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Distribution and dietary regulation of an associated facultative Rhizobiales-related bacterium in the omnivorous giant tropical ant, *Paraponera clavata*

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Abstract We document a facultative *Bartonella*-like Rhizobiales bacterium in the giant tropical ant, *Paraponera clavata*. In a lowland tropical rainforest in Costa Rica, 59 colonies were assayed for the prevalence of the *Bartonella*-like bacterium (BLB), 14 of which were positive. We addressed three questions: First, how does the prevalence of BLB within colonies vary with environmental conditions? Second, how does diet affect the prevalence of BLB in *P. clavata*? Third, how does the distribution of BLB among colonies reflect ambient differences in food resources and foraging habits? A variety of environmental variables that may be predictive of the presence of BLB were measured, and diet manipulations were conducted to test whether the prevalence of BLB responded to supplemental carbohydrate or prey. The ambient frequency of BLB is much higher in young secondary forests, but is nearly absent from older secondary forests. The prevalence of BLB inside field colonies increased over the duration of a 2-week carbohydrate supplementation; however, water and prey supplementation did not alter the prevalence of BLB. The diets of the colonies located in young secondary forest, compared to other habitats, have a diet richer in carbohydrates and lower in prey. The abundance of carbohydrate, or the relative lack of N, in a colony's diet influences the occurrence of the BLB microbe in *P. clavata*. As experimental diet manipulations can affect the facultative presence of an N-cycling microbe, a consistent diet shift in diet may facilitate the emergence of tighter symbioses.

Keywords Carbohydrate · Extrafloral nectar · N cycling · Secondary forest · Symbiosis · Tropical rainforest

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Introduction

Nutritional symbionts affect the evolution of their hosts, often allowing for expanded niches enabling changes in diet breadth or trophic position (Gil et al. 2004; Gibson and Hunter 2010). Despite their widespread occurrence, the ecology of facultative nutritional symbioses is poorly described. The spatiotemporal dynamics of facultative symbionts in natural populations and the factors affecting the distribution of the symbiont are important in the development of functional hypotheses that explain how obligate symbioses emerge.

In ants, endosymbiotic microbial communities track the trophic position of their hosts (Russell et al. 2009; Anderson et al. 2012). Many ants live on diets with stochastic access to protein and carbohydrate to the extent that some omnivores may, at times, live as functional herbivores (Tillberg and Breed 2004; Cook and Davidson 2006). Arboreal-foraging "herbivorous" ant species do not overtly consume plant tissues; instead, they are dependent on N-poor liquids such as

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honeydew, nectar, and sap (Blüthgen et al. 2003; Davidson et al. 2004). Despite the large disparity in consumption of nitrogen-rich nutrients, the tissue C and N content of ants varies little, suggesting an alternative source of N for herbivorous ants (Blüthgen et al. 2004).

Endosymbiotic bacteria are likely to play a role in ameliorating N limitation in arboreal-foraging ants. Within *Tetraponera nigra* ants, Van Borm et al. (2002) described bacteria related to N-fixing *Rhizobium*, *Pseudomonas*, and *Burkholderia*; these microbes were located within a special gut pouch, suggesting a nutritional mutualism. *Tetraponera pilosa* and two congeneric ant species were described more extensively by Stoll et al. (2007), and all three species harbored bacteria related to known N fixers. Of note, the Rhizobiales bacterium in *T. nigra* was the first *Bartonella*-like endosymbiont to be described in ants (Stoll et al. 2007). Among 283 ant species representing 141 genera, members of the order Rhizobiales were found to be the most common "symbiont," and the Rhizobiales identified were also most closely related to the genus *Bartonella* (Russell et al. 2009). Ants with Rhizobiales symbionts were predominantly herbivorous (Russell et al. 2009). The association between microbes from a known N-cycling lineage and an herbivorous diet in their ant hosts may be more than coincidental and suggests a functional role.

The relationships that mediate facultative nutritional symbioses are unknown even though these microbes are likely to affect the nutritional ecology of their hosts (Douglas 2009). An understanding of the environmental and dietary factors that shape the distribution of N-cycling microbes is a critical foundation in the understanding of the role of facultative mutualisms in the diet and foraging ecology of omnivores with stochastic N limitation in their diets.

In the present study, we document a facultative *Bartonella*-like bacterium (BLB) within a Costa Rican population of the omnivorous giant tropical ant, *Paraponera clavata*. We aim to understand how dietary and environmental conditions account for the pattern of BLB prevalence within *P. clavata* colonies. We evaluate three questions using a combination of observational and manipulative approaches: First, how does the prevalence of BLB in *P. clavata* vary with environmental conditions? Second, how does diet affect the prevalence of BLB in *P. clavata*? Third, how does the distribution of BLB in *P. clavata* reflect ambient differences in food resources?

Materials and methods

This study was conducted between January 2011 and August 2012 at La Selva Biological Station located in a tropical wet forest of northeastern Costa Rica (10° 26′ N, 83° 59′ W) that receives ca. 4 m of rain annually (McDade et al. 1994), and more information about La Selva is available from ots.ac.cr. The giant tropical ant, *P. clavata*, facultatively hosts a BLB in this population. *P. clavata* is restricted to wet tropical forests of the Neotropics, nests primarily at the base of trees and forages within the canopy using multiple trees and lianas to access nectar, and prey found in the arboreal environment (Janzen and Carroll 1983; Breed and Bennett 1985; Belk et al. 1989). *P. clavata* is omnivorous, with a diet consisting principally of arthropod prey and sugar solution, the latter of which is often derived from extrafloral nectaries (EFN) (Bennett and Breed 1985; Young and Herman 1980). *P. clavata* has been found to preferentially nest at the base of the most common tree at La Selva, the EFN-bearing *Pentaclethra macroloba* (Bennett and Breed 1985, but see Belk et al. 1989). *P. clavata* hunts opportunistically, though not randomly (Dyer and Floyd 1993), utilizing a potent venom, which is excruciatingly painful to humans (Schmidt et al. 1984) and debilitating to prey (Dyer 2002). At our particular field site, Dyer (2002) observed that nearly all proteinaceous prey items were arthropods, with leafcutter ants *Atta cephalotes* comprising the majority of prey items.

Fifty-nine colonies of *P. clavata* were selected along the trail systems of La Selva, across a range of forest ages, including old growth, early secondary (<30 years), and late secondary (>30 years) forests. Nests were identified by their characteristic excavated dirt mounds and foraging entrances at the base of trees (Janzen and Carroll 1983; Bennett and Breed 1985; Belk et al. 1989). They were located along the following trails: Sendero Occidental, Sendero Holdridge, Sendero Sábalo-Esquina, Sendero Sin Nombre, Sendero Tres Rios, Sendero Surá, Camino Circular Cercano, Camino Circular Lejano, and Sendero Atajo (Online Appendix A). The sites of all nests were mapped to an accuracy of 5 m and aligned to the La Selva Geographic Information System.

All fifty-nine colonies were screened for *Bartonella*-like bacteria via diagnostic polymerase chain reaction. The prevalence of BLB was estimated using the relative frequency of BLB in colonies, ranging from 0 to 3, among the three individuals screened per colony. Scoring was based on 16S ribosomal RNA (rRNA) sequence identification, which was initiated using DNA extracted from the gaster of each individual using the Qiagen DNeasy Kit (Qiagen, Valencia, CA, USA). For PCR amplification, we used a 16S forward primer (Bac_27F 5′-AGA GTT TGA TCC TGG CTC AG-3′) (Lane 1991) and a Rhizobiales-specific reverse primer (Rhiz1181R 5′-CGC TGC CCA CTG TCA CCA CC-3′) known to target *Bartonella*, *Rhizobium*, and "*Bartonella*-like" symbionts found in *Tetroponera binghami* (Stoll et al. 2007). The PCR mixture contained 1 μL of each primer (10 μM), 2.5 μL 10× Taq buffer, 2.5 μL dNTP Mix (2 mM), 0.5 μL Taq DNA polymerase, 15.5 μL sterile H_2O, and 2 μL of the extracted DNA for a final reaction volume of 25 μL. The cycling protocol was as follows: 5 min of initial denaturating at 94 °C, followed by 25 cycles of denaturation for 1 min at 94 °C, primer annealing for 1 min at 58 °C, primer extension

for 1 min at 72 °C, and a final elongation step of 6 min at 72 °C. Negative and positive controls were used for each PCR and electrophoretic run, and successful PCR amplification was considered to indicate the presence of BLB. Polymerase chain reaction products were cleaned prior to sequencing with MultiScreen HTS plates (Millipore Corporation, Bedford, MA) and sequenced directly using primers 27F and 1181R. Sequencing reactions were performed using the Genome Laboratory DTCS Quick Start Kit (Beckman Coulter, Fullerton, CA) precipitated with glycogen and sodium acetate, resuspended in 40 μL of formamide, and run on a Ceq 8800 Genetic Analysis System (Beckman Coulter, Fullerton, CA). Sequences were assembled and edited using Sequencher v4.10.1 (GeneCodes Corp.). Obtained sequences were edited with MEGA 5 and aligned using GUIDANCE (Penn et al. 2010), employing a MAFFT algorithm and 100 bootstrap repeats. Final editing was made in MEGA 5 (Tamura et al. 2011). Phylogenetic analyses were performed through Bayesian inference using MrBayes 3.2 (Ronquist and Huelsenbeck 2003) and the sequence evolution model SYM+G+I estimated by jMODELTEST (Posada 2008). All analyses employed one cold chain and three incrementally heated chains (temperature parameter=0.1). Four separate Markov Chain Monte Carlo runs were performed, with two million generations each, discarding the initial 500,000 generations from each run as burn-in and sampling 1 in every 100 generations to calculate posterior probabilities for each branch. All trees remaining after burn-in were used to construct a majority rule consensus tree. Final editing of the phylogenetic tree was made in MEGA 5. Additional sequences were obtained from GenBank and compiled and aligned with our 16S rRNA sequences using the ARB-automated alignment tool with subsequent manual refinements (Ludwig et al. 2004). For near full-length representatives and closest relatives, neighbor-joining analysis was conducted with Olsen distance correction.

Do environmental conditions predict the prevalence of BLB?

The nesting and foraging environment of each colony was characterized with a battery of environmental variables measured between March and July 2011. Colonies nest at the bases of individual trees or multiple adjacent trees, and they often use trees near the nest to access the canopy for foraging. The tree with largest and greatest number of nest entrances was denoted as the primary nest tree, and all trees and lianas within 1 m of the primary nest tree were considered the "nest site," as these typically are used for additional access to the canopy. All primary nest trees were identified to species and categorized with respect to the presence of extrafloral nectaries (EFNs), bark texture (smooth or rough), distance to trails of the principal prey species (*A. cephalotes*), and diameter at breast height (DBH). Bark texture was included as this influences the efficiency of movement on foraging trails as well as the density of competing species (Yanoviak et al. 2012). Nest sites were described by the presence of EFNs on the lianas and tree species present, canopy cover and forest age. Forests were classified into four classes: old growth, early secondary (< 30 years), late secondary (>30 years), and the arboretum, which is a managed environment with many mature trees but lacking an understory (four colonies in this study were in the arboretum though not used in all experiments). DBH was measured of each primary nest tree following methods of Clark (2002). Canopy cover was estimated with a convex spherical densitometer. Temperatures at the nesting sites were recorded simultaneously for 1 week at 10-min intervals using dataloggers (iButtons, Maxim Integrated Products, Sunnyvale, CA, USA). The dataloggers were mounted ca. 1 m above the foraging entrance on the primary nest tree of each colony. Route connectivity towards the canopy was measured using an estimate of connecting pathways between the canopy and nest site. In this estimate, we counted all individual plants taller than 1 m within a 3-m radius of the primary nest tree; as the biomass of large-stature forests is predominantly made up of trees with diameters greater than 10 cm (Clark et al. 2001), all lianas and stems with a DBH≥10 cm were designated as canopy connections, and all others were designated as understory connections. The total route connectivity was recorded as a mean of the canopy connections and understory connections.

How does diet affect the prevalence of BLB?

We conducted an in situ diet manipulation in July 2011 to determine whether diet, independent of other environmental characteristics, influences the prevalence of BLB. Colonies were stratified by trail and forest age and assigned to one of three diet manipulations: sucrose, protein, or control. All colonies, including controls, received supplemental water ad libitum. Colonies were provided dietary supplements along an established foraging trail on the primary nest tree at a site located 1–2 m above the nest entrance. Colonies receiving sucrose supplements received 25 mL of a 0.7-M sucrose solution replaced every 48 h; this is the median concentration of foraged nectar observed by Breed et al. (1987). Colonies in the protein treatment received 2.5 g of frozen *A. cephalotes* workers replaced every 48 h. *A. cephalotes* workers were collected using a modified hand vacuum, then frozen and sorted from debris, and broken into pieces to prevent clumping and facilitate collection by foraging *P. clavata* workers. The supplemental water, nectar, and prey were provided using apparatuses that permit the collecting of the materials without spillage and permit handling for materials to safely reach the nest. Each apparatus consists of a wire rack holding three interchangeable 50 mL centrifuge tubes attached to the tree with two pins to minimize contