to account for correlations between factors. *Indicates p-value significant at alpha level of 0.05

Alpha Diversity for Bacteria		
Factor	**t value**	**p-value**
Elevation	1.096	0.287
pH	4.259	4.24E-04*
Percent Open	0.044	0.966
Fluid Volume	-0.532	0.601
Pitcher Length	0.870	0.395
Pitcher Morph (Uppers relative to Lowers)	0.219	0.829
Pitcher Color (Red relative to Green)	-0.753	0.461

Table 3.3. Results of generalized linear model test of factors correlating with eukaryotic alpha diversity (Shannon Index). All factors included in one model to account for correlations between factors.
*Indicates p-value significant at alpha level of 0.05

Alpha Diversity for Eukaryotes		
Factor	**t value**	**p-value**
Elevation	0.187	0.854
pH	1.46	0.161
Percent Open	-0.835	0.414
Fluid Volume	1.235	0.232
Pitcher Length	1.991	0.061
Pitcher Morph (Uppers relative to Lowers)	-3.434	0.003*
Pitcher Color (Red relative to Green)	-1.356	0.191

Table 3.4. Results of generalized linear model test of factors correlating with eukaryotic alpha diversity excluding Metazoa (Shannon Index). All factors included in one model to account for correlations between factors.
*Indicates p-value significant at alpha level of 0.05

Alpha Diversity for Eukaryotes without Metazoa		
Factor	**t value**	**p-value**
Elevation	0.987	0.336
pH	2.1	0.049*
Percent Open	-0.304	0.764
Fluid Volume	1.087	0.291
Pitcher Length	1.738	0.098
Pitcher Morph (Uppers relative to Lowers)	-1.988	0.062
Pitcher Color (Red relative to Green)	-1.69	0.107

ANCOM

One bacterial OTU and one eukaryotic OTU were significantly differentially abundant by elevation. The bacterial OTU is assigned to Acetobacteriaceae (unclassified to genus). This OTU appears at low elevation, but not at mid or high. However, this trend is not representative of Acetobacteriaceae or even all unassigned Acetobacteriaceae in general, which show no differences in relative abundance across elevation categories. The eukaryotic OTU is assigned to Chrysophyceae (Stramenopiles, “uncultured

marine eukaryote E222") and is present at mid and high elevation, but not at low. This reflects the trend in Stramenopiles in general, as all Stramenopile OTUs together are much less abundant at low elevation than mid and high elevation. While no Cryptophyceae OTUs were determined to be differentially abundant by elevation through the ANCOM test, Cryptophyceae in general (all classified as the genus *Goniomonas*) tend to be more abundant at low elevation, in contrast to the Chrysophyceae (Figure 3.4).

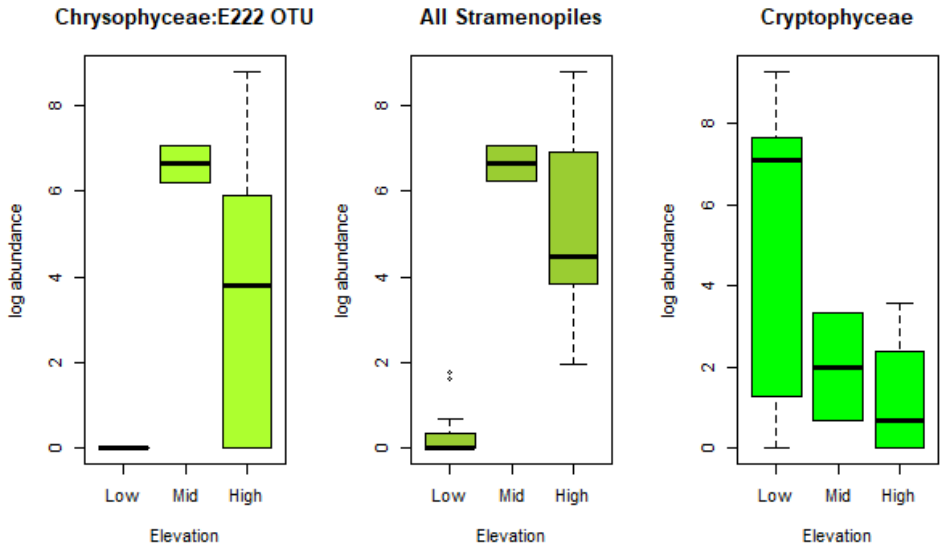

Figure 3.4. Boxplots showing results of ANCOM analysis for eukaryotes by elevation category.

Two bacterial OTUs and one eukaryotic OTU are significantly differentially abundant by pH. The bacterial OTUs include one classified in the genus *Acidisoma* and one in *Acidocella* (both Acetobacteriaceae), which both tend to increase with increasing pH. This trend reflects what can be seen for all Acetobacteriaceae in general, with lower mean log relative abundance in the high pH category. As Acetobacteriaceae are the dominant representatives of Alphaproteobacteria, the trend also holds for Alphaproteobacteria in general. This can be contrasted against Betaproteobacteria, which have higher mean log relative abundance at higher pH, or Gammaproteobacteria which exhibit no clear trend with pH (Figure 3.5).

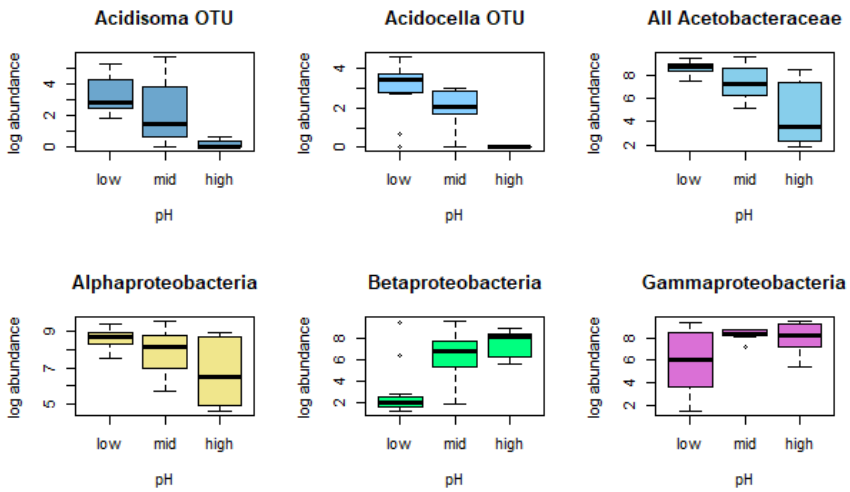

Figure 3.5. Results of ANCOM analysis for bacteria by pH category.

The eukaryotic OTU that is significantly differentially abundant by pH is classified as belonging to *Termitomyces* (Agaricomycotina: Basidiomycota); the trend of higher relative abundance at high pH compared to low and mid pH categories is generalizable to Agaricomycotina. This can be contrasted with Saccharomycetes (Ascomycota) which have lower relative abundance at high pH compared to low and mid pH categories (Figure 3.6).

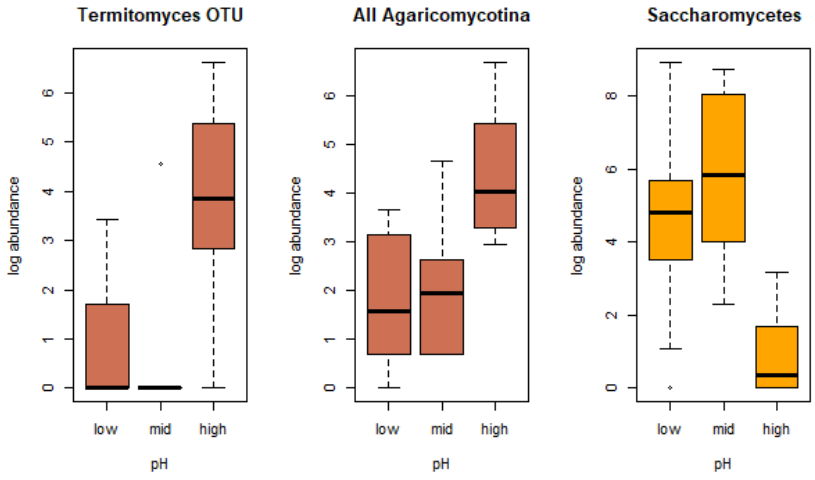

Figure 3.6. Results of ANCOM analysis for eukaryotes by pH category.

One bacterial OTU, classified as *Acidocella* (Acetobacteraceae) is significantly differentially abundant by canopy openness, having low abundance in closed canopy, higher abundance in the "semi-open" category, and higher mean abundance still in the "open" category. This trend is generalizable to Acetobacteraceae, with increasing mean log relative abundance with canopy openness.

One bacterial OTU and one eukaryotic OTU are significantly differentially abundant by pitcher morph. The bacterial OTU is assigned to the genus *Acidisoma* (Acetobacteraceae: Alphaproteobacteria) and is relatively more abundant in upper pitchers. This trend is not generalizable to genus or to family. The eukaryotic OTU is assigned to the genus *Voromonas* (Protalveolata: Alveolata) and is relatively more abundant in lower pitchers. This trend is generalizable to Alveolata as a whole.

Richness and abundance of arthropods

By examining physical specimens, we were able to determine the composition and abundance of both prey and inquiline arthropods. The insect inquiline community we found consists of dipteran larvae belonging primarily to Culicidae (mosquitoes), secondarily to Ceratopogonidae (midges), and very few individuals belonging to the Brachyceran sub-order (possibly representatives of the family Phoridae). Mites in the family Histostomatidae are known *Nepenthes* inquilines. Interestingly, while our 18S data reveals the presence of histostomatid mites in our samples, the physical mite specimens we were able to count are from the order Oribatida. This is likely because oribatids are relatively large-bodied and pigmented, while histostomatids are generally smaller and transparent, and thus more likely to be missed or lost when being filtered from the fluid. So, we are uncertain whether the oribatid mites function as inquilines or prey. The prey spectrum is dominated by ants; there were a total of 1172 ants across all samples (mean ± standard deviation= 75.4 ± 35.5) compared to a total of 93 prey items identified as other insects (mean ± standard deviation= 0.31 ± 0.76). The non-ant prey spectrum consists of 11 identified orders of insects: Blattodea (both cockroaches and termites), Coleoptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera, Mantodea, Neuroptera, Orthoptera, Psocoptera, and Thysanoptera.

Hymenoptera (primarily represented by Chalcidoidea) is the most frequently occurring order (found in 18 samples), followed by Diptera (13 samples), Coleoptera (12 samples), and Hemiptera (10 samples); the remaining orders individually occur in four or fewer pitchers. We identified 42 morphospecies (3 identifiable to named species) of ants in 5 subfamilies (Dolichoderinae, Dorylinae, Formicinae, Myrmicinae, and Ponerinae) and 15 genera, including *Aenictus*, *Brachyponera*, *Camponotus*, *Cardiocondyla, Colobopsis*, *Crematogaster*, *Echinopla*, *Iridomyrmex*, *Leptogenys*, *Monomorium*, *Nylanderia*, *Oecophylla*, *Pheidole*, *Tapinoma*, and *Tetramorium* (Supplemental Figure 3.1). All of the culicid larvae in our samples were identified as belonging to the genus *Tripteroides*, which could be separated into four separate morphospecies based on characteristics of hairs, spines, and setae (Supplemental Figure 3.2).

Morphospecies-level richness of ants, culicids, and order-level richness of non-ant insect prey did not significantly correlate with elevation (Table 3.5, $p>0.05$ in all cases).There were no significant effects of the other measured factors on richness (Table 3.6, $p>0.05$ for ants and non-ant insect prey), except that culicid morphospecies-level richness increases with decreasing pH (glm, z value= -2.89, $p=0.004$).

Table 3.5. Relationship between arthropod richness and elevation, for ant and culicid morphospecies and order-level richness for prey insects other than ants. Each is calculated with a separate Poisson regression and significance is determined at a Bonferroni-corrected alpha level of 0.0167.

Arthropod Taxon	z value	p-value
Ant Morphospecies	1.234	0.217
Culicid Morphospecies	-1.253	0.210
Prey Insect Orders	0.349	0.727

Table 3.6. Results of tests on relationships between arthropod richness and the other examined factors. All factors were included in a single generalized linear model (along with elevation) for each individual arthropod category (ant morphospecies, culicid morphospecies, and prey insect orders not including ants), using a Poisson regression. *Significant at a Bonferroni-corrected alpha level of 0.0167.

	Ant Morphospecies		Culicid Morphospecies		Prey Insect Orders	
Factor	**z value**	**p-value**	**z value**	**p-value**	**z value**	**p-value**
pH	-1.933	0.053	-2.890	0.004*	-0.878	0.380
Canopy Openness	-1.706	0.088	0.030	0.976	-1.172	0.241
Fluid Volume	0.535	0.593	2.030	0.042	-0.696	0.486
Pitcher Length	-1.784	0.074	-1.714	0.086	-0.235	0.814
Pitcher Morph (Uppers relative to Lowers)	0.116	0.908	0.401	0.688	-0.550	0.583
Pitcher Color (Red relative to Green)	0.188	0.851	0.251	0.802	-2.044	0.041

Culicids, ceratopogonids, mites, and ants all show a significant decrease in abundance with rising elevation (Table 3.7, $p<0.001$ in all cases). There is no significant trend for brachyceran larvae or non-ant insect prey, however there is a slight increasing trend for the latter (Poisson regression, z value= 2.368, p= 0.0168).

