# Preference for mammalian urine is higher in the canopy than on the ground in a tropical rainforest ant community in Yunnan, China

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### Abstract

Ants are among the most abundant groups of arthropods, and approximately half of all ant species are associated with forest canopies. The forest canopy environment is distinct from the understory and forest floor, and vertical stratification in environmental conditions shapes species assembly and organismal traits and behaviors across taxa in forest communities. Canopy ants are faced with a more nitrogen-limited environment compared with ground ants because of their reliance on nitrogen-poor plant and insect exudates. Despite prior work suggesting that some ant species consume mammalian urine and use symbiotic bacteria to extract nitrogen, we have little knowledge about the consumption of urine in canopy ants or the relative preference for urine between ground and canopy ants. We conducted an observational field experiment in a lowland tropical rainforest in southern China to test for vertical stratification in ant preference for sugar and urine, setting ground and canopy baited pitfall traps with the use of a canopy crane. We found distinct vertical stratification in the use of urine, with higher richness and abundance in sugar baits on the ground, and a higher abundance in urine baits in the canopy. Furthermore, the composition of captured ants differentiated according to both vertical stratum and bait type. This distinct vertical stratification of niche preference may represent an important case of niche partitioning that contributes to high ant species diversity in tropical rainforests as well as high species turnover between ground and canopy strata. The preference of canopy ants for mammalian urine also highlights the importance of interspecific interactions across highly unrelated animal taxa and emphasizes the need for a holistic understanding of biological networks to effectively conserve threatened tropical forest communities.

**Key words:** Carbohydrate, diet, Formicidae, Hymenoptera, nitrogen, nutrient preference, tropical forest, vertical stratification.

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## Introduction

Arthropods, the most abundant and diverse phylum on Earth, are widely distributed across most global ecosystems (Sabrosky 1988, Stork 2018). Arthropods provide key essential services such as pollination, decomposition, pest control, and seed dispersal (Del Toro & al. 2012, Saunders & Rader 2020). However, climate change and increased anthropogenic disturbance (e.g., pollution and habitat destruction) are fueling recent biodiversity loss,

including arthropods, at an alarming rate (Del Toro & al. 2012, Amendt 2021, Wagner & al. 2021). Although the rate of terrestrial arthropod abundance decline has been estimated to be around 9% per decade (Van Klink & al. 2020), necessary data on the ecological distribution, niche preferences, and community structure of arthropods in terrestrial ecosystems like tropical forests are still lacking (Van Klink & al. 2022).

Besides harboring an estimated two-thirds of global biodiversity (Raven 1988), tropical forests are key habitats that ecologists have explored to better understand the impact of the vertical dimension – formed by the ground, understory, and canopy – on ecological communities (Nakamura & al. 2017). Such a vertical dimension allows taxa to inhabit a "3D niche", and elucidating the differences in 3D niche occupancy between species is crucial for understanding species interactions and community assembly (Gámez & Harris 2022). A recent meta-analysis of four vertebrate groups (bats, small mammals, amphibians, and birds) found significant variation in patterns of vertical stratification of abundance and diversity in tropical forests, with higher abundance of bats in the canopy, decreasing small mammal and amphibian abundance and richness towards the canopy, and no consistent vertical trend in bird abundance or richness (Basham & al. 2022). No such meta-analysis yet exists for arthropods, but vertical stratification has been documented for several arthropod groups including beetles (Grimbacher & Stork 2007), spiders (Oguri & al. 2014), and ants (Hoenle & al. 2023), among many others. Beyond documenting patterns of vertical stratification, identifying causal mechanisms that produce and maintain stratification in tropical forests, such as differential diet preferences, remains a key challenge in community ecology, especially for hyperdiverse taxonomic groups such as insects.

Among arthropod families, ants (Formicidae) are one of the dominant groups in terms of abundance and diversity in terrestrial ecosystems. Ant abundance and diversity are highest in the tropics and subtropics (Davidson & Patrell-Kim 1996, Del Toro & al. 2012, Saunders & Rader 2020, Schultheiss & al. 2022). Ant global biomass has been estimated to be approximately 12 megatons of dry carbon, which is equivalent to 20% of total human biomass (Schultheiss & al. 2022). In forests, ant distribution varies vertically from the forest floor to the high canopy, with as much as 50% of total local diversity found in the tropical forest canopy (Davidson & Patrell-Kim 1996, Floren & al. 2014). Furthermore, ants have been reported to be the dominant taxon in forest canopies when compared with other arthropod groups (Stork & Eggleton 1992, Tobin & al. 1995, Floren & al. 2014). Ants can also account for over 50% of animal biomass in tropical forest canopies and approximately 90% of canopy individuals (Dejean & al. 2007).

Canopy ants rely significantly on carbohydrate-rich exudates for their nutritional requirements, and most rainforest ant species living in the canopy are primarily or partially herbivores (Davidson & al. 2003). Herbivorous ants feed mainly on extrafloral nectar, ant-plant food bodies, and exudates from sap-sucking trophobionts (e.g., Hemipterans) (Webber & al. 2007, Pringle & al. 2017) or plant wounds (Staab & al. 2017), because unlike many other arthropod groups, ants are unable to feed directly on plant leaves. However, the exudates from Hemipterans and plants include relatively low amounts of essential proteins that are paramount for ant colony growth (Davidson & Patrell-Kim 1996). Therefore, ants in the canopy tend to be nitrogen-limited because the available nitrogen is lower than on the forest floor (Davidson 1997, Yanoviak & Kaspari 2000).

Some canopy ants have evolved alternative diets that increase nitrogen access, like *Pseudomyrmex* twig ants feeding on specialized protein-rich food bodies provided by their ant-plant mutualists (Orona-Tamayo & al. 2013) or *Cephalotes* turtle ants consuming pollen (Urbani & De Andrade 1997). One understudied yet widespread potential source of nitrogen for nitrogen-limited canopy ants is mammalian urine and bird feces (Blüthgen & Feldhaar 2010). A detailed metabolic analysis found that human (mammalian) urine primarily consists of water, urea, and sodium, in addition to uric acid, creatinine, chlorine, potassium, and other compounds at lower concentrations (Bouatra & al. 2013). Bird excreta (often a combination of feces and urine) is believed to typically contain uric acid in much higher concentrations than urea (Bird & al. 2008), although a recent study suggests that uric acid may be converted to other compounds shortly prior to excretion (Crouch & al. 2020). While accessing nitrogen from urea and uric acid presents a challenge for many animals, including ants, some ant species may have evolved mutualistic bacteria (e.g., the gut symbiont *Blochmannia*) that convert such waste products into useable amino acids for their ant hosts (Feldhaar & al. 2007, Hu & al. 2018, Petit & al. 2020).

Urine and feces may be relatively scarce in the canopy environment when compared with honeydew-producing Hemipteran insects or other carbohydrate-rich food sources yet still carry great potential as a nitrogen-rich dietary supplement or even specialized primary food source (e.g., *Wasmannia auropunctata* (Roger, 1863), see Rosumek 2017; *Camponotus terebrans* (Lowne, 1865), see Petit & al. 2020). The availability of urine and feces on leaves and other arboreal surfaces is not well-studied, but excreta of large vertebrates such as the harpy eagle can cover substantial portions of the canopy surface around the nest-tree (de Miranda & al. 2023). Many arboreal ant species are often observed foraging on the surface of leaves for stray droplets and other dispersed foods (Davidson & al. 2004), suggesting the importance of low-density liquids for arboreal ant diets. In support of this observation, *Camponotus* carpenter ants living in a nitrogen-poor sandy soil environment continued to forage at dry sand even 29 days after the application of mammalian urine (Petit & al. 2020). The potential attractiveness of vertebrate urine and feces to at least some ant species is highlighted by a recent study demonstrating the attraction of *Camponotus*

*modoc* Wheeler, 1910 carpenter ants to bird feces semiochemicals (Renyard & al. 2022). With distinctly higher selection pressure to evolve the use of this food source in nitrogen-limited tropical canopies, even if such sources are present at lower densities than on the forest floor, urine and feces could contribute to vertical stratification in tropical forests and represent an important niche differentiation that contributes to the maintenance of high ant diversity in such habitats. The few existing empirical studies directly testing nutrient preference in ants across vertical strata have not yet established a general pattern (Davidson & al. 2003, Weiser & al. 2010, Arcila Hernández & al. 2012, Lasmar & al. 2023), and thus potential differences in preference for urine and other excreta between ground and canopy ants are currently unknown.

To explore how the incorporation of mammalian urine into ant diets varies between the ground and canopy, we conducted an observational field experiment in a tropical rainforest in southern Yunnan in Southwest China. We aimed to answer the following questions: Do ground and canopy ants show different preferences for mammalian urine in the tropical forest? Do ground and canopy ant communities differentiate according to differential preference for mammalian urine? We hypothesized that, because of the nitrogen-limited canopy environment, canopy ants would prefer urine baits over sugar (carbohydrates) baits. In contrast, we hypothesized that ground ants would prefer sugar over urine, given reduced access to carbohydrate-rich food sources when compared with canopy ants. We further hypothesized that, because of these food preference differences, the ground ant and canopy ant communities would clearly differentiate according to vertical strata and bait type.

## Material and methods

### Study site

The study was conducted in a 1.44 ha square plot in a lowland seasonal rainforest in Xishuangbanna Dai Autonomous Prefecture, Yunnan Province, China, in one of the five subdistricts that together form the Xishuangbanna National Nature Reserve (101° 34' 59.1'' E, 21° 37' 2.6'' N, 634 m above sea level). This region is characterized by a typical monsoon climate that alternates between a dry season from November to February and a rainy season from May to October, with a transition period from March to May (Sharma & al. 2022). The mean annual temperature is 21.8 °C, and the mean annual precipitation is 1493 mm (Cao & al. 2008). An 81 m high canopy crane (TCT7015-10E, Zoomlion Heavy Industry, Changsha, China) was built at the center of the square plot in December 2014 and is managed by the Xishuangbanna Tropical Botanical Garden (XTBG). The crane's 60 m long arm provides access to the canopy and sub-canopy within a 1.13 ha circular area (Deng & al. 2020).

The Xishuangbanna National Natural Reserve covers an area of 242,510 ha that comprises the largest tropical forest area in China. The 20 ha plot established in the nature reserve contains 95,834 free-standing trees with diameter at breast height (DBH) ≥ 1 cm, belonging to 468 species in 213 genera and 70 families (Cao & al. 2008). Within the 1.44 ha plot investigated, the forest is dominated by *Parashorea chinensis* Wang Hsie, 1977, more than 30 m high in the canopy layer. It is accompanied by *Semecarpus reticulata* Lecomte, 1907, *Pometia tomentosa* Teijsm & Binn, 1866, *Sloanea tomentosa* Rehder & Wilson, 1915, and *Canarium album* Raeusch, 1825. The sub-canopy layer (16 - 30 m) is dominated by *Pseuduvaria trimera* Su & Saunders, 2006, *Barringtonia fusicarpa* Hu, 1963, *Litsea dilleniifolia* Pai & Huang, 1978, *Diospyros atrotricha* Li, 1965, and *Ficus langkokensis* Drake, 1896. The understory layer (6 - 16 m) is dominated by *Mitrephora maingayi* Hook & Thomson, 1872, *Baccaurea ramiflora* Lour, 1790, and *Pittosporopsis kerrii* Craib, 1911 (Shen & al. 2022).

### Experiment design

Ants were sampled from two tree species, *Parashorea chinensis* and *Pseuduvaria trimera*, in the canopy / sub-canopy (henceforth "canopy") and on the ground below selected tree individuals. As noted above, these two tree species are common dominant tree species in the XTBG plot. Neither of these tree species is a nitrogen-fixing species, reducing the potential indirect influence of nitrogen-fixing on host ant communities via herbivore mutualists. For each tree species, 6 individuals (12 total trees) were selected. All sampled trees were at least 5 m apart to ensure that none of the tree branches were touching or connected by lianas. The DBH of each tree was measured at 1.3 m above ground, and the height of each tree was estimated relative to known, measured reference points when sampling ants in the canopy.

Baited pitfall traps included two food treatments that reflected natural resources available to ants in a seasonal rainforest: sugar (carbohydrate [COH]; 7:1 water-honey ratio by volume; commercial honey) and mammalian urine (nitrogen [N] and salt [NaCl]; 2:1 water-urine ratio by volume; urine provided by B.D.B. over 48 hours and combined to avoid potential effects of dietary variation over the urine collection period). Plain water was used as the control. 50 ml of each solution were added into 120 ml vials (4.0 cm diameter × 10.5 cm length) with a drop of detergent added to each trap to facilitate ant drowning and capture. In the canopy, three traps (sugar, urine, and plain water) were set at a similar height from the ground, with two replicates at approximately opposite sides of the tree crown (a total of six traps per tree). For each replicate, three traps were wired on the same main branch with two or three sub-branches depending on availability. If three sub-branches were available, each trap was placed on a separate sub-branch. If only two were available, a trap was placed on each sub-branch and the remaining trap on the main branch (Fig. S1, as digital supplementary material to this article, at the journal's web pages). On the ground, for each of the two replicates per tree, three traps were

set 1 m from the base of the tree trunk and each replicate set of traps were placed at opposite ends of the tree. In both strata, the traps were set approximately 0.4 m apart from each other (direct linear distance on the ground, and branch-length distance in the canopy) to allow for food choice while minimizing interference between different baits. To reduce the chance of ants unwittingly falling into the traps and to prevent the vial liquid from pouring out, the traps were vertically placed with the opening of the trap set approximately 1.5 cm above the ground or branch edge, rather than flush with the surface (Fig. S1). This baited pitfall trap design allowed for foragers to taste the bait and potentially recruit nestmates while also collecting attracted workers over time through falls while feeding and / or death due to the neutral detergent added to the baits.

After 48 hours, the traps were collected and brought to the lab. Three canopy traps spilled (two urine and one sugar), likely because of vertebrate interference in the canopy, and so a total of 141 traps were retained for specimen sorting and identification. All sampled individuals were identified to genus and species (or confirmed morphospecies) using images and descriptions available from online databases (AntWeb 2022, antweb.org accessed November 2022; Antwiki 2022, antwiki.org accessed November 2022) and two regional references (Xu 2002, Zhou & al. 2020). Mounted vouchers for each species / morphospecies identified in each pitfall trap are deposited in the Forest Canopy Ecology Lab at XTBG.

### Statistical analyses

Generalized linear mixed models (GLMMs) were constructed using glmmTMB (Brooks & al. 2017) to analyze the differences in ant richness and abundance between food treatments and vertical strata. Six potential predictor variables were initially considered: food treatment, strata, tree species, tree height, trap height, and tree DBH. Individual tree identity was also included as a random effect. First, for the richness data, potential overdispersion was tested for a model using a Poisson distribution including all potential predictors using the "check_overdispersion" function in the R package performance (Lüdecke & al. 2021). Zero-inflation was also checked using the "check_zeroinflation" function in the performance package. Because no overdispersion or zero inflation was detected, the Poisson distribution was used for the richness model. Abundance data were log-transformed and a Gaussian model including all potential predictors was assigned for the abundance model. For ant community diversity studies, abundance is often avoided because of the eusocial nature of ants that can render individual worker abundance less ecologically meaningful. However, in this study, the focus is food preference, and abundance numbers should be ecologically meaningful in this case (e.g., for a given replicate of three traps placed near each other, one worker each in the water and sugar traps versus 100 workers in the urine trap demonstrates meaningful differences in preference).

Using these models for richness and abundance, multicollinearity of predictors was then tested by calculating each predictor's variance inflation factor (VIF) using the "check_collinearity" function in the performance package and excluding those with a VIF greater than 5 (James & al. 2013), which included tree height and tree DBH for both models and trap height for the abundance model. Next, model selection was conducted using the corrected Akaike Information Criterion (AICc), with a delta AICc cut-off value of 2, to select the simplest model and exclude any predictors that failed to add meaningful information. The "dredge" function in the R package MuMIn (Barton & Barton 2018) was used to compare each model, including every possible combination of predictors and predictor interactions. Lastly, for the selected richness model and log-transformed abundance model, the "simulateResiduals" function in the R package DHARMa (Hartig 2022) was implemented to confirm adequate model fits (slight deviation from homoscedasticity was detected for low values in the abundance model, but other assumptions were well met). The "Anova" function in the R package car (Fox & Weisberg 2018) was then implemented to assess the significance of each predictor in each model. The "emmeans" function in the R package emmeans (Lenth & al. 2019), which incorporates a Tukey adjustment for multiple comparisons, was used to assess pairwise differences (e.g., ground sugar vs. canopy sugar).

Lastly, the "adonis" function with Bray-Curtis dissimilarity matrix and 999 permutations in the R package vegan (PERMANOVA: permutational multivariate analysis of variance; Oksanen & al. 2013) was used to test whether the sampled ant community composition differed between nutrient preferences and vertical strata. Separations among treatments were visualized with non-metric multidimensional scaling (NMDS) using a Bray-Curtis dissimilarity matrix with logarithmic transformation and the default 20 random restarts using the "metaMDS" function in vegan.

For all statistical tests involving calculation of a p-value (p), an alpha of 0.05 was used to assess statistical significance. All statistical analyses were performed with R version 4.2.2 (R Core Team 2022) in the RStudio graphical user interface (Posit Team 2023).

## Results

### Sampling

We collected 5568 individuals representing 21 genera and 47 species (Fig. 1, Tab. S1). The maximum abundance of a single trap was more than 1500 individuals, with a maximum richness of 9 species. Including control traps, mean ground richness was 67.3% higher than mean canopy richness (1.819 species / trap and 1.087 species / sample, respectively). Genera found on the ground were largely distinct from those in the canopy. Based on moderate or high abundance, the ground community was characterized by *Acanthomyrmex*, *Aphaenogaster*, *Brachyponera*, *Ectomomyrmex*, *Kartidris*, *Leptogenys*, *Myrmecina*, *Nylanderia*, *Odontoponera*, *Pheidole*, *Technomyrmex*,
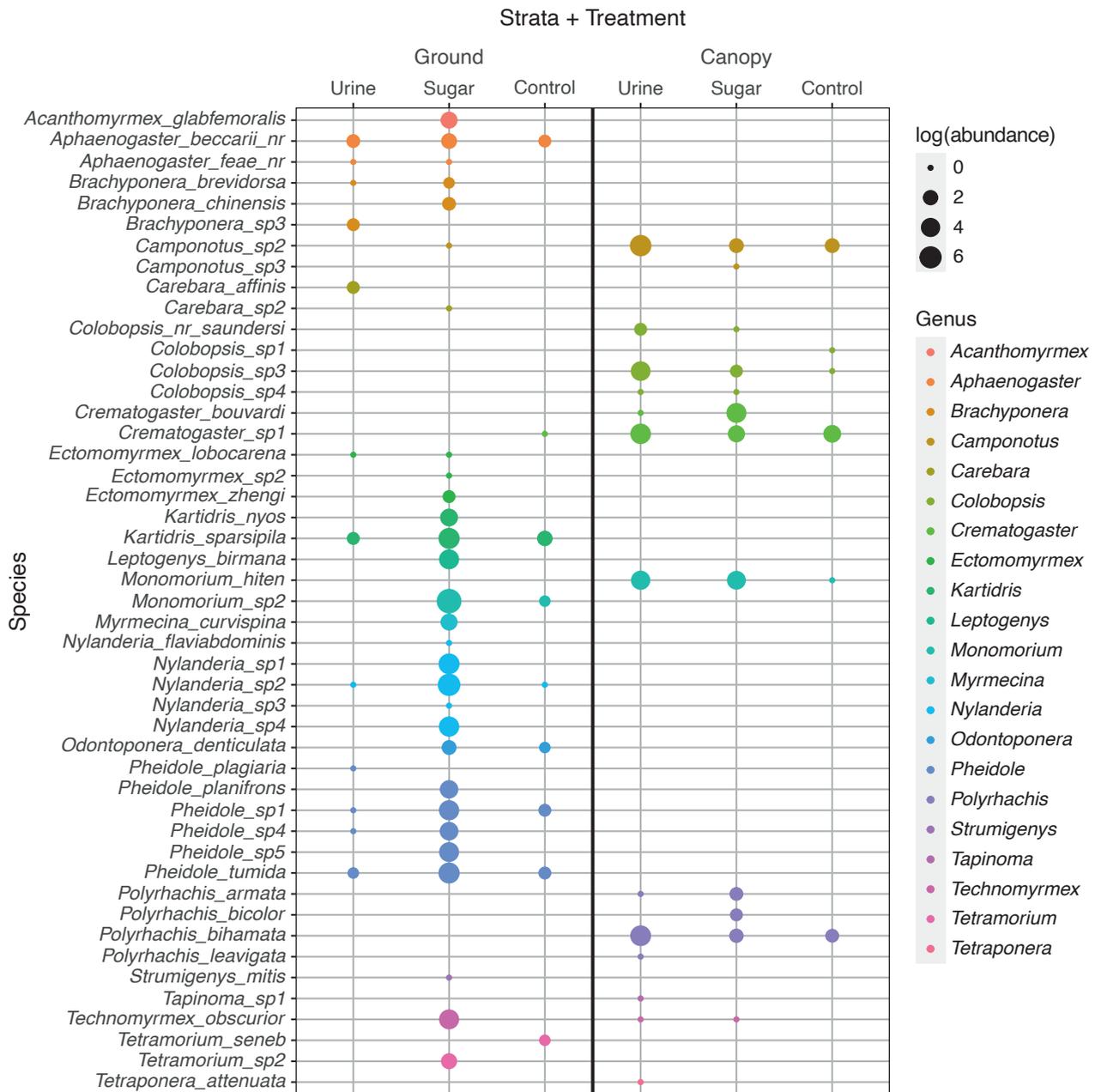
**Fig. 1:** Log-abundance of each ant species collected, separated by combinations of strata and treatment. Color corresponds to genus, and bubble size corresponds to log-abundance.

and *Tetramorium*, while the canopy community was characterized by *Camponotus*, *Colobopsis*, *Crematogaster*, and *Polyrhachis* (Fig. 1). Only *Monomorium* was found at moderate or high abundance in both ground and canopy strata (*Monomorium* sp. 2 on the ground and *Monomorium hiten* TERAYAMA, 1996 in the canopy; Fig. 1).

### Strata and treatment effects

Our selected GLMMs for richness and abundance included only stratum, food treatment, and their interaction (Tabs. 1 and 2). Richness was significantly higher in ground traps than in canopy traps and significantly higher in sugar traps than in urine traps (p < 0.01; Fig. 2a,

Tab. 3). There was also a significant interaction between stratum and food treatment, with higher richness in sugar traps on the ground and in urine traps in the canopy (p < 0.001; Fig. 2a, Tab. 3). Analyses of pairwise differences demonstrated that ground sugar trap richness was significantly higher than both ground urine trap richness and canopy sugar trap richness (p < 0.001 in each case; Tab. 4), whereas higher canopy urine trap richness than canopy sugar trap richness was not statistically significant (p = 0.932). There was also a non-significant trend of higher richness in canopy urine traps versus ground urine traps (p = 0.111; Fig. 2a, Tab. 4). Abundance was only marginally significantly higher on the ground than in the
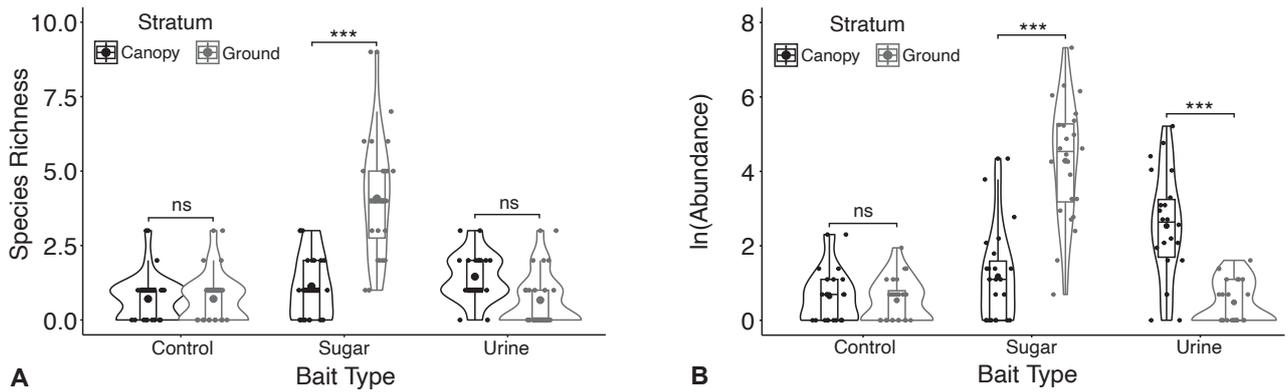
**Fig. 2:** Violin plots with bar graphs and pitfall trap sample points displayed for (a) species richness and (b) natural log-abundance. Pairwise comparisons between canopy and ground strata for each bait type are displayed (ns = "not significant", *** = $p < 0.001$; statistics based on estimated marginal means calculated based on generalized linear mixed models using "emmeans"; Lenth & al. 2019).
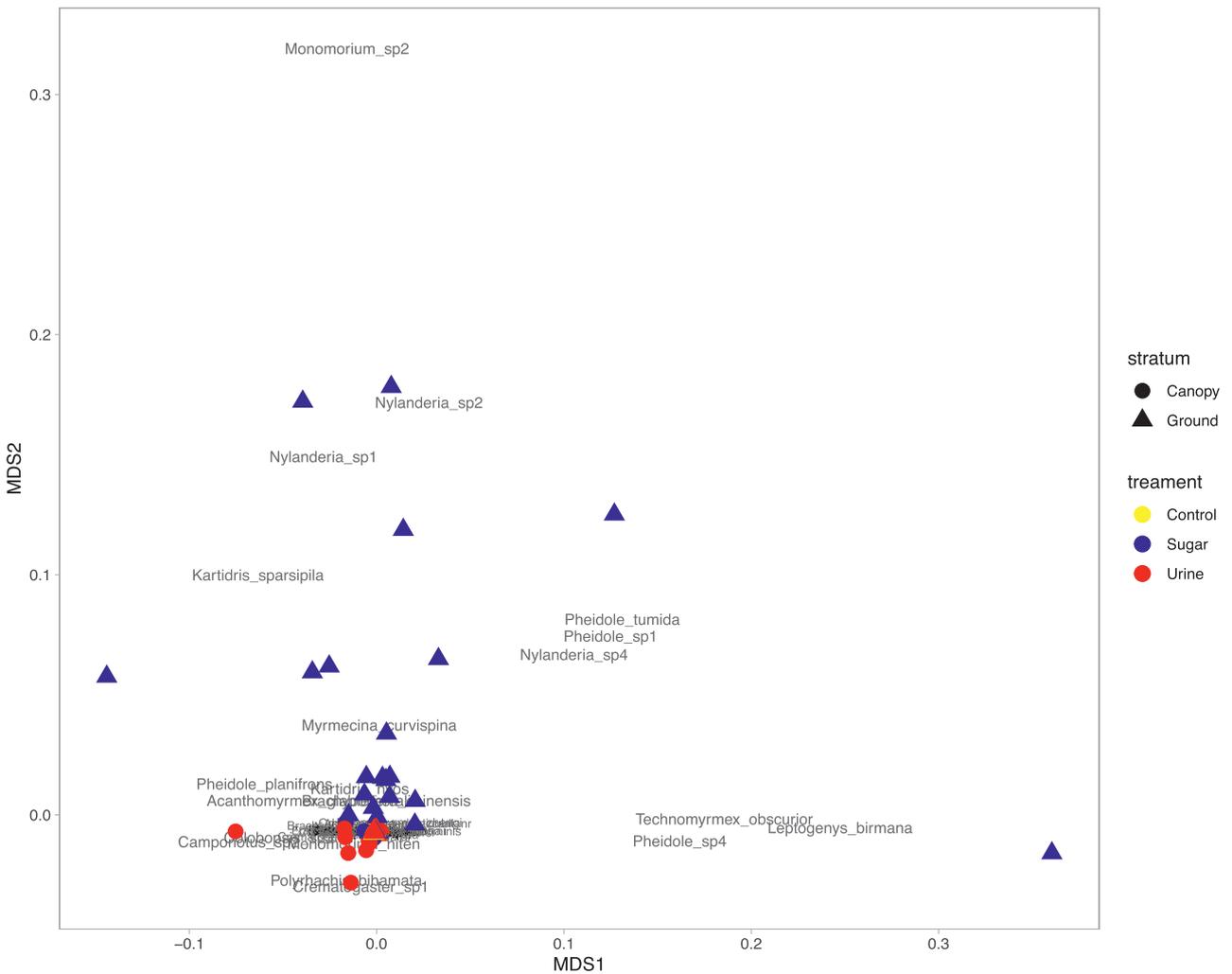


**Fig. 3:** Non-metric multidimensional scaling plot produced using a Bray-Curtis dissimilarity matrix with logarithmic transformation. Point shape corresponds to stratum (circle = canopy, triangle = ground) and color corresponds to treatment (yellow = control, blue = sugar, red = urine). Note that the control data points cluster around the origin, and are obscured by shared sugar and urine data points.

**Tab. 1:** Corrected Akaike Information Criterion (AICc) model comparison for species richness, conducted among generalized linear mixed models with individual tree identity as a random effect in all models. The selected model (delta AICc < 2; lowest-parameter model among available options) is in bold. Trap_Ht = Trap height (meters from the ground); Tree_Sp = Tree species (*Parashorea chinensis* or *Pseuduvaria trimera*). Log-likelihood (logLik) is also displayed; df = degrees of freedom.

| Model | df | logLik | AICc | delta | weight |
|---|---|---|---|---|---|
| Stratum*Treatment + Trap_Ht | 8 | -185.633 | 388.356 | 0.000 | 0.453 |
| Stratum*Treatment + Trap_Ht + Tree_Sp | 9 | -184.867 | 389.109 | 0.752 | 0.311 |
| **Stratum*Treatment** | **7** | **-187.701** | **390.244** | **1.887** | **0.176** |
| Stratum*Treatment + Tree_Sp | 8 | -187.661 | 392.413 | 4.057 | 0.060 |
| Treatment + Trap_Ht | 5 | -203.338 | 417.120 | 28.764 | 0.000 |
| Treatment + Trap_Ht + Tree_Sp | 6 | -202.599 | 417.825 | 29.469 | 0.000 |
| Stratum + Treatment + Trap_Ht | 6 | -203.309 | 419.244 | 30.888 | 0.000 |
| Stratum + Treatment + Trap_Ht + Tree_Sp | 7 | -202.585 | 420.011 | 31.655 | 0.000 |
| Stratum + Treatment | 5 | -205.450 | 421.345 | 32.989 | 0.000 |
| Stratum + Treatment + Tree_Sp | 6 | -205.420 | 423.468 | 35.111 | 0.000 |
| Treatment | 4 | -211.929 | 432.153 | 43.796 | 0.000 |
| Treatment + Tree_Sp | 5 | -211.875 | 434.195 | 45.838 | 0.000 |
| Trap_Ht | 3 | -235.983 | 478.141 | 89.784 | 0.000 |
| Trap_Ht + Tree_Sp | 4 | -235.260 | 478.815 | 90.458 | 0.000 |
| Stratum + Trap_Ht | 4 | -235.919 | 480.131 | 91.775 | 0.000 |
| Stratum + Trap_Ht + Tree_Sp | 5 | -235.260 | 480.964 | 92.607 | 0.000 |
| Stratum | 3 | -237.883 | 481.941 | 93.585 | 0.000 |
| Stratum + Tree_Sp | 4 | -237.857 | 484.008 | 95.652 | 0.000 |
| Null | 2 | -244.446 | 492.979 | 104.623 | 0.000 |
| Tree_Sp | 3 | -244.400 | 494.974 | 106.618 | 0.000 |

**Tab. 2:** Corrected Akaike Information Criterion (AICc) model comparison for log-abundance, conducted among generalized linear mixed models with individual tree identity as a random effect in all models. The selected model (delta AICc < 2; lowest-parameter model among available options) is in bold. Tree_Sp = Tree species (*Parashorea chinensis* or *Pseuduvaria trimera*). Log-likelihood (logLik) is also displayed; df = degrees of freedom.

| Model | df | logLik | AICc | delta | weight |
|---|---|---|---|---|---|
| Stratum*Treatment + Tree_Sp | 9 | -208.232 | 435.838 | 0.000 | 0.606 |
| **Stratum*Treatment** | **8** | **-209.804** | **436.700** | **0.862** | **0.394** |
| Treatment | 5 | -258.610 | 527.664 | 91.826 | 0.000 |
| Stratum + Treatment | 6 | -257.781 | 528.189 | 92.352 | 0.000 |
| Treatment + Tree_Sp | 6 | -257.866 | 528.360 | 92.522 | 0.000 |
| Stratum + Treatment + Tree_Sp | 7 | -257.064 | 528.969 | 93.132 | 0.000 |
| Null | 3 | -279.838 | 565.851 | 130.014 | 0.000 |
| Stratum | 4 | -279.178 | 566.649 | 130.812 | 0.000 |
| Tree_Sp | 4 | -279.333 | 566.960 | 131.122 | 0.000 |
| Stratum + Tree_Sp | 5 | -278.693 | 567.830 | 131.992 | 0.000 |

**Tab. 3:** Type II analysis of variance results for richness and abundance models (generalized linear mixed models) based on the likelihood-ratio chi-squared test (LR Chisq); df = degrees of freedom.

| | Richness | | | Abundance | | |
|---|---|---|---|---|---|---|
| Factor | LR Chisq | df | p-value | LR Chisq | df | p-value |
| Stratum | 8.221 | 1 | 0.004 | 3.292 | 1 | 0.070 |
| Treatment | 51.932 | 2 | < 0.001 | 98.738 | 2 | < 0.001 |
| Stratum:Treatment | 31.165 | 2 | < 0.001 | 137.462 | 2 | < 0.001 |

**Tab. 4:** Pairwise comparisons (emmeans) of strata-treatment combinations for the richness model (generalized linear mixed model), with lower (LCL) and upper (UCL) asymptotic confidence intervals displayed; SE = standard error, df = degrees of freedom.

| Stratum-Treatment | emmean | SE | df | Asymp LCL | Asymp UCL |
|---|---|---|---|---|---|
| Canopy Control | -0.345 | 0.243 | Inf | -0.820 | 0.131 |
| Ground Control | -0.345 | 0.243 | Inf | -0.820 | 0.131 |
| Canopy Sugar | 0.123 | 0.196 | Inf | -0.262 | 0.507 |
| Ground Sugar | 1.407 | 0.101 | Inf | 1.209 | 1.605 |
| Canopy Urine | 0.375 | 0.177 | Inf | 0.028 | 0.721 |
| Ground Urine | -0.405 | 0.250 | Inf | -0.895 | 0.085 |
| **contrast** | **estimate** | **SE** | **df** | **z-ratio** | **p-value** |
| Canopy Control - Ground Control | 0.000 | 0.343 | Inf | 0.000 | 1.000 |
| Canopy Control - Canopy Sugar | -0.467 | 0.312 | Inf | -1.499 | 0.665 |
| Canopy Control - Ground Sugar | -1.752 | 0.263 | Inf | -6.667 | <.0001 |
| Canopy Control - Canopy Urine | -0.720 | 0.300 | Inf | -2.397 | 0.157 |
| Canopy Control - Ground Urine | 0.061 | 0.348 | Inf | 0.174 | 1.000 |
| Ground Control - Canopy Sugar | -0.467 | 0.312 | Inf | -1.499 | 0.665 |
| Ground Control - Ground Sugar | -1.752 | 0.263 | Inf | -6.668 | <.0001 |
| Ground Control - Canopy Urine | -0.720 | 0.300 | Inf | -2.397 | 0.157 |
| Ground Control - Ground Urine | 0.061 | 0.348 | Inf | 0.174 | 1.000 |
| Canopy Sugar - Ground Sugar | -1.284 | 0.221 | Inf | -5.822 | <.0001 |
| Canopy Sugar - Canopy Urine | -0.252 | 0.264 | Inf | -0.955 | 0.932 |
| Canopy Sugar - Ground Urine | 0.528 | 0.318 | Inf | 1.662 | 0.557 |
| Ground Sugar - Canopy Urine | 1.032 | 0.204 | Inf | 5.070 | <.0001 |
| Ground Sugar - Ground Urine | 1.812 | 0.270 | Inf | 6.722 | <.0001 |
| Canopy Urine - Ground Urine | 0.780 | 0.306 | Inf | 2.548 | 0.111 |

canopy (p = 0.070; Tab. 3) but significantly higher in sugar traps than in urine traps (p < 0.001; Tab. 3). There was also a significant stratum-treatment interaction, with higher abundance in sugar traps on the ground and urine traps in the canopy (p < 0.001; Tab. 3). Analyses of pairwise differences found that abundance was significantly higher in ground sugar traps than in ground urine traps, canopy urine traps than in canopy sugar traps, ground sugar traps than in canopy sugar traps, and canopy urine traps than in ground urine traps (p < 0.001 in all cases; Fig. 2b, Tab. 5).

### Community composition

NMDS ordination demonstrated a distinct differentiation between canopy ants at urine baits and ground ants at sugar baits (Fig. 3). Additionally, the dispersion of ground ants at sugar baits is much larger than that of canopy ants at urine baits. Only *Camponotus* sp. 2, *Colobopsis* sp. 3, *Crematogaster* sp. 1, *Monomorium hiten*,

**Tab. 5:** Pairwise comparisons (emmeans) of strata-treatment combinations for the abundance model (generalized linear mixed model), with lower and upper asymptotic confidence intervals (CL) displayed; SE = standard error, df = degrees of freedom.

| Stratum-Treatment | emmean | SE | df | Lower CL | Upper CL |
|---|---|---|---|---|---|
| Canopy Control | 0.647 | 0.219 | 133 | 0.214 | 1.080 |
| Ground Control | 0.536 | 0.219 | 133 | 0.104 | 0.969 |
| Canopy Sugar | 1.182 | 0.223 | 133 | 0.741 | 1.624 |
| Ground Sugar | 4.285 | 0.219 | 133 | 3.852 | 4.718 |
| Canopy Urine | 2.535 | 0.228 | 133 | 2.083 | 2.987 |
| Ground Urine | 0.481 | 0.219 | 133 | 0.049 | 0.914 |
| **Contrast** | **Estimate** | **SE** | **df** | **t-ratio** | **p-value** |
| Canopy Control - Ground Control | 0.111 | 0.309 | 133 | 0.358 | 0.999 |
| Canopy Control - Canopy Sugar | -0.536 | 0.313 | 133 | -1.713 | 0.526 |
| Canopy Control - Ground Sugar | -3.638 | 0.309 | 133 | -11.762 | <.0001 |
| Canopy Control - Canopy Urine | -1.888 | 0.316 | 133 | -5.969 | <.0001 |
| Canopy Control - Ground Urine | 0.166 | 0.309 | 133 | 0.536 | 0.995 |
| Ground Control - Canopy Sugar | -0.646 | 0.313 | 133 | -2.067 | 0.311 |
| Ground Control - Ground Sugar | -3.749 | 0.309 | 133 | -12.120 | <.0001 |
| Ground Control - Canopy Urine | -1.999 | 0.316 | 133 | -6.319 | <.0001 |
| Ground Control - Ground Urine | 0.055 | 0.309 | 133 | 0.177 | 1.000 |
| Canopy Sugar - Ground Sugar | -3.103 | 0.313 | 133 | -9.923 | <.0001 |
| Canopy Sugar - Canopy Urine | -1.352 | 0.320 | 133 | -4.232 | 0.001 |
| Canopy Sugar - Ground Urine | 0.701 | 0.313 | 133 | 2.243 | 0.226 |
| Ground Sugar - Canopy Urine | 1.750 | 0.316 | 133 | 5.534 | <.0001 |
| Ground Sugar - Ground Urine | 3.804 | 0.309 | 133 | 12.297 | <.0001 |
| Canopy Urine - Ground Urine | 2.054 | 0.316 | 133 | 6.493 | <.0001 |

**Tab. 6:** Permutational multivariate analysis of variance results testing significant differences in composition between groups, df = degrees of freedom, SumOfSqs = sum of squares.

| Factor | df | SumOfSqs | R² | F | p-value |
|---|---|---|---|---|---|
| Treatment | 2 | 0.727 | 0.087 | 7.502 | 0.001 |
| Stratum | 1 | 0.352 | 0.042 | 7.267 | 0.001 |
| Treatment:Stratum | 2 | 0.774 | 0.092 | 7.989 | 0.001 |
| Residual | 135 | 6.542 | 0.779 | | |
| Total | 140 | 8.396 | 1.000 | | |

and *Polyrhachis bihamata* (Fabricius, 1775) are clearly associated with canopy urine baits, whereas 14 species are associated with ground sugar baits, including *Acanthomyrmex glabfemoralis* Zhou & Zheng, 1997, *Brachyponera chinensis* (Emery, 1895), two *Kartidris* species, *Leptogenys birmana* Forel, 1900, *Myrmecina curvispina* Zhou & al., 2008, three *Nylanderia* species, four *Pheidole* species, and *Technomyrmex obscurior* Wheeler, 1928 (Fig. 3). Consistent with this qualitative pattern, the PERMANOVA found significant differences in the composition according to stratum and treatment and their interaction (p < 0.01 in all cases; Tab. 6).

## Discussion

We used an observational field experiment to investigate the hypothesis that mammalian urine is an alternative source of nitrogen for nitrogen-limited canopy ants and a potential contributing factor to vertical stratification in tropical forests. We compared the attraction of ants to traps baited with urine, sugar, and water on the ground

and in the canopy in a lowland tropical forest in southern China. Our results indicate that the abundance and richness of ground ants were significantly higher at sugar baits, whereas the abundance of canopy ants was significantly higher at urine baits (Figs. 1 - 2). Richness did not significantly differ between bait types in the canopy, although there was a trend of higher richness in urine baits. Furthermore, the tropical forest ant community significantly differentiated according to vertical strata and food preference (Fig. 3). The results from our study, which is to our knowledge unique in its inclusion of urine across vertical strata over an extended sampling period (48 hours) with water included as a control, support the conclusion that mammalian urine is more strongly preferred as an alternative food source for canopy than for ground ants. This distinct vertical stratification of food preference may represent an important case of niche partitioning that contributes to high ant species diversity in tropical rainforests (SCHULTHEISS & al. 2022) as well as high species turnover between ground and canopy strata (BRÜHL & al. 1998).

The canopy ant species found in moderate to high abundance in urine traps included three genera of the subfamily Formicinae (*Camponotus*, *Colobopsis*, *Polyrhachis*), and two genera of the subfamily Myrmicinae (*Crematogaster* and *Monomorium*) (Fig. 1). Two genera of Dolichoderinae, *Tapinoma* and *Technomyrmex*, and one genus of Pseudomyrmecinae, *Tetraponera*, were also captured in urine traps, but these were in such low abundances (only one worker each) that they likely represent incidental / accidental capture (Tab. S1). Our finding that multiple formicine genera are attracted to urine is consistent with previous work, particularly on *Camponotus*. For example, FELDHAAR & al. (2007) demonstrated that *Blochmannia floridanus* SAUER & al., 2000 endosymbionts upgrade urea diets of *Camponotus floridanus* (BUCKLEY, 1866) host ants and noted the presence of the endosymbiont in other genera of the tribe Camponotini, which includes *Polyrhachis* and *Colobopsis*. Furthermore, SHETTY (1982) found that *Camponotus compressus* (FABRICIUS, 1787) ants were attracted to and ingest mammalian urine, while RENYARD & al. (2022) showed that *Camponotus modoc* ants are attracted to bird feces semiochemicals.

In contrast, our finding of two separate myrmicine genera in the canopy urine traps is somewhat more surprising, as such feeding behavior has not been documented frequently in Myrmicinae, the most diverse ant subfamily. However, SPRENGER & al. (2020) found that the parabiotic species *Crematogaster levior* LONGINO, 2003 fed at uric acid baits at a higher frequency than their *Camponotus femoratus* (FABRICIUS, 1804) mutualists. Intriguingly, MENZEL & al. (2012) reported the opposite result for the parabiotic species *Crematogaster modiglianii* EMERY, 1900 and *Camponotus rufifemur* EMERY, 1900, with *Crematogaster modiglianii* avoiding urea and uric acid baits while *Camponotus rufifemur* foraged at both baits. HU & al. (2018) also demonstrated that *Cephalotes* turtle ant endosymbiotic bacteria are capable of recycling urea. To our knowledge, the consumption of urine or uric acid

has not been previously documented for any *Monomorium* species. It is notable that in our study, the canopy species *Monomorium hiten* was found in moderate numbers in urine and sugar baits while the ground species *Monomorium* sp. 2 was found in high numbers in sugar baits and completely absent from urine baits (Fig. 1, Tab. S1). Our results suggest that potential urea- and uric acid-upgrading endosymbiotic bacteria may exist more broadly across arboreal myrmicine genera even outside of specialized taxa like parabiotic *Crematogaster* species and *Cephalotes* turtle ants.

We believe it is plausible that canopy ants primarily utilize urine to extract nitrogen rather than salt, based on the few existing studies that involve urine or urea as opposed to putative artificial proxies for these substances. As noted earlier, previous work has demonstrated that both *Cephalotes* turtle ants (HU & al. 2018) and ground foraging *Camponotus* carpenter ants (PETIT & al. 2020) are capable of extracting nitrogen from urea. In the latter study, the results further showed that the ants avoided salt baits. In a separate study with sampling over two, one-hour diurnal periods, ARCILA HERNÁNDEZ & al. (2012) found that both ground and canopy ants were disproportionately attracted to salt baits while the abundance of ants at urine baits was indistinguishable from that at water (control), sugar, and protein baits. These studies suggest that ant usage of urine is distinct from usage of salt. However, it is certainly possible that canopy ants derive salt in addition to – or instead of – nitrogen from urine. For example, KASPARI & al. (2009), conducting sampling over a one-hour diurnal period, found that urine baits were visited by ground-foraging ants at the statistically same frequency as salt baits, although this frequency was not compared with a water control. Furthermore, a recent study by LASMAR & al. (2023) found higher salt preference among arboreal ants than among subterranean and epigaeic ants in two of the six biomes studied (Amazon and Atlantic), a finding that is consistent with a study in the Brazilian Cerrado biome (VIEIRA & VASCONCELOS 2015). Overall, we posit that our controlled experiment, involving a time-extensive sampling that captures both diurnal and nocturnal foragers in the canopy and on the ground, provides uniquely robust evidence that mammalian urine is an alternative source of nitrogen for multiple genera of tropical canopy ants while being largely avoided by the diverse tropical ground ant community. Future work that incorporates sodium baits combined with an extended (24 hours or longer) sampling design across vertical strata would be beneficial to confirm these results and interpretation.

Nitrogen limitation has long been proposed as a selection pressure that exists for ants in the canopy more strongly than those on the ground, because of canopy ants' reliance on relatively nitrogen-poor plant and insect mutualist exudates (DAVIDSON & PATRELL-KIM 1996). This selection pressure may be high enough to have promoted the evolution of symbioses with bacterial mutualists that provision nitrogen for host ants through recycling of urea (HU & al. 2018), while the higher availability of nitrogen

on the ground (e.g., large insect carcasses) lessens such selection pressure for ground ants. Notably, Petit & al. (2020) found that ground dwelling *Camponotus* ants mine sand for urine in a sandy soil environment that is unusually nitrogen-poor compared with typical ground habitats. In our study, with only a few exceptions, the relatively high urine:sugar foraging ratio in the canopy and relatively low ratio on the ground is highly consistent across different genera representing multiple different subfamilies (Fig. 1), further supporting the mechanistic explanation of nitrogen as a general limitation for canopy ants.

### Study limitations

Our study utilized urine from only one of this study's authors (B.D.B.), and thus our urine sampling does not account for potential differences in urine between people or for potential differences in urine between different mammal species. However, it is not clear how such differences would result in the vertical stratification in preference for urine that is apparent from our results – any such effect should equally impact the canopy and ground urine traps. Furthermore, Petit & al. (2020) report from experimental and personal observations that *Camponotus* ants consume urine from a diversity of mammal species including humans, kangaroos, and cats. Nevertheless, future studies could incorporate urine from multiple sources when conducting baited pitfall trap sampling.

We interpret our findings as a potential preference for urine among some canopy ants as an alternative source of nitrogen. As also noted above, ant preference for urine in the canopy could instead be due to sodium preference (Kaspari & al. 2009, Vieira & Vasconcelos 2015), another component in urine. To address this limitation in future studies with similarly long sample periods (> 24 hours), sodium baits could be included in addition to urine, sugar, and water baits (Arcila Hernández & al. 2012).

One general limitation of food preference studies for inferring overall foraging behavior is that the food preferred in experiments could represent nutrients that are lacking for the organism rather than ones that constitute the primary components of the organism's diet. In our case, there may already be ample urine on the forest floor and substantial carbohydrate sources in the canopy – ground ants could in fact consume urine frequently but have less access to sugar, and thus flock to the resource when it is available. One method of differentiating these alternative mechanisms could be testing food preferences of collected canopy and ground ant species' colonies in the lab after a starvation period of several days. However, notably, if ground ants frequently consume urine as a component of their diets, there would surely still be some moderate number of ground ants in our urine traps. Instead, despite high abundance numbers in ground sugar traps and significantly more species on the ground than in the canopy, we found exceedingly low numbers of ground ants in urine traps, even below those found in the control water traps (Figs. 1 - 2), consistent with accidental falls rather than foraging for consumption.

### Conclusions

The data in our study highlight the uniqueness of the canopy environment in tropical forests, a threatened habitat in southeast Asia (Sodhi & al. 2004) and around the globe (Hoang & Kanemoto 2021). Importantly, mammalian urine is a food source provided by taxa only distantly related to Formicidae. Thus, the conservation of a wide range of taxonomic groups is likely necessary to effectively conserve diverse insect communities like ants. With increasing evidence of a global decline in insect communities (Van Klink & al. 2020, Wagner & al. 2021), it is abundantly clear that both expanding and applying our understanding of the complex species interactions that support insect diversity is necessary to avoid accelerating local and total extinction of innumerable insect species.

## Conflicts of interest

The authors have no conflicts of interest to declare.

## Author contributions

B.D.B., Q.D., M.D., Y.N., D.M.N., and A.N. conceived the ideas and designed methodology; B.D.B., Q.D., M.D., Y.N., and D.M.N. collected the data; B.D.B., Q.D., D.M., Y.N., and D.M.N. identified individuals to genus and species / morphospecies, with B.D.B. confirming initial identifications; B.D.B., D.M., and Y.N. analyzed the data; B.D.B. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

## Data availability

All data files associated with this study, including raw data and R script, are available via the Dryad data repository at <https://doi.org/10.5061/dryad.73n5tb33w>.

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