Table 3.7. Relationship between arthropod counts and elevation, using Poisson regression. The examined arthropods include 6 categories: culicids (mosquito larvae), ceratopogonids (midge larvae), brachyceran larvae (dipteran inquiline families other than mosquitoes and midges), mites, ants, and all other insects combined. *Indicates significance at Bonferroni-corrected alpha level of 0.008.

Arthropod Taxon	**z value**	**p-value**
Culicids	-12.02	2.00E-16*
Ceratopogonids	-3.45	5.61E-04*
Brachyceran Larvae	1.81	0.070
Mites	-3.586	3.35E-04*
Ants	-16.17	2.00E-16*
Other Insects	2.368	0.018

The abundance of culicids, brachyceran larvae, ants, other insect prey, and mites all decreases with increasing pH (Table 3.8; culicids, mites, ants, other insects: $p<0.001$; brachyceran larvae, p= 0.002). The abundance of culicids, ceratopogonids, and mites all significantly increase with canopy openness

(Table 3.8; $p<0.001$ for culicids and mites, $p= 0.002$ for ceratopogonids), whereas ants decrease with increasing canopy openness (Table 3.8, $p<0.001$). Culicids and mites both significantly increase with fluid volume (Table 3.8, $p<0.001$ for both). Ant abundance decreases with increasing fluid volume (Table 3.8, $p<0.001$). Ant abundance increases with increasing pitcher length (Table 3.8, $p<0.001$). Culicid and ceratopogonid abundance decrease with increasing pitcher length (Table 3.8, $p<0.001$ for both). In terms of correlation between pitcher morph and abundance, mite abundance is greater in lower pitchers while ant abundance is greater in upper pitchers (Table 3.8, $p<0.001$ for both). In terms of correlation between pitcher color and abundance, ceratopogonid abundance is greater in red pitchers while ant abundance is greater in green pitchers (Table 3.8, $p<0.001$ for both).

Table 3.8. Results of tests on relationships between arthropod abundance and the other examined factors. All factors were included in a single generalized linear model (along with elevation) for each individual arthropod category (culicids, ceratopogonids, brachyceran larvae, mites, ants, and other insects), using a Poisson regression. *Significant at a Bonferroni-corrected alpha level of 0.008.

	Culicids		Ceratopogonids		Brachyceran Larvae	
Factor	**z value**	**p-value**	**z value**	**p-value**	**z value**	**p-value**
pH	-12.329	2.00E-16*	-2.057	0.040	-3.166	0.002*
Canopy Openness	10.661	2.00E-16*	3.158	0.002*	-1.317	0.188
Fluid Volume	14.559	2.00E-16*	-1.421	0.155	0.715	0.475
Pitcher Length	-13.491	2.00E-16*	-6.373	1.86E-10*	-0.646	0.518
Pitcher Morph (Uppers relative to Lowers)	-0.356	0.722	2.533	0.011	-1.206	0.228
Pitcher Color (Red relative to Green)	-0.906	0.365	4.091	4.30E-05*	-2.015	0.044

	Mites		Ants		Other Insects	
Factor	**z value**	**p-value**	**z value**	**p-value**	**z value**	**p-value**
pH	-3.624	2.90E-04*	-4.244	2.19E-05*	-4.148	3.36E-05*
Canopy Openness	3.284	0.001*	-16.015	2.00E-16*	-1.592	0.111
Fluid Volume	3.219	0.001*	-10.619	2.00E-16*	-0.201	0.841
Pitcher Length	-2.539	0.011	6.928	4.25E-12*	-1.622	0.105
Pitcher Morph (Uppers relative to Lowers)	-6.109	1.00E-09*	3.278	0.001*	-0.883	0.377
Pitcher Color (Red relative to Green)	-0.245	0.806	-7.363	1.80E-13*	-2.608	0.009

Discussion

Elevation

We investigated bacterial and eukaryotic communities within pitchers of *Nepenthes mindanaoensis* along an elevational gradient to determine whether elevation has any influence on the structure and composition of these phytotelm communities, and whether microbes and macrobes are differentially influenced by the relative contributions of the external environment (e.g. elevation) and plant-regulated traits (e.g. pH). We found that while bacterial community similarity was much more strongly influenced by pH (a plant-regulated trait) than by elevation, elevation was the factor with the greatest effect on eukaryotic community similarity. Not only this, but removing Metazoa from the analysis of community similarity shows that eukaryotes in general are most strongly impacted by elevation in our study; the trend is not strictly driven by the macroscopic/multicellular members. On the other hand, elevation has no significant effect on the alpha diversity of bacteria or eukaryotes, or on the morpospecies-/order-level richness of arthropod groups. Our beta diversity analysis shows no difference in sample-to-sample turnover between low and high elevation samples for either bacteria or eukaryotes, meaning the effect of elevation on community similarity cannot be attributed to greater compositional heterogeneity in one elevation category over the other; community similarity is fairly stable within elevations though different between elevations. A caveat to all our analyses of elevation is that ground distance between pitchers perfectly correlates with elevation, so it is not statistically possible to disentangle the effects of elevation from potential effects of spatial distance. However, our results reveal biologically meaningful patterns that are consistent with effects of elevation found in other studies.

A particularly striking change in the eukaryotic taxonomic composition along our elevational transect is that Cryptophyceae seems to be replaced by Stramenopiles (primarily Chrysophyceae) at high elevation (Figure 3.4). Our ANCOM results support this observation; the sole eukaryotic OTU that is significantly differentially abundant by elevation is one assigned to the E222 clade within Chrysophyceae, and its relative abundance is negligible at low elevation. Stramenopiles in general are much less abundant at low

elevation here compared to mid and high elevations. This can be contrasted with Cryptophyceae which show the opposite pattern. This result for the distribution of these two algal taxa is notable; studies of algal communities in high-altitude lakes, using either molecular or morphological methods, found Chrysophyceae to be the most important members of those communities and Cryptophyceae were relatively less important both in terms of richness and abundance (Tolotti et al. 2003, 2006; Grossmann et al. 2016). This is in contrast to lower altitude lakes where Cryptophyceae are common and abundant (Debroas et al. 2017)—in fact a large-scale analysis of freshwater protists suggests that Cryptophyceae can be considered an indicator taxon for the biogeographical patterns of eukaryotic microbes in general (Boenigk et al. 2018). That phytotelmata such as pitchers can reflect the macroecology of freshwater lakes is intriguing. Tolotti et al. (2003) show that Chrysophyceae thrive in high altitude lakes because they are well-adapted to the oligotrophic conditions common to such lakes. In our study, the abundance of ants, which are the main insect prey, significantly decreases with elevation—which is a well-established macroecological phenomenon (Hölldobler and Wilson 1990). It is thus quite possible that high elevation pitchers are less nutrient-rich than low elevation ones.

Culicids, ceratopogonids, mites, and ants all show a significant decrease in abundance with rising elevation. As with insects in general, aquatic insects like dipteran larvae have been shown to decrease with elevation, partly due to decreasing temperature (Madsen et al. 2015). In accordance with the general macroclimatic trend of decreasing temperature with elevation, WorldClim data show that the peak of Mt. Hamiguitan is much cooler than lower elevations, about 5ºC lower in monthly mean and minimum temperatures.

Brachyceran larvae were the only inquilines in our dataset that did not significantly decrease with elevation; the lack of a significant trend might be explained by their rarity, as they were found in only five pitchers. Non-ant prey abundance also lacked the elevational decrease in abundance, but more surprisingly, showed a slight trend of increase with elevation (Poisson regression, z value= 2.368, p= 0.0168). Rather than acting as neutral pitfall traps that simply reflect the broader patterns of the insect fauna of their surroundings, pitchers can target a particular prey spectrum. This is true of specialist

species like the termite-trapping *N. albomarginata* (Moran et al. 2010). Ants are often the most abundant arthropods in terrestrial ecosystems (Hölldobler and Wilson 1990), as such many *Nepenthes* species count on ants as their main source of prey (Juniper et al. 1989). Whether a diet of ants is achieved solely by a generalist strategy, or whether these species have evolved to specifically target them has not yet been determined (Chin et al. 2014). For species like *N. mindanaoensis* that rely mostly on ants, growing at high elevations may necessitate a shift to alternative prey as the abundance of ants can drop off much more steeply than other insects (Hölldobler and Wilson 1990); thus, it would certainly be advantageous for pitchers to increase their ability to capture non-ant prey at higher elevations.

Canopy openness

Canopy openness (which includes shading not only by canopy trees, but also understory vegetation covering pitchers as well) decreases with increasing elevation. This is interesting because canopy trees decrease in height with elevation, ultimately reaching "bonsai" stature in the pygmy mossy forest found at 1200 m a.s.l. Despite this, *N. mindanaoensis* pitchers grew in more shaded light microenvironments at high elevation, including under dense herbaceous foliage and within nooks and crevices in the ground with overhanging projections of earth. We expected that photoautotrophic microbes such as cyanobacteria and algal eukaryotes would be influenced by canopy openness, but this is not what we found in our ANCOM results. Instead, we found that the only OTU significantly influenced by canopy openness was classified as *Acidocella* (Acetobacteraceae: Rhodospirillales), which was more abundant under more open canopy. We found this trend to be generalizable for Acetobacteraceae as a whole. This trend is sensible however, as the family contains some known photoheterotrophs (Komagata et al. 2014). Interestingly, two of the major "algal" taxa in our dataset, Chrysophyceae and Cryptophyceae (Stramenopiles) may largely consist of members that are non-photosynthetic or possibly even heterotrophic (McFadden et al. 1994; Grujcic et al. 2018). We found that the abundance of culicids, ceratopogonids, and mites all significantly increase with canopy openness, and ants decrease with increasing canopy openness. This might reflect the light environment preferences of these arthropods. The result for ants makes sense

considering that many of the ant genera we found are at least partly arboreal (General and Alpert 2012), such as the most abundant genus *Crematogaster* (total count= 420 workers classified into six morphospecies); this is also supported by the significantly higher abundance of ants in the upper pitcher morph.

Fluid pH

The plant-regulated trait with the strongest effects in our study was pH. *Nepenthes mindanaoensis* exhibits a wide pH range in our study, ranging from 6.5 at highest down to the very acidic pH 1.5. This matches previous observations that note that the *Nepenthes* species which achieve very low pH levels also tend to have greater variance in pH (Bittleston 2018). Fluid pH is the only measured factor significantly structuring bacterial community similarity, and it also has a role in structuring eukaryotic community similarity. Interestingly, however, elevation is still a more significant factor for community similarity of microbial (non-Metazoan) eukaryotes. The pH seems to be the primary factor responsible for the alpha diversity of microbes in general, though, as both bacterial and non-Metazoan eukaryotic alpha diversity significantly increases with rising pH, albeit only marginally significant for the latter. The strong response of microbes, especially bacteria, to pH fits expectation given the well-known narrow pH requirements of bacteria (Rabotnova 1963). Higher pH levels may be less harsh, thus allowing for greater diversity.

In our study, Acetobacteraceae dominates at low pH levels. This observation is supported by our ANCOM test results, which determined that an *Acidocella* OTU and an *Acidisoma* OTU are significantly differentially abundant across pH categories, increasing in relative abundance as pH becomes more acidic. Being characteristically acidophilic (Komagata et al. 2014), the greater abundance of Acetobacteraceae at low pH is sensible. Interestingly Acetobacteraceae, especially the genus *Acidocella*, has been found to be regularly associated with *Nepenthes* pitchers in the wild (Sickel et al. 2016) and even in an indoor greenhouse environment (Gilbert et al. in prep). Pitchers could plausibly maintain low pH levels in order to favor the growth of key symbionts, whereas high pH conditions would also allow less specialized bacteria to establish. The less frequent Alphaproteobacteria appear in the least acidic

pitchers, for example the Ellin329 candidate order (Harbison et al. 2016). Also, the only pitchers where *Acidocella* is not the dominant representative of Acetobacteraceae are pitchers with high pH (Figure 3.3).

Additionally, our ANCOM results show that an OTU classified as *Termitomyces* (Agaricomycotina: Basidiomycota) is significantly more abundant at the high pH category as opposed to the low and mid categories. While further BLAST results show that this OTU's exact generic placement is equivocal (note: termites were rare in our samples, only appearing in one pitcher), it can be confidently placed within Agaricomycotina. Agaricomycotina in general appear more frequently in the high pH samples. In contrast, we found that Saccharomycetes (Ascomycota) in general appear more frequently in the low pH samples. Saccharomycete yeasts appear to be common in pitchers in general (Bittleston 2018). It might be that Saccharomycetes and Agaricomycotina have different functions in pitchers.

Interestingly the abundances of all arthropod counts significantly increase with increasing acidity, except ceratopogonids for which the trend is still negative but not statistically significant. As prey capture induces fluid acidification (Lloyd and others 1942; Saganová et al. 2018), this might explain the greater abundance of ants, other insect prey, and possibly the oribatid mites. The dipteran inquilines are likely specially adapted to the pitcher phytotelm environment, as many pitcher-associated larvae are specialized (Bittleston 2018), especially *Tripteroides* where all described species found in *Nepenthes* are obligate pitcher-breeders (Belkin 1955). However, it is still somewhat surprising that not only the abundance, but also the richness of culicids increases with decreasing pH. This indicates that all four morphospecies are equally tolerant of acidic conditions, as opposed to the occurrence of multiple species with different physiological tolerances that only coexist in more moderate conditions. This contrasts with the case for bacterial alpha diversity.

Fluid volume and pitcher length

Phytotelmata act as aquatic "islands" within a terrestrial matrix, as such, one might expect the communities to follow the predictions of island biogeography theory (IBT) (MacArthur and Wilson 1963). For example, IBT predicts that larger fluid volume (e.g. greater habitat area) would lead to greater

species richness or abundance. Past studies have indeed found such an effect of fluid volume in phytotelmata, including for microbes (Bell et al. 2005). However, volume in our study had no influence on the community similarity of either bacteria or eukaryotes (with or without Metazoa), nor on the morphospecies-level richness of ants and culicids or the order-level richness of insect prey. However, the abundance of both culicids and mites significantly increase with increasing volume, following the expectation based on habitat size. However, ant abundance shows the opposite pattern, decreasing with increasing volume. This is difficult to explain other than considering that ants are prey, not living inquilines, and thus fluid volume does not represent habitat size in their case. On the other hand, ant abundance increases with increasing pitcher length, a proxy for pitcher size, which shows that bigger pitchers can hold more prey. The abundance of culicid and ceratopogonid larvae both decrease with increasing pitcher length. One possible hypothesis for how pitcher length may affect influence inquilines relates to length as a proxy for depth. Given two pitchers of equal volume, the longer pitcher will have a deeper fluid column; one effect of this is that oxygen will diffuse more easily in the shallower container. If the larvae rely heavily on cutaneous respiration (Macfie 1917) and if pitchers do not produce enough oxygen at the bottom to counteract this, then ovipositing females might prefer shallower containers to reduce the risk of hypoxia.

Pitcher morph and color

Pitcher morph and color are two morphological factors that may influence insect inquilines and prey through signaling. In the case of morph, this can encompass both visual and olfactory modalities depending on species (Moran 1996), but also involves the microhabitat differences between the morphs. In the case of color, red pigmentation may be involved in visual signaling of prey (Schaefer and Ruxton 2008) and inquilines, although the possible signaling roles of red pigmentation in *Nepenthes* are not well understood (Gilbert et al. 2018). In this study, we found that ceratopogonid abundance is greater in red pitchers and ant abundance is greater in green pitchers, which contradicts hypotheses of red coloration as a prey attractant. Perhaps this is informative of the visual ecology of these inquiline and prey taxa, but

future experimental work is needed. Regarding pitcher morph, mite abundance is greater in lower pitchers and ant abundance is greater in upper pitchers. These results are sensible as the mites counted from our samples are largely oribatids, which are typically soil- and leaf litter-dwelling, thus occurring in closer proximity to the typically terrestrial lower pitchers. Many of the ants, on the other hand, are at least partly arboreal and thus in closer proximity to the twining upper pitchers. Interestingly, pitcher morph is the one factor that correlates significantly with overall eukaryotic alpha diversity, with greater alpha diversity in lower pitchers. This shows that the ecological differences between pitcher morphs can have community-level effects (Clarke 1997; Rembold et al. 2010). The one eukaryotic OTU with significant differential abundant by pitcher morph via the ANCOM test is one assigned to *Voromonas* (Protalveolata: Alveolata), which is relatively more abundant in lower pitchers, a trend we found to be generalizable to Alveolata as a whole. Alveolata in our dataset includes gregarines, which are obligate parasites of arthropods (Chen 1999); this has potential implications for the inquilines (Baker et al. 2016).

Conclusions

For over a century, ecologists have studied elevational patterns of biodiversity in plants and animals, yet only in the past decade have microbes received similar treatment. The extent to which microbes follow the macroecological patterns of macroscopic organisms has been an unsettled issue. In our study, cell type was a more important distinction than the microbe-macrobe divide. We found evidence that bacteria and eukaryotes living in the same specialized microhabitat respond differently to increasing elevation (elevation being a proxy for external environmental factors). Whereas eukaryotes are most strongly influenced by elevation, bacteria are most strongly influenced by their immediate chemical environment (i.e. pH). It is interesting to contemplate the biological differences between the two domains that could lead to such stark differences. Perhaps the small size of bacteria, typically at least an order of magnitude smaller than the smallest eukaryotic microbes, is at play (Soininen et al. 2011), or perhaps their faster generation times are key. Another fundamental difference is that the diversity of metabolic strategies in bacteria far outstrip those of eukaryotes; the majority of the pathways that bacteria implement are ones

that do not even exist for eukaryotes (e.g., photoheterotrophy). Likely a constellation of factors is involved in the bacteria-eukaryote divide. In any case, a major contribution of our study is the demonstration that phytotelmata can be useful systems for elevational gradient studies. Through metabarcoding, we were able to compare patterns of diversity in a large number of taxa simultaneously. Macroecological trends are best generalizable when a variety of trophic levels are included, so there is great value in attempting to compare *all* eukaryotes within a community to *all* bacteria within the same system—this is relatively feasible to achieve in phytotelmata yet fairly intractable for larger communities. Another major advantage of phytotelmata is that they are readily amenable to experimental manipulation, as noted by Kitching (2001).

To our knowledge, very few elevational gradient studies have utilized phytotelmata thus far (Sota and Mogi 1996; Richardson et al. 2000). Littlefair et al. (2019) recently applied metabarcoding to study prey in *Sarracenia* along an altitudinal gradient. Aquatic microbial systems have been understudied in macroecology relative to soil microbes (Wang et al. 2017), and protists are also understudied relative to bacteria and fungi (Boenigk et al. 2018). So, our work contributes to advancing knowledge of microbial macroecology in multiple ways, which has led to novel insights. For instance, we have learned that high elevation *Nepenthes* algal communities are analogous to those of alpine lakes. Additionally, we have identified patterns of the living inquiline phytotelm community and how they interact with the external environment and the plant, and we can compare this with the prey composition as well. Thus, our study provides insight into a wide range of taxa within a small aquatic ecosystem, both living members and prey, and the various effects of external conditions and plant-regulated traits on the system.

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Chapter 3 References:

Adlassnig W, Peroutka M, Lendl T. **2011**. Traps of carnivorous pitcher plants as a habitat: composition of the fluid, biodiversity and mutualistic activities. *Annals of Botany* **107**: 181–94.

Amoroso VB, Lagunday NE, Coritico FP, Colong RD. **2017**. *Nepenthes alfredoi* (Caryophyllales, Nepenthaceae), a new species of pitcher plant from Mindanao, Philippines. *Philippine Journal of Systematic Biology* **11**: 14–19.

Bahram M, Põlme S, Kõljalg U, Zarre S, Tedersoo L. **2012**. Regional and local patterns of ectomycorrhizal fungal diversity and community structure along an altitudinal gradient in the Hyrcanian forests of northern Iran. *New Phytologist* **193**: 465–473.

Baker CC, Bittleston LS, Sanders JG, Pierce NE. **2016**. Dissecting host-associated communities with DNA barcodes. *Phil. Trans. R. Soc. B* **371**: 20150328.

Belkin JN. **1955**. The *Tripteroides caledonica* complex of mosquitoes in Melanesia (Diptera: Culicidae).

Bell T, Ager D, Song J-I, Newman JA, Thompson IP, Lilley AK, van der Gast CJ. **2005**. Larger islands house more bacterial taxa. *Science (New York, N.Y.)* **308**: 1884.

Bittleston LS. **2018**. Carnivorous Plants: Physiology, Ecology, and Evolution.Ellison, Aaron M and Adamec, Lubom'\ir ed. Oxford University Press, 314–332.

Bittleston LS, Wolock CJ, Yahya BE, Chan XY, Chan KG, Pierce NE, Pringle A. **2018**. Convergence between the microcosms of Southeast Asian and North American pitcher plants. *eLife* **7**.

Boenigk J, Wodniok S, Bock C, Beisser D, Hempel C, Grossmann L, Lange A, Jensen M. **2018**. Geographic distance and mountain ranges structure freshwater protist communities on a European scale. *Metabarcoding and Metagenomics* **2**: e21519.

Bryant JA, Lamanna C, Morlon H, Kerkhoff AJ, Enquist BJ, Green JL. **2008**. Colloquium paper: microbes on mountainsides: contrasting elevational patterns of bacterial and plant diversity. *Proceedings of the National Academy of Sciences of the United States of America* **105 Suppl 1**: 11505–11.

Cheek M, Jebb M. **2013**. Recircumscription of the *Nepenthes alata* group (Caryophyllales: Nepenthaceae), in the Philippines, with four new species. *European Journal of Taxonomy*.

Chen W. **1999**. The life cycle of *Ascogregarina taiwanensis* (Apicomplexa: Lecudinidae). *Parasitology Today* **15**: 153–156.

Chin L, Chung AY, Clarke C. **2014**. Interspecific variation in prey capture behavior by co-occurring Nepenthes pitcher plants: evidence for resource partitioning or sampling-scheme artifacts? *Plant signaling \& behavior* **9**: e27930.

Clarke C. **1997**. The effects of pitcher dimorphism on the metazoan community of the carnivorous plant *Nepenthes bicalcarata* Hook. f. *The Malayan Nature Journal (Malaysia)*.

Coince A, Cordier T, Lengellé J, Defossez E, Vacher C, Robin C, Buée M, Marçais B. **2014**. Leaf and root-associated fungal assemblages do not follow similar elevational diversity patterns. *PloS one* **9**:

e100668.

Das I, Haas A. **2010**. New species of *Microhyla* from Sarawak: Old World's smallest frogs crawl out of miniature pitcher plants on Borneo (Amphibia: Anura: Microhylidae). *Zootaxa* **2571**: 37–52.

Debnath R, Yadav A, Gupta VK, Singh BP, Handique PJ, Saikia R. **2016**. Rhizospheric bacterial community of endemic *Rhododendron arboreum Sm. Ssp. delavayi* along eastern Himalayan slope in Tawang. *Frontiers in plant science* **7**: 1345.

Debroas D, Domaizon I, Humbert J-F, Jardillier L, Lepère C, Oudart A, Ta"\ib N. **2017**. Overview of freshwater microbial eukaryotes diversity: a first analysis of publicly available metabarcoding data. *FEMS microbiology ecology* **93**: fix023.

Fick SE, Hijmans RJ. **2017**. WorldClim 2: new 1-km spatial resolution climate surfaces for global land areas. *International journal of climatology* **37**: 4302–4315.

Fierer N, McCain CM, Meir P, Zimmermann M, Rapp JM, Silman MR, Knight R. **2011**. Microbes do not follow the elevational diversity patterns of plants and animals. *Ecology* **92**: 797–804.

General DM, Alpert GD. **2012**. A synoptic review of the ant genera (Hymenoptera, Formicidae) of the Philippines. *ZooKeys*: 1–111.

Gilbert KJ, Nitta JH, Talavera G, Pierce NE. **2018**. Keeping an eye on coloration: ecological correlates of the evolution of pitcher traits in the genus Nepenthes (Caryophyllales). *Biological Journal of the Linnean Society* **123**: 321–327.

Gronemeyer T, Suarez W, Nuytemans H, Calaramo M, Wistuba A, Mey F, Amoroso V. **2016**. Two New *Nepenthes* species from the Philippines and an emended description of *Nepenthes ramos*. *Plants* **5**: 23.

Grossmann L, Jensen M, Pandey RV, Jost S, Bass D, Psenner R, Boenigk J. **2016**. Molecular investigation of protistan diversity along an elevation transect of alpine lakes. *Aquatic Microbial Ecology* **78**: 25–37.

Grujcic V, Nuy JK, Salcher MM, Shabarova T, Kasalicky V, Boenigk J, Jensen M, Simek K. **2018**. Cryptophyta as major bacterivores in freshwater summer plankton. *The ISME journal*: 1.

Hall R. **2002**. Cenozoic geological and plate tectonic evolution of SE Asia and the SW Pacific: computer-based reconstructions, model and animations. *Journal of Asian Earth Sciences* **20**: 353–431.

Harbison AB, Carson MA, Lamit LJ, Basiliko N, Bräuer SL. **2016**. A novel isolate and widespread abundance of the candidate alphaproteobacterial order (Ellin 329), in southern Appalachian peatlands. *FEMS microbiology letters* **363**.

Hua Y, Li H. **2005**. Food web and fluid in pitchers of *Nepenthes mirabilis* in Zhuhai, China. *Acta botanica gallica* **152**: 165–175.

Hölldobler B, Wilson EO. **1990**. *The ants*. Harvard University Press.

Juniper BE, Robins RJ, Joel DM. **1989**. *The carnivorous plants*. London, etc.: Academic Press.

Kanokratana P, Mhuanthong W, Laothanachareon T, Tangphatsornruang S, Eurwilaichitr L, Kruetreepradit T, Mayes S, Champreda V. **2016**. Comparative Study of Bacterial Communities in *Nepenthes*. *Microbial ecology* **72**: 381–393.

Kitching R. **2001**. Food webs in phytotelmata:"bottom-up" and "top-down" explanations for community structure. *Annual Review of Entomology* **46**: 729–760.

Komagata K, Iino T, Yamada Y. **2014**. The family acetobacteraceae. *The Prokaryotes: Alphaproteobacteria and Betaproteobacteria*: 3–78.

Li H, Zeng J, Ren L, Wang J, Xing P, Wu QL. **2017**. Contrasting patterns of diversity of abundant and rare bacterioplankton in freshwater lakes along an elevation gradient. *Limnology and Oceanography* **62**: 1570–1585.

Lim K, Ng P. **1991**. Nepenthiphilous larvae and breeding habits of the sticky frog, *Kalophrynus pleurostigma* Tschudi (Amphibia: Microhylidae). *Raffles Bulletin of Zoology* **39**: 209–214.

Littlefair JE, Zander A, de Sena Costa C, Clare EL. **2019**. DNA metabarcoding reveals changes in the contents of carnivorous plants along an elevation gradient. *Molecular ecology* **28**: 281–292.

Liu L, Hart MM, Zhang J, Cai X, Gai J, Christie P, Li X, Klironomos JN. **2015**. Altitudinal distribution patterns of AM fungal assemblages in a Tibetan alpine grassland. *FEMS microbiology ecology* **91**.

Lloyd F, others. **1942**. The carnivorous plants. *The carnivorous plants.*

MacArthur RH, Wilson EO. **1963**. An equilibrium theory of insular zoogeography. *Evolution* **17**: 373–387.

Macfie JS. **1917**. The limitations of kerosene as a larvicide, with some observations on the cutaneous respiration of mosquito larvae. *Bulletin of Entomological Research* **7**: 277–295.

Madsen PB, Morabowen A, Andino P, Espinosa R, CAUVY-FRAUNIÉ S, Dangles O, Jacobsen D. **2015**. Altitudinal distribution limits of aquatic macroinvertebrates: an experimental test in a tropical alpine stream. *Ecological entomology* **40**: 629–638.

Malkmus R, Dehling JM. **2008**. Anuran amphibians of Borneo as phytotelm-breeders-a synopsis. *Herpetozoa* **20**: 165–172.

Mandal S, Van Treuren W, White RA, Eggesbø M, Knight R, Peddada SD. **2015**. Analysis of composition of microbiomes: a novel method for studying microbial composition. *Microbial ecology in health and disease* **26**: 27663.

McFadden G, Gilson P, Hill D. **1994**. *Goniomonas*: rRNA sequences indicate that this phagotrophic flagellate is a close relative of the host component of cryptomonads. *European Journal of Phycology* **29**: 29–32.

McPherson S, Robinson A, Fleischmann A. **2009**. *Pitcher plants of the Old World*. Redfern Natural History Productions Dorset.

Moran JA. **1996**. Pitcher dimorphism, prey composition and the mechanisms of prey attraction in the pitcher plant *Nepenthes rafflesiana* in Borneo. *Journal of Ecology*: 515–525.

Moran JA, Hawkins BJ, Gowen BE, Robbins SL. **2010**. Ion fluxes across the pitcher walls of three Bornean Nepenthes pitcher plant species: flux rates and gland distribution patterns reflect nitrogen sequestration strategies. *Journal of Experimental Botany*: erq004.

Ni Y, Yang T, Zhang K, Shen C, Chu H. **2018**. Fungal communities along a small-scale elevational gradient in an alpine tundra are determined by soil carbon nitrogen ratios. *Frontiers in microbiology* **9**: 1815.

Nottingham AT, Fierer N, Turner BL, Whitaker J, Ostle NJ, McNamara NP, Bardgett RD, Leff JW, Salinas N, Silman MR, others. **2018**. Microbes follow Humboldt: temperature drives plant and soil microbial diversity patterns from the Amazon to the Andes. *Ecology* **99**: 2455–2466.

O'Malley MA. **2008**. "Everything is everywhere: but the environment selects": ubiquitous distribution and ecological determinism in microbial biogeography. *Studies in History and Philosophy of Science Part C: Studies in History and Philosophy of Biological and Biomedical Sciences* **39**: 314–325.

Pelser P, Barcelona J, Nickrent D. **2011**. Co's Digital Flora of the Philippines. *URL: http://www. philippineplants. org. Accessed: March 2019*.

Qian X, Chen L, Guo X, He D, Shi M, Zhang D. **2018**. Shifts in community composition and co-occurrence patterns of phyllosphere fungi inhabiting *Mussaenda shikokiana* along an elevation gradient. *PeerJ* **6**: e5767.

Rabotnova IL. **1963**. *The importance of physical-chemical factors (pH and rH2) for the life activity of microorganisms*.

Rahbek C. **1995**. The Elevational Gradient of Species Richness: A Uniform Pattern? *Ecography* **18**: 200–205.

Rasband W. **2012**. ImageJ: Image processing and analysis in Java. *Astrophysics Source Code Library*.

Rattanarithikul R. **1982**. A guide to the genera of mosquitoes (Diptera: Culicidae) of Thailand with illustrated keys, biological notes and preservation and mounting techniques. *Mosq Syst* **14**: 139–208.

RCoreTeam. **2013**. R: A Language and Environment for Statistical Computing. **55**: 275–286.

Rembold K, Fischer E, Wetzel MA, Barthlott W. **2010**. Prey composition of the pitcher plant *Nepenthes madagascariensis*. *Journal of Tropical Ecology* **26**: 365–372.

Richardson BA, Richardson M, Scatena F, McDowell WH. **2000**. Effects of nutrient availability and other elevational changes on bromeliad populations and their invertebrate communities in a humid tropical forest in Puerto Rico. *Journal of Tropical Ecology* **16**: 167–188.

Saganová M, Bokor B, Stolárik T, Pavlovič A. **2018**. Regulation of enzyme activities in carnivorous pitcher plants of the genus *Nepenthes*. *Planta*: 1–14.

Schaefer HM, Ruxton GD. **2008**. Fatal attraction: carnivorous plants roll out the red carpet to lure insects. *Biology letters* **4**: 153–5.

Schön ME, Nieselt K, Garnica S. **2018**. Belowground fungal community diversity and composition associated with Norway spruce along an altitudinal gradient. *PloS one* **13**: e0208493.

Shen C, Xiong J, Zhang H, Feng Y, Lin X, Li X, Liang W, Chu H. **2013**. Soil pH drives the spatial distribution of bacterial communities along elevation on Changbai Mountain. *Soil Biology and Biochemistry* **57**: 204–211.

Sickel W, Grafe TU, Meuche I, Steffan-Dewenter I, Keller A. **2016**. Bacterial diversity and community structure in two Bornean *Nepenthes* species with differences in nitrogen acquisition strategies. *Microbial ecology* **71**: 938.

Soininen J, Korhonen JJ, Karhu J, Vetterli A. **2011**. Disentangling the spatial patterns in community composition of prokaryotic and eukaryotic lake plankton. *Limnology and Oceanography* **56**: 508–520.

Sota T, Mogi M. **1996**. Species richness and altitudinal variation in the aquatic metazoan community in bamboo phytotelmata from North Sulawesi. *Researches on population ecology* **38**: 275–281.

Teittinen A, Kallajoki L, Meier S, Stigzelius T, Soininen J. **2016**. The roles of elevation and local environmental factors as drivers of diatom diversity in subarctic streams. *Freshwater Biology* **61**: 1509–1521.

Tolotti M, Manca M, Angeli N, Morabito G, Thaler B, Rott E, Stuchlik E. **2006**. Phytoplankton and zooplankton associations in a set of Alpine high altitude lakes: geographic distribution and ecology. *Hydrobiologia* **562**: 99–122.

Tolotti M, Thies H, Cantonati M, Hansen CM, Thaler B. **2003**. Flagellate algae (Chrysophyceae, Dinophyceae, Cryptophyceae) in 48 high mountain lakes of the Northern and Southern slope of the Eastern Alps: biodiversity, taxa distribution and their driving variables. *Hydrobiologia* **502**: 331–348.

Vos M, Wolf AB, Jennings SJ, Kowalchuk GA. **2013**. Micro-scale determinants of bacterial diversity in soil. *FEMS microbiology reviews* **37**: 936–954.

Wang J-T, Cao P, Hu H-W, Li J, Han L-L, Zhang L-M, Zheng Y-M, He J-Z. **2015**. Altitudinal distribution patterns of soil bacterial and archaeal communities along Mt. Shegyla on the Tibetan Plateau. *Microbial ecology* **69**: 135–145.

Wang J, Meier S, Soininen J, Casamayor EO, Pan F, Tang X, Yang X, Zhang Y, Wu Q, Zhou J, others. **2017**. Regional and global elevational patterns of microbial species richness and evenness. *Ecography* **40**: 393–402.

Wang J, Soininen J, Zhang Y, Wang B, Yang X, Shen J. **2011**. Contrasting patterns in elevational diversity between microorganisms and macroorganisms. *Journal of Biogeography* **38**: 595–603.

Wang J, Soininen J, Zhang Y, Wang B, Yang X, Shen J. **2012**. Patterns of elevational beta diversity in micro-and macroorganisms. *Global Ecology and Biogeography* **21**: 743–750.

Yang H, Lü G, Jiang H, Shi D, Liu Z. **2017**. Diversity and distribution of soil micro-fungi along an elevation gradient on the north slope of Changbai Mountain. *Journal of Forestry Research* **28**: 831–839.

Yang T, Weisenhorn P, Gilbert JA, Ni Y, Sun R, Shi Y, Chu H. **2016**. Carbon constrains fungal endophyte assemblages along the timberline. *Environmental microbiology* **18**: 2455–2469.

Yuan Y, Si G, Wang J, Luo T, Zhang G. **2014**. Bacterial community in alpine grasslands along an altitudinal gradient on the Tibetan Plateau. *FEMS microbiology ecology* **87**: 121–132.

Zeng J, Zhao D, Li H, Huang R, Wang J, Wu QL. **2016**. A monotonically declining elevational pattern of bacterial diversity in freshwater lake sediments. *Environmental microbiology* **18**: 5175–5186.

Zhang B, Liang C, He H, Zhang X. **2013**. Variations in soil microbial communities and residues along an altitude gradient on the northern slope of Changbai Mountain, China. *PLoS One* **8**: e66184.

Zhao Y, Song C, Dong H, Luo Y, Wei Y, Gao J, Wu Q, Huang Y, An L, Sheng H. **2018**. Community structure and distribution of culturable bacteria in soil along an altitudinal gradient of Tianshan Mountains, China. *Biotechnology \& Biotechnological Equipment* **32**: 397–407.

Observations on the regulation of dissolved oxygen by *Nepenthes* pitchers

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Introduction:

Most phytotelmata are ephemeral resources prone to desiccation and hypoxia. Large communities of heterotrophic organisms living within water-filled tree holes or bromeliad tanks, for example, will eventually deplete the oxygen dissolved in the phytotelm unless photosynthetic algae are also present. The host plant tissues lack the ability to maintain the dissolved oxygen (DO) of their phytotelm. Pitcher plants appear to be the exception. A few papers have demonstrated that *Sarracenia* pitchers maintain high DO despite containing large communities of heterotrophs (Cameron et al. 1977; Bradshaw 1983). *Sarracenia* pitchers were discovered to contain chloroplasts in the epidermal cells of the pitcher interior, which is unusual as this is non-stomatal tissue that would typically lack chloroplasts in terrestrial plant species (Joel and Gepstein 1985). Bradshaw (1983) demonstrated that *Sarracenia* pitchers assimilate dissolved CO_2 in the presence of light while maintaining stable DO without much regard to light. The photosynthetic efficiency of *Nepenthes* pitchers and lamina has been examined for four species (*N. talangensis, N. alata, N. mirabilis*, and *N. ventricosa*), showing that pitchers seem to exhibit extremely low net assimilation rates (Pavlovič et al. 2007, 2009; Adamec 2010). That the functions of digestion and photosynthesis are carried out by two distinct organs in *Nepenthes* as opposed to the dual role of the pitchers of the Sarraceniaceae presents an interesting complication when trying to draw parallels between what was shown for *Sarracenia purpurea* and what may be the case in *Nepenthes*. Relative to *Sarracenia*, *Nepenthes* pitchers do not have as much photosynthetic tissue, but there are increased chloroplasts in the digestive zone, surrounding the glands (Pavlovič et al. 2007). Thus, I sought to determine whether *Nepenthes* pitchers oxygenate their fluid as has been shown for *Sarracenia*. I was interested in whether there might be species or ontogenetic differences between pitchers kept in the same conditions.

Methods:

In January 2014, I took opportunistic measurements of dissolved oxygen levels in unopened pitchers of wild *Nepenthes gracilis* in Singapore using the Shriwastav et al. (2010) micro-Winkler titration method.

In August 2014, I measured dissolved oxygen concentrations in *Nepenthes* pitchers in a Singapore horticultural greenhouse with a well-controlled indoor environment. I used a fiber-optic probe and spectrometer-based sensing system (Ocean Optics, Inc., Dunedin, FL, USA). The probe was calibrated on-site using saturated and deoxygenated ddH_2O standards. Pitchers were covered with a dark opaque fabric during measurements to mitigate noisy measurements due to ambient light. I recorded point measurements taken from the middle of the fluid column; I made sure to keep the probe in the same relative position for each measurement as I had observed possible differences in oxygen level depending on the depth of the probe within individual pitchers.

In February 2016, I collected fluid from several wild *N. gracilis* in Singapore, homogenized and filtered it in a sterile bottle-top vacuum filter, and added 1.0 mL allotments of this stock fluid to open glass vials (with similar dimensions to the *N. gracilis* pitchers) and pitchers from potted *N. gracilis* which had been emptied out and cleaned beforehand. Each vial or pitcher also received an equal-sized mealworm segment and each container type was equally divided into four treatments: one mosquito larva added, one *Endonepenthia* (Diptera: Phoridae) larva added, a combination of one mosquito and one *Endonepenthia* larva added, and no larvae added. I measured oxygen levels with an oxygen optrode (Ocean Optics) after about 18 hours. This experiment was done at the greenhouse at the Raffles Institution, with the primary goal of observing the effects of inquilines on pitcher ammonia levels, but this setup incidentally allowed for oxygen measurements.

Results and Discussion:

I obtained data from 14 *Nepenthes* species/hybrids in the horticultural greenhouse: *N. chaniana* x *veitchii*, *N. clipeata* x *eymae*, *N. copelandii*, *N. densiflora*, *N. fusca*, *N. inermis* x *bongso*, *N. jamban*, *N. khasiana*,

N. muluensis x *lowii*, *N. petiolata*, *N. sanguinea*, *N. spectabilis* x *talangensis*, *N. tentaculata*, and *N. ventricosa*, with 1-3 pitchers per plant and up to 4 plants per species.

I did not find significant differences in dissolved oxygen concentrations by species (Figure A.1, Kruskal-Wallis test, p>0.05), but there was notable variation across pitchers, ranging from ~5% to ~30% oxygen (where atmospheric oxygen in the greenhouse is 21%).

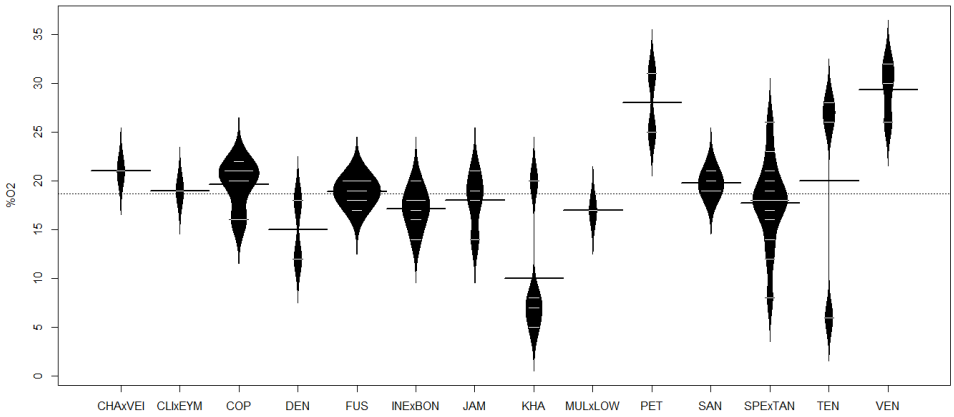


Figure A.1. Results of oxygen measurements in 14 *Nepenthes* species and hybrids in the Singapore horticultural greenhouse. CHAxVEI= *N. chaniana* x *veitchii*, CLIxEYM= *N clipeata* x *eymae*, COP= *N. copelandii*, DEN= *N. densiflora*, FUS= *N. fusca*, INExBON= *N. inermis* x *bongso*, JAM= *N. jamban*, KHA= *N. khasiana*, MULxLOW= *N. muluensis* x *lowii*, PET= *N. petiolata*, SAN= *N. sanguinea*, SPExTAN= *N. spectabilis* x *talangensis*, TEN= *N. tentaculata*, and VEN= *N. ventriosa*.

I noticed a trend of upper pitchers having lower oxygen levels than lower pitchers (Figure A.2). Most species did not have multiple pitcher morphs available, and in most cases, there either was not a significant difference or enough replication to be meaningful, but I obtained several datapoints for multiple *N. spectabilis x talangensis* plants containing upper, lower, and intermediate pitcher morphs, and the difference in means is significant in this case (Kruskal-Wallis, p=0.03). Note that most of the points that fall below 15% in the Figure A.1 are from uppers (except the one from *N. densiflora*). Upper pitchers are necessarily younger than lower pitchers on the same plant, thus I believe that this trend may indicate that younger pitchers do not oxygenate their fluid as much as older pitchers (some of the lower pitchers with low oxygen levels appeared to be more newly opened). My observations of unopened pitchers in the field support this; fluid from unopened pitchers were all relatively hypoxic (five samples: 7.8%, 11.4%, 11.4%, 8.4%, and 10.2%, where atmospheric oxygen is 21%). A possible explanation may be that since

unopened and newly opened pitchers release large magnitudes of carbon dioxide (Baby et al. 2017), the younger pitchers flush out oxygen and this may stop happening as the pitchers mature, possibly giving way to more input of oxygen from photosynthesis.

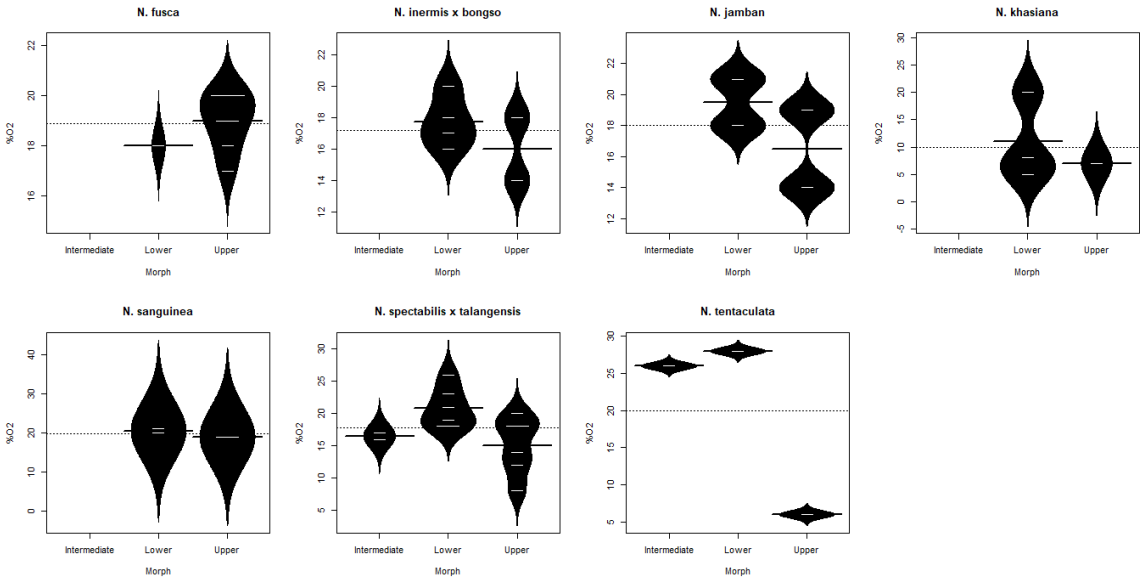
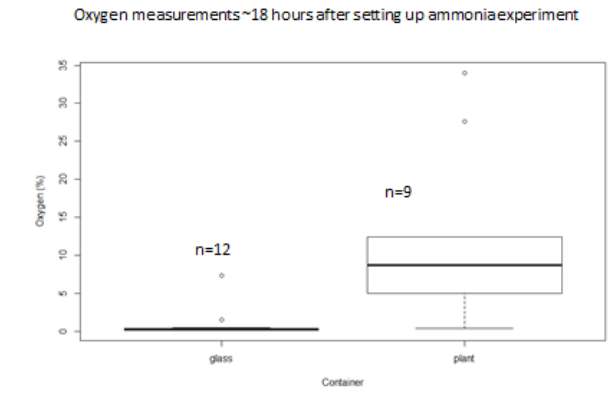


Figure A.2. Data from Singapore horticultural greenhouse *Nepenthes* dissolved oxygen measurements, broken up by species.

In the 2016 experiment, I found significant differences between the glass vial and plant treatments (Figure A.3), showing both how quickly microbial respiration can lead to hypoxia in pitcher fluid in glass vials (final O_2 levels falling mostly between 0.11% and 0.42%, where atmospheric oxygen in this greenhouse is 22.8%), and suggesting that the plants keep fluid oxygenated with photosynthesis that outpaces microbial respiration. There were no significant differences between the different inquiline treatments (Kruskal-Wallis test, $p>0.05$).

In summary, I suspect there may be some active oxygenation in *Nepenthes* and while I see no evidence for interspecific differences, ontogenetic differences seem plausible.



Kruskal-Wallis chi-squared = 12.158, df = 1, p-value = 0.0004888

Figure A.3. Results of the 2016 experiment at the Raffles Institute.

Appendix A Acknowledgements:
I thank Shawn Lum for his hospitality in Singapore, Patrick Hayes and the staff of HortPark for their help in 2014, and Jeffrey Lee for his generous help in 2016. I also thank Missy Holbrook and Tony Rockwell for helpful conversations and help with operating and troubleshooting oxygen optrodes.

Appendix A References:

Adamec L. **2010**. Ecophysiological look at organ respiration in carnivorous plants: a review. *Cell respiration and cell survival: processes, types and effects. New York: Nova Science Publishers, Inc*: 225–235.

Baby S, Johnson AJ, Zachariah EJ, Hussain AA. **2017**. *Nepenthes* pitchers are CO2-enriched cavities, emit CO2 to attract preys. *Scientific Reports* **7**.

Bradshaw WE. **1983**. Interaction between the mosquito *Wyeomyia smithii*, the midge *Metriocnemus knabi*, and their carnivorous host *Sarracenia purpurea*. *Phytotelmata: terrestrial plants as hosts for aquatic insect communities*: 161–189.

Cameron C, Donald G, Paterson C. **1977**. Oxygen-fauna relationships in the pitcher plant *Sarracenia purpurea* L. with reference to the chironomid *Metriocnemus knabi* Coq. *Canadian Journal of Zoology* **55**: 2018–2023.

Joel DM, Gepstein S. **1985**. Chloroplasts in the epidermis of *Sarracenia* (the American pitcher plant) and their possible role in carnivory-An immunocytochemical approach. *Physiologia Plantarum* **63**: 71–75.

Pavlovič A, Masarovičová E, Hudák J. **2007**. Carnivorous syndrome in Asian pitcher plants of the genus *Nepenthes*. *Annals of Botany* **100**: 527–536.

Pavlovič A, Singerová L, Demko V, Hudák J. **2009**. Feeding enhances photosynthetic efficiency in the carnivorous pitcher plant *Nepenthes talangensis*. *Annals of Botany*: mcp121.

Shriwastav A, Sudarsan G, Bose P, Tare V. **2010**. Modification of Winkler's method for determination of dissolved oxygen concentration in small sample volumes. *Analytical Methods* **2**: 1618–1622.

Supplementary Material for Chapter 1

Supplemental Table 1.1. List of taxa with Genbank accessions used in phylogenetic reconstructions. *This sample has been renamed as "*N. chaniana 3*" in this paper.

Taxon	*trnK*	*PTR1*	*ITS*
Ancistrocladus abbreviatus	AF315939.1		
Nepenthes adnata	AF315866.1	DQ840220.1	AB675864.1
Nepenthes alata	AF315891.1		AB675865.1
Nepenthes albomarginata	AF315908.1	DQ840224.1	
Nepenthes ampullaria	AF315888.1		AB675914.1
Nepenthes anamensis		DQ840225.1	
Nepenthes aristolochioides	DQ007088.1		
Nepenthes bellii	AF315926.1		AB675868.1
Nepenthes bicalcarata	DQ007089.1		AB675715.1
Nepenthes bongso	AF315865.1		AB675703.1
Nepenthes boschiana	AF315903.1	DQ840226.1	
Nepenthes burbidgeae	AF315921.1	DQ840227.1	AB675869.1
Nepenthes burkei	DQ840247.1	DQ840216.1	AB675870.1
Nepenthes cf petiolata HM2001	AF315902.1		
Nepenthes chaniana	KP152384.1		AB675872.1
Nepenthes chaniana 2	KP152385.1		
Nepenthes clipeata	AF315878.1	DQ840212.1	AB675873.1
Nepenthes danseri	DQ007087.1		AB675915.1
Nepenthes densiflora	AF315927.1	DQ840234.1	AB675875.1
Nepenthes diatas	AF315915.1	DQ840235.1	AB675876.1
Nepenthes distillatoria	AF315886.1	DQ840204.1	AB675877.1
Nepenthes dubia	AF315869.1		AB675698.1
Nepenthes edwardsiana	DQ840248.1	DQ840236.1	
Nepenthes ephippiata	AF315906.1	DQ840237.1	AB675878.1
Nepenthes eustachya	AF315867.1	DQ840238.1	AB675702.1
Nepenthes eymae	AF315930.1		AB675696.1
Nepenthes faizaliana	AF315917.1	DQ840239.1	AB675879.1
Nepenthes fusca	AF315936.1	DQ840240.1	AB675880.1
Nepenthes glabrata	AF315928.1	DQ840222.1	AB675881.1
Nepenthes gracilis	AF315937.1	DQ840241.1	AB675882.1
Nepenthes gracillima	DQ007086.1		
Nepenthes gymnamphora	AF315864.1	DQ840214.1	AB675694.1
Nepenthes hamata	AF315914.1	DQ840221.1	
Nepenthes hirsuta	AF315889.1	DQ840242.1	AB675916.1
Nepenthes inermis	AF315870.1	DQ840243.1	AB675701.1
Nepenthes insignis	AF315881.1	DQ840210.1	
Nepenthes insignis 2	AF315882.1		

Supplemental Table 1.1 cont.

Nepenthes khasiana	AF315887.1	DQ840208.1	AB675883.1
Nepenthes lamii	AF315905.1		
Nepenthes lavicola	AF315935.1	DQ840219.1	
Nepenthes longifolia	AF315871.1		AB675885.1
Nepenthes lowii	AF315875.1		AB675695.1
Nepenthes macfarlanei	AF315894.1		
Nepenthes macrophylla	AF315931.1		
Nepenthes macrovulgaris	AF315934.1		
Nepenthes madagascariensis	AF315883.1	DQ840207.1	AB769064.1
Nepenthes mapuluensis	AF315918.1	DQ840206.1	
Nepenthes masoalensis	AF315884.1		
Nepenthes maxima	AF315913.1	DQ840244.1	AB675697.1
Nepenthes merrilliana	AF315912.1		AB675887.1
Nepenthes mikei	AF315911.1		AB675700.1
Nepenthes mira	DQ007085.1		AB675711.1
Nepenthes mirabilis	AF315920.1		AB675889.1
Nepenthes muluensis	AF315933.1		
Nepenthes murudensis	DQ007084.1	DQ840223.1	
Nepenthes neoguineensis	AF315896.1		AB675917.1
Nepenthes northiana	AF315901.1		
Nepenthes ovata	AF315873.1		AB675892.1
Nepenthes pectinata	AF315909.1		AB675708.1
Nepenthes pervillei	AF315885.1		AB675893.1
*Nepenthes pilosa**	AF315919.1	DQ840209.1	
Nepenthes rafflesiana	AF315910.1		
Nepenthes rajah	AF315880.1		AB675895.1
Nepenthes ramispina	DQ007083.1		
Nepenthes reinwardtiana	AF315907.1		AB675896.1
Nepenthes rhombicaulis	AF315874.1		AB675897.1
Nepenthes sanguinea	AF315923.1		AB675898.1
Nepenthes sibuyanensis	DQ840246.1	DQ840218.1	
Nepenthes singalana	DQ007082.1	DQ840228.1	
Nepenthes sp HHM 2001 1	AF315938.1		
Nepenthes sp HHM 2001 2	AF315929.1	DQ840230.1	
Nepenthes sp HHM 2001 3	DQ840245.1		
Nepenthes spathulata	DQ007081.1	DQ840229.1	AB675900.1
Nepenthes spectabilis	AF315868.1		AB675901.1
Nepenthes stenophylla	AF315922.1	DQ840231.1	AB675903.1
Nepenthes sumatrana	AF315872.1	DQ840215.1	AB675904.1
Nepenthes talangensis	AF315924.1		AB675905.1
Nepenthes tentaculata	AF315932.1		AB675920.1

Supplemental Table 1.1 cont.

Nepenthes thorelii	AF315890.1	DQ840232.1	AB675712.1
Nepenthes tobaica	AF315899.1	DQ840233.1	AB675907.1
Nepenthes tomoriana	AF315898.1	DQ840205.1	AB675706.1
Nepenthes treubiana	AF315893.1		
Nepenthes truncata	AF315904.1		AB675908.1
Nepenthes veitchii	AF315895.1		AB675909.1
Nepenthes ventricosa	AF315892.1		AB675910.1
Nepenthes vieillardii	AF315897.1		
Nepenthes villosa	AF315925.1	DQ840211.1	AB675911.1
Nepenthes xiphioides	DQ007080.1	DQ840213.1	
Triphyophyllum peltatum	AF315940.1		

Supplemental Table 1.2. Summary of collections of pitcher infauna for Singapore summer 2014, numbers presented are means ± standard deviation.
†Four pitchers that became severely damaged are excluded from the second date's counts.

	First Collection Date			**Second Collection Date**				
Pitcher Type	**Culicidae**	**Other Larvae**	**Ants**	**Culicidae**	**Other Larvae**	**Ants**	**Mites**	**Flying Prey**
Lower Green	0.33 ± 0.50	0.11 ± 0.33	1.33 ± 1.80	0.50 ± 0.76	0 ± 0	6.13 ± 7.85	0 ± 0	0 ± 0
Lower Red†	2.93 ± 5.09	0.64 ± 1.25	12.36 ± 20.31	1.05 ± 1.86	0.27 ± 0.55	11.32 ± 17.82	1.73 ± 2.85	0.36 ± 0.73
Upper Green	2.10 ± 2.47	0.57 ± 1.19	6.17 ± 7.11	1.74 ± 3.19	0.29 ± 0.80	3.18 ± 6.20	0.18 ± 0.63	0.94 ± 1.58
Upper Red	2.80 ± 2.39	1.80 ± 2.95	22.00 ± 40.87	0.40 ± 0.55	0 ± 0	12.60 ± 15.60	0.40 ± 0.55	1.80 ± 1.79
All Pitchers	2.13 ± 3.60	0.59 ± 1.32	8.59 ± 16.95	1.21 ± 2.47	0.22 ± 0.63	6.42 ± 12.12	0.63 ± 1.76	0.67 ± 1.30

Supplemental Table 1.3. Results of Poisson regressions conducted on *N. gracilis* pitcher infauna counts. For each prey item type (ants, mites, and flying prey) and symbiont (culicids and other larvae), the counts were tested in a linear mixed model with all traits as fixed effects in one model and with plant nested with site as random effects. The numbers presented are the estimate for the fixed effect with p-values in parentheses.
*Significant at $p<0.05$

Trait	Ants	Culicid Larvae	Other Larvae	Mites	Flying Prey
Pitcher color	1.01 (0.35)	-0.28 (0.80)	1.61 (0.51)	1.59 (0.15)	1.21 (0.44)
Pitcher morph	0.75 (0.49)	-0.18 (0.88)	1.72 (0.47)	0.87 (0.37)	2.67 (0.10)
Connectedness	-0.56 (0.06)	-0.16 (0.48)	-0.58 (0.11)	0.39 (0.25)	-0.20 (0.48)
Pitcher size	0.02 (0.01)*	0.01 (0.55)	-0.01 (0.82)	-0.01 (0.52)	-0.04 (0.07)
Distance from the ground	-0.01 (0.18)	0.01 (0.18)	0.03 (0.13)	-0.01 (0.10)	0.01 (0.15)

Supplemental Table 1.4. Contingency table for field survey of *N. gracilis* color polymorphism. $p=8*10^{-25}$

	Upper	Lower	Total
Red	9	189	198
Green	334	290	624
Total	343	479	822

Supplemental Table 1.5: Results of test of correlated evolution (binary PGLMM) between color polymorphism (converted to binary state, presence or absence of "similar coloration") and dimorphism, reduced lower pitchers, and the six color pattern-related traits: upper and lower lid contrast, upper and lower peristome contrast, and upper and lower peristome stripes. Provided for each trait is the s2 value and correlation estimate, with p-values in parentheses. The s2 value is a measure of the phylogenetic signal in the residuals; s2 values further from zero indicate more significant phylogenetic signal in the residual, p-values are in parentheses. Values that are significant at the Bonferroni-corrected alpha value of 0.00625 are indicated with an asterisk.

Dependent Trait	s2	Correlation Estimate
Pitcher Dimorphism	5.32E-14 (0.500)	-2.64 (0.016)
Reduced Lower Pitchers	1.45 (0.013)	1.54 (0.009)
Lower Lid Contrast	1.90 (0.164)	0.39 (0.573)
Lower Peristome Contrast	3.50 (0.007)	0.15 (0.818)
Lower Peristome Stripes	4.64 (1.33E-12*)	-0.53 (0.358)
Upper Lid Contrast	3.52 (1.09E-09*)	-0.35 (0.585)
Upper Peristome Contrast	4.34 (8.63E-04*)	0.22 (0.744)
Upper Peristome Stripes	4.85 (1.42E-15*)	-0.68 (0.231)

Supplemental Table 1.6: Results of test of correlated evolution (binary PGLMM) between reduced lower pitcher production trait and dimorphism, three binary color polymorphism traits (presence/absence of "similar coloration", "redder lowers", and "redder uppers"), and the six color pattern-related traits: upper and lower lid contrast, upper and lower peristome contrast, and upper and lower peristome stripes. Provided for each trait is the s2 value and correlation estimate, with p-values in parentheses. The s2 value is a measure of the phylogenetic signal in the residuals; s2 values further from zero indicate more significant phylogenetic signal in the residual, p-values are in parentheses. Values that are significant at the Bonferroni-corrected alpha value of 0.00625 are indicated with an asterisk.

Dependent Trait	s2	Correlation Estimate
Pitcher Dimorphism	2.63E-14 (2.32E-06*)	3.42 (0.074)
Similar Coloration	1.45E-13 (0.500)	1.70 (0.003*)
Redder Lowers	1.83E-09 (0.500)	-1.72 (0.003*)
Redder Uppers	4.11 (0.056)	0.15 (0.909)
Lower Lid Contrast	2.78 (0.132)	-0.10(0.908)
Lower Peristome Contrast	3.53 (0.005*)	0.37 (0.623)
Lower Peristome Stripes	3.81 (3.50E-10*)	0.04 (0.946)
Upper Lid Contrast	4.51 (2.78E-12*)	0.73 (0.322)
Upper Peristome Contrast	5.14 (1.37E-04*)	1.25 (0.104)
Upper Peristome Stripes	4.89 (3.21E-15*)	-0.78 (0.258)

Supplemental Tables 1.7: Select results of tests of correlated evolution (binary PGLMM) against habit (terrestrial/epiphyte/lithophyte) and habitat (dipterocarp forest, peat swamp, heath forest, montane forest, scrub, cliff, mangrove, seasonal grassland, and degraded). Provided for each response trait is the s2 value, p-value of the s2 value, correlation estimate, and p-vale for the correlation estimate. The s2 value is a measure of the phylogenetic signal in the residuals; s2 values further from zero indicate more significant phylogenetic signal in the residual, p-values are in parentheses. P-values that are significant at the Bonferroni-corrected alpha value of 0.00417 are indicated with an asterisk.

Pitcher Dimorphism

Dependent Trait	s2	s2 p-value	Correlation Estimate	Correlation Estimate p-value
Terrestrial	3.53E-09	8.95E-18*	-0.17	0.876
Epiphyte	2.65	1.57E-09*	23.60	0.999
Lithophyte	1.79E-07	0.002*	15.04	0.993
Dipterocarp Forest	3.11	3.89E-05*	1.36	0.235
Peat Swamp	9.08	0.001*	0.01	0.995
Heath Forest	0.20	0.361	0.95	0.385
Montane Forest	2.68	3.86E-06*	-0.89	0.431
Scrub	3.49	5.64E-09*	0.44	0.573
Cliff	1.08	1.71E-04*	-0.54	0.462
Mangrove	0.19	3.72E-15*	1.57	0.363
Seasonal Grassland	0.09	0.002*	13.62	0.990
Degraded	1.67	0.028*	1.61	0.147

Supplemental Tables 1.7 cont.

Reduced Lowers

Dependent Trait	s2	s2 p-value	Correlation Estimate	Correlation Estimate p-value
Terrestrial	1.23E-12	1.10E-05*	14.09	0.991
Epiphyte	0.28	1.04E-05*	5.90	0.002*
Lithophyte	1.92E-12	1.10E-05*	-18.22	0.997
Dipterocarp Forest	3.01	4.26E-05*	-1.07	0.158
Peat Swamp	5.75	3.23E-09*	-2.77	0.155
Heath Forest	0.64	0.187	-0.22	0.743
Montane Forest	1.91	3.19E-08*	4.33	0.024
Scrub	3.40	3.10E-08*	-0.10	0.876
Cliff	0.78	0.008	-1.94	0.016
Mangrove	0.28	5.82E-06*	-3.73	0.234
Seasonal Grassland	9.10E-13	1.10E-05*	-14.09	0.991
Degraded	1.23	0.055	-1.38	0.054

Color Polymorphism (Presence/absence of "Similar Coloration")

Dependent Trait	s2	s2 p-value	Correlation Estimate	Correlation Estimate p-value
Terrestrial	1.02E-12	3.62E-08*	15.33	0.993
Epiphyte	1.56	8.24E-05*	0.35	0.484
Lithophyte	8.84E-12	3.62E-08*	-16.05	0.992
Dipterocarp Forest	2.90	9.42E-05*	-0.41	0.479
Peat Swamp	9.38	0.001*	-0.83	0.419
Heath Forest	0.42	0.267	-0.06	0.916
Montane Forest	2.51	3.02E-05*	0.90	0.181
Scrub	3.41	8.67E-08*	-0.06	0.907
Cliff	1.28	4.53E-05*	0.16	0.755
Mangrove	0.27	3.57E-08*	-5.01	0.221
Seasonal Grassland	1.54E-12	3.62E-08*	-15.33	0.993
Degraded	1.50	0.055	-1.01	0.064

Lower Lid Contrast

Dependent Trait	s2	s2 p-value	Correlation Estimate	Correlation Estimate p-value
Terrestrial	3.14E-15	1.79E-19*	0.47	0.779
Epiphyte	1.89	7.24E-06*	0.32	0.672
Lithophyte	3.99E-07	0.497	1.34	0.292
Dipterocarp Forest	2.64	3.32E-04*	0.56	0.481
Peat Swamp	9.34	0.001*	-0.34	0.813
Heath Forest	0.74	0.181	-0.30	0.727
Montane Forest	2.88	4.40E-06*	-0.60	0.458
Scrub	3.56	2.00E-07*	2.19	0.061
Cliff	1.61	1.82E-06*	-1.37	0.141

Supplemental Tables 1.7 cont.

Mangrove	1.78	4.71E-04*	-11.77	0.960
Seasonal Grassland	2.97E-14	1.79E-19*	-0.47	0.779
Degraded	2.15	0.003*	-0.25	0.748

Upper Lid Contrast

Dependent Trait	s2	s2 p-value	Correlation Estimate	Correlation Estimate p-value
Terrestrial	8.24E-11	6.54E-06*	14.13	0.991
Epiphyte	1.54	1.89E-04*	-0.46	0.453
Lithophyte	7.94E-09	0.495	1.13	0.431
Dipterocarp Forest	3.00	1.55E-05*	0.37	0.587
Peat Swamp	9.65	0.001*	-0.42	0.732
Heath Forest	0.74	0.257	-0.76	0.295
Montane Forest	1.97	0.003*	-1.42	0.032
Scrub	3.39	3.10E-07*	0.37	0.593
Cliff	1.47	1.11E-06*	-0.69	0.288
Mangrove	0.57	0.329	0.63	0.525
Seasonal Grassland	5.94E-16	6.54E-06*	-14.13	0.991
Degraded	1.84	0.006	-0.21	0.734

Upper Peristome Contrast

Dependent Trait	s2	s2 p-value	Correlation Estimate	Correlation Estimate p-value
Terrestrial	3.33E-13	3.17E-05*	14.08	0.991
Epiphyte	1.97	9.03E-07*	0.43	0.504
Lithophyte	2.67	2.66E-05*	-13.90	0.986
Dipterocarp Forest	3.15	9.01E-06*	1.35	0.057
Peat Swamp	8.57	0.002*	0.68	0.518
Heath Forest	0.51	0.193	0.44	0.492
Montane Forest	2.82	2.87E-06*	-0.54	0.449
Scrub	3.30	9.63E-08*	0.38	0.604
Cliff	0.94	0.001*	0.04	0.951
Mangrove	1.81	0.101	-0.44	0.727
Seasonal Grassland	5.39E-13	3.17E-05*	-14.08	0.991
Degraded	1.76E-09	0.500	1.12	0.046

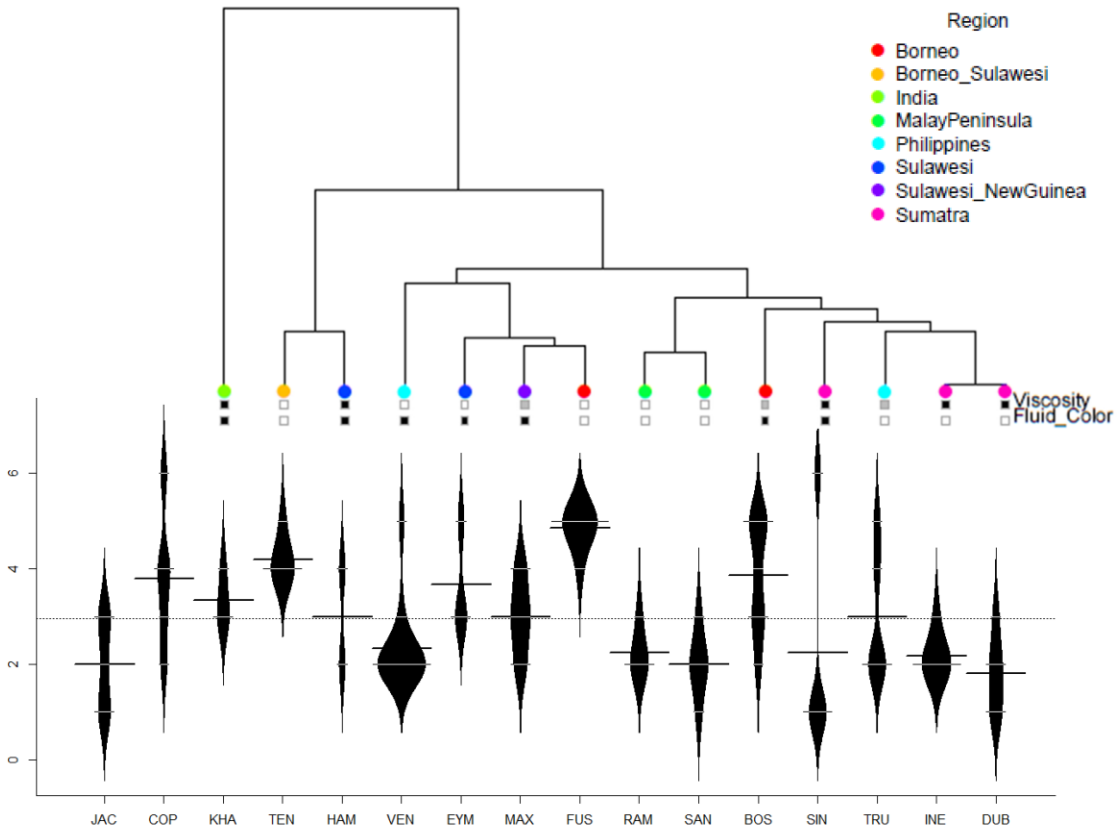
Upper Peristome Stripes

Dependent Trait	s2	s2 p-value	Correlation Estimate	Correlation Estimate p-value
Terrestrial	1.81E-09	2.13E-12*	-16.80	0.994
Epiphyte	1.59	7.66E-05*	0.40	0.438
Lithophyte	17.56	5.63E-16*	6.56	0.221
Dipterocarp Forest	3.71	8.34E-05*	0.49	0.422
Peat Swamp	7.82	0.003*	-0.83	0.497

Supplemental Tables 1.7 cont.

Heath Forest	0.05	0.484	1.04	0.056
Montane Forest	4.23	1.42E-09*	-0.82	0.235
Scrub	3.01	4.08E-06*	-0.69	0.221
Cliff	0.94	0.001*	0.00	0.996
Mangrove	0.54	0.328	-0.90	0.435
Seasonal Grassland	5.87E-14	2.16E-08*	-15.33	0.992
Degraded	0.66	0.165	-0.53	0.309

Supplementary Material for Chapter 2



Supplemental Figure 2.1. Pitcher traits viewed in a phylogenetic context, using the Gilbert et al. (2018) topology for all previously sequenced species included in this study. *Nepenthes jacquelineae* and *N. copelandii* have not been sequenced. For the purpose of this illustration, *N. ventricosa* x "Bill Bailey" is coded according to the parent species *N. ventricosa*. Beanplot shows pH by species; the species have been reorganized to fit the phylogeny. For viscosity and fluid color, a white square denotes absence and a filled square denotes presence. Tips are additionally colored by the region of origin for each species.

Supplemental Discussion for Chapter 2: Taxonomic composition of experimental pitchers

Bacteria

Previous studies indicate a characteristic set of bacterial taxa associate with pitchers of *Nepenthes* species, as well as with the convergently evolved pitchers of New World pitcher plants in the genus *Sarracenia* (Bittleston et al. 2018). Some of the common bacterial taxa in our samples match what appear to be common associates of other pitcher plant taxa, especially the orders Rhodospirillales, Actinomycetales, and Rhizobiales, but also Sphingobacteriales (including the family Chitinophagaceae), Burkholderiales, Enterobacteriales, and Xanthomonadales (Bittleston 2018). While not dominant in terms of relative abundance, Acidobacteria and Caulobacterales occur fairly frequently across multiple samples, and these taxa have also been found to associate with *Nepenthes* in nature (Sickel et al. 2016; Bittleston 2018). Some of the common taxa in our study may also be common inhabitants of plant surfaces in general, such as Sphingomonadales, Pseudomonadales, and Xanthomonadales that have all been found in earlier studies of phyllosphere bacteria in other plant species (Vorholt 2012; Vacher et al. 2016). Other abundant or frequent taxa in our study include bacteria that do not appear to be frequent associates of pitchers, and these could represent environmental bacteria that are a consequence of the experimental glasshouse setting: Chlamydiae, Rickettsiales, TM6, and Verrucomicrobia. Most samples also possess a small fraction of OTUs that cannot be assigned at the phylum level. Overall, while the bacterial communities in our experimental plants may not be typical of those found in wild *Nepenthes*, we nevertheless recovered many commonalities in the diversity and abundance of certain groups.

Acetobacteriaceae, particularly the genus *Acidocella* (and to a lesser extent, *Acidisoma*) show up as frequent associates of *Nepenthes* pitchers in most microbiome studies so far, from natural or semi-natural settings (Chou et al. 2014; Kanokratana et al. 2016; Sickel et al. 2016; Bittleston et al. 2018). Considering *Acidocella* sp.'s apparent relationship with *Nepenthes* and given that it is not known to be a common environmental bacterium, previously isolated from only a few extreme habitats (Kishimoto et al. 1995; Belova et al. 2009; Kimoto et al. 2010; Jones et al. 2013), it would be worthwhile to probe the function of taxa from this genus in relation to its host.

The possibility of vertical transmission cannot be completely ruled out regarding important *Nepenthes* symbionts. Vertical transmission has been seen in other phyllosphere systems (Vorholt 2012), and although it was initially established that pitchers are sterile prior to opening (Buch et al. 2012), more recent microbiome studies were able to find bacterial DNA in unopened pitchers (Chou et al. 2014; Takeuchi et al. 2015; Kanokratana et al. 2016), including *Acidocella* (Chou et al. 2014; Kanokratana et al. 2016). In each case, only a minority of unopened pitchers examined yielded detectable amounts of DNA, so the possible occurrence of bacteria in unopened pitchers is unresolved. In known cases of vertical transmission in plants, seed-associated bacteria can spread systemically throughout the developing plant (Vorholt 2012), so it is interesting to note that Sickel et al. (2016) found that bacterial composition did not differ significantly between pitcher fluid, pitcher external surfaces, and leaf lamina in their study. As evidence against vertical transmission of *Acidocella* sp., a previous greenhouse *Nepenthes* microbiome study did not find *Acidocella* as a prominent taxon (Takeuchi et al. 2015). However, the Takeuchi et al. (2015) study took place in temperate Germany, and it seems likely that key associates of pitchers such as *Acidocella* sp. are themselves geographically restricted, which could explain its presence both in the wild and in the Singapore greenhouse, without needing to invoke vertical transmission.

Eukaryotes

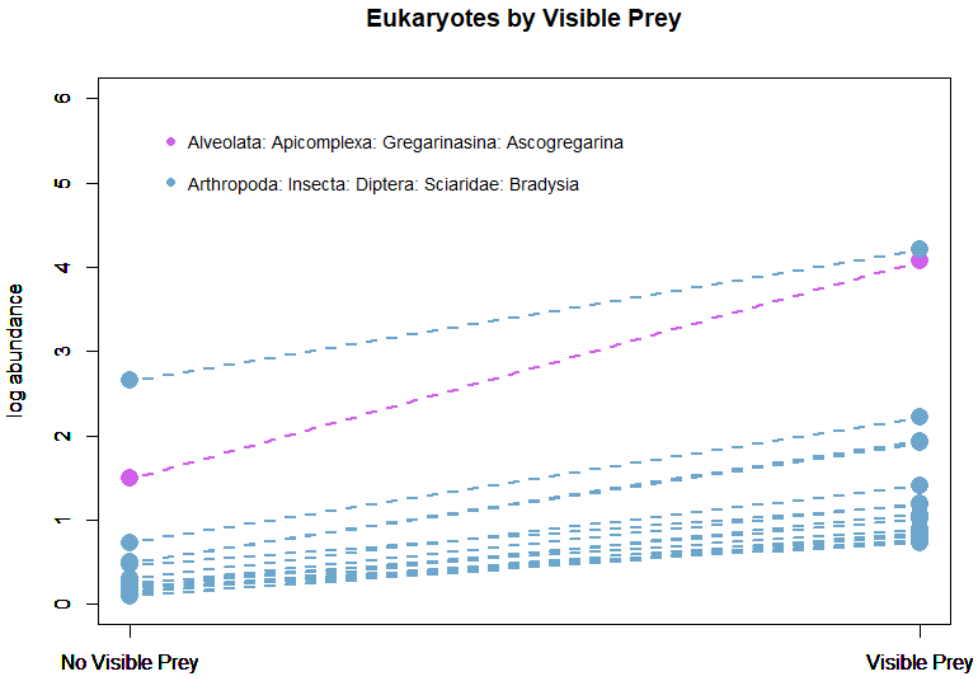
Symbiotic arthropod communities in pitcher plants, including several families of dipteran larvae and mites, have been well-characterized over decades of research (Beaver 1979). Being in an enclosed glasshouse, however, our experimental plants exhibited no evidence of being colonized by symbiotic arthropods. Additionally, microfauna such as nematodes and rotifers that may be expected to be fairly common members of wild pitcher communities (Quisado 2013; Bittleston et al. 2016; Bittleston 2018) were not common in our samples. Nevertheless, insect prey was available in the glasshouse, primarily fungus gnats (Sciaridae), which are common indoor plant pests that can be found in potting media. This prey DNA was detectable in our samples, enabling us to examine the impact of prey capture on microbiome dynamics.

Few studies have documented the composition of eukaryotic microbial communities in wild *Nepenthes* (Bittleston et al. 2016; Bittleston 2018; Bittleston et al. 2018). Fungi such as Saccharomycetes, Agaricomycetes, and basal fungi (e.g. Mucoromycotina, Chytridiomycota) have been previously found in wild (Bittleston 2018; Bittleston et al. 2018), and these taxa also were present in our samples. However, while ascomycetous yeasts in Saccharomycetes appear to be the dominant fungi in wild communities (Bittleston 2018), our samples were dominated by Basidiomycota in Ustilaginomycotina and in Agaricomycotina (especially Tremellomycetes). Tremellomycetes can exist in single-celled yeast form, so these fungi could hypothetically occupy a similar ecological niche to the fungal yeasts in wild pitchers. The Ascomycota in our samples include Trichocomaceae (including the well-known genus *Penicillium*), which are common members of indoor microbial communities (Barberán et al. 2015); Chaetothyriales ("black yeasts" including the family Herpotrichiellae), which were previously found in wild pitchers (Bittleston 2018); as well as Sordariomycetes and Leotiomycetes, also members of Pezizomycotina. A single pitcher sample was dominated by Microsporidia, a basal fungus and obligate intracellular parasite of animals. So while certain fungal taxa have been found in wild pitchers, the overall taxonomic composition of fungi in our study appears to have had some major influence from the microbial pool of the greenhouse environment.

Pitcher plant microbiomes in this and previous studies also include Protista. Algae have been found to be common inhabitants of pitchers in the wild (Bittleston et al. 2016, Bittleston 2018), and we found a diverse community of algae throughout our samples, including members of Archaeplastida (Trebouxiophyceae and Chlorophyceae), Stramenopiles (Chrysophyceae and diatoms), and Discoba (Euglenozoa). Rhizaria and Amoebozoa are two abundant taxa in our samples that have also been found associated with pitchers in other studies (Bittleston et al. 2016, Bittleston 2018). Another common taxon across our samples was Alveolata, particularly Gregarinasina (gregarines). These are obligate arthropod parasites, especially of larval mosquitoes (Chen 1999; Tseng 2007) and they have been previously found in pitcher communities in association with the symbiotic larval dipterans (Baker et al. 2016; Bittleston et al. 2016). As there were no mosquitoes or dipteran larvae of any kind inhabiting our pitchers, the frequent

occurrence of gregarines here is somewhat surprising. These gregarines may have been parasitizing the adult fungus gnats trapped by the pitchers; this is supported by ANCOM analysis showing that gregarine relative abundance is higher in pitchers with visible prey than those without visible prey (Supplemental Figure 2.2) Perhaps more culture-independent surveys of eukaryotic diversity will reveal that gregarines are an even more important component of ecosystems than currently appreciated, perhaps infecting a wide assortment of arthropods in a variety of ecological contexts (Dabert and Dabert 2008; Criado-Fornelio et al. 2017).

Overall, the eukaryotic community composition appears to be somewhat uneven at the broad taxonomic level compared to the bacteria. Multiple phyla generally can be seen co-occurring within most samples for bacteria. However, for eukaryotes, most samples appear to be dominated by a single broad taxon, i.e. fungus-, metazoan-, or protist-dominated communities.



Supplemental Figure 2.2: Results of ANCOM analysis showing slopes of change in log relative abundance for eukaryote OTUs in relation to presence of visible prey.

Chapter 2 Supplemental Discussion References:

Baker CC, Bittleston LS, Sanders JG, Pierce NE. **2016**. Dissecting host-associated communities with DNA barcodes. *Phil. Trans. R. Soc. B* **371**: 20150328.

Barberán A, Dunn RR, Reich BJ, Pacifici K, Laber EB, Menninger HL, Morton JM, Henley JB, Leff JW, Miller SL, others. **2015**. The ecology of microscopic life in household dust. *Proc. R. Soc. B* **282**: 20151139.

Beaver R. **1979**. Biological studies of the fauna of pitcher plants [*Nepenthes*] in west Malaysia. *Annales de la Société Entomologique de France*.3–17.

Belova SE, Pankratov TA, Detkova EN, Kaparullina EN, Dedysh SN. **2009**. *Acidisoma tundrae* gen. nov., sp. nov. and *Acidisoma sibiricum* sp. nov., two acidophilic, psychrotolerant members of the Alphaproteobacteria from acidic northern wetlands. *International Journal of Systematic and Evolutionary Microbiology* **59**: 2283–2290.

Bittleston LS. **2018**. Commensals of *Nepenthes* Pitchers, in *Carnivorous Plants: Physiology, Ecology, and Evolution*. Ellison, Aaron M and Adamec, Lubomír ed. Oxford University Press, 314–332.

Bittleston LS, Baker C, Strominger LB, Pringle A, Pierce NE. **2016**. Metabarcoding as a tool for investigating arthropod diversity in *Nepenthes* pitcher plants. *Austral Ecology* **41**: 120–132.

Bittleston LS, Wolock CJ, Yahya BE, Chan XY, Chan KG, Pierce NE, Pringle A. **2018**. Convergence between the microcosms of Southeast Asian and North American pitcher plants. *eLife* **7**.

Buch F, Rott M, Rottloff S, Paetz C, Hilke I, Raessler M, Mithöfer A. **2012**. Secreted pitfall-trap fluid of carnivorous *Nepenthes* plants is unsuitable for microbial growth. *Annals of Botany*: mcs287.

Chen W. **1999**. The life cycle of *Ascogregarina taiwanensis* (Apicomplexa: Lecudinidae). *Parasitology Today* **15**: 153–156.

Chou LY, Clarke CM, Dykes GA. **2014**. Bacterial communities associated with the pitcher fluids of three *Nepenthes* (Nepenthaceae) pitcher plant species growing in the wild. *Archives of Microbiology* **196**: 709–717.

Criado-Fornelio A, Verdú-Expósito C, Martin-Pérez T, Heredero-Bermejo I, Pérez-Serrano J, Guàrdia-Valle L, Panisello-Panisello M. **2017**. A survey for gregarines (Protozoa: Apicomplexa) in arthropods in Spain. *Parasitology Research* **116**: 99–110.

Dabert M, Dabert J. **2008**. Ribosomal DNA sequences reveal gregarine pathogens (Apicomplexa: Gregarinia) in mites and other arachnids (Arachnida). *Soil Org* **80**: 197–204.

Jones RM, Hedrich S, Johnson DB. **2013**. *Acidocella aromatica* sp. nov.: an acidophilic heterotrophic alphaproteobacterium with unusual phenotypic traits. *Extremophiles : Life Under Extreme Conditions* **17**: 841–50.

Kanokratana P, Mhuanthong W, Laothanachareon T, Tangphatsornruang S, Eurwilaichitr L, Kruetreepradit T, Mayes S, Champreda V. **2016**. Comparative Study of Bacterial Communities in *Nepenthes*. *Microbial Ecology* **72**: 381–393.

Kimoto K, Aizawa T, Urai M, Ve NB, Suzuki K, Nakajima M, Sunairi M. **2010**. *Acidocella aluminiidurans* sp. nov., an aluminium-tolerant bacterium isolated from *Panicum repens* grown in a highly acidic swamp in actual acid sulfate soil area of Vietnam. *International Journal of Systematic And Evolutionary Microbiology* **60**: 764–768.

Kishimoto N, Kosako Y, Wakao N, Tano T, Hiraishi A. **1995**. Transfer of *Acidiphilium facilis* and *Acidiphilium aminolytica* to the genus *Acidocella* gen. nov., and emendation of the genus *Acidiphilium*. *Systematic and Applied Microbiology* **18**: 85–91.

Quisado JS. **2013**. Nematode diversity of phytotelmata of *Nepenthes* spp. in Mount Hamiguitan Range Wildlife Sanctuary, Philippines. (thesis)

Sickel W, Grafe TU, Meuche I, Steffan-Dewenter I, Keller A. **2016**. Bacterial diversity and community structure in two Bornean *Nepenthes* species with differences in nitrogen acquisition strategies. *Microbial Ecology* **71**: 938.

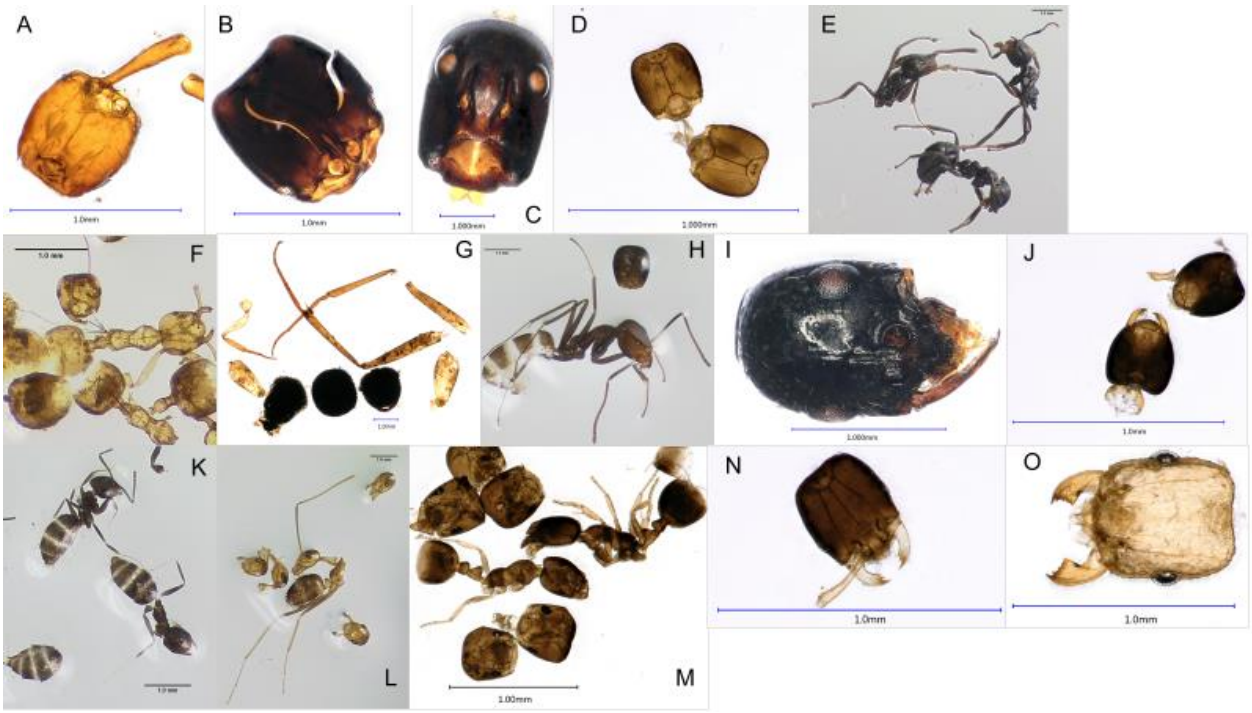
Takeuchi Y, Chaffron S, Salcher MM, Shimizu-Inatsugi R, Kobayashi MJ, Diway B, von Mering C, Pernthaler J, Shimizu KK. **2015**. Bacterial diversity and composition in the fluid of pitcher plants of the genus *Nepenthes*. *Systematic and Applied Microbiology* **38**: 330–339.

Tseng M. **2007**. Ascogregarine parasites as possible biocontrol agents of mosquitoes. *Journal of the American Mosquito Control Association* **23**: 30–34.

Vacher C, Hampe A, Porté AJ, Sauer U, Compant S, Morris CE. **2016**. The phyllosphere: microbial jungle at the plant-climate interface. *Annual Review of Ecology, Evolution, and Systematics* **47**: 1–24.

Vorholt JA. **2012**. Microbial life in the phyllosphere. *Nature Reviews. Microbiology* **10**: 828–40.

Supplementary Material for Chapter 3



Supplemental Figure 3.1. Representative specimens of the ant genera identified from *Nepenthes mindanaoensis* fluid samples: (A) *Aenictus*, (B) *Brachyponera*, (C) *Camponotus*, (D) *Cardiocondyla,* (E) *Colobopsis*, (F) *Crematogaster*, (G) *Echinopla*, (H) *Iridomyrmex*, (I) *Leptogenys*, (J) *Monomorium*, (K) *Nylanderia*, (L) *Oecophylla*, (M) *Pheidole*, (N) *Tapinoma*, and (O) *Tetramorium*. All scale bars denote 1.0 mm.

Supplemental Figure 3.2. Representative specimens of the culicid morphospecies identified from *Nepenthes mindanaoensis* fluid samples: (A) *Tripteroides* sp.1, (B) *Tripteroides* sp.2, (C) *Tripteroides* sp.3, and (D) *Tripteroides* sp.4. All scale bars denote 1.0 mm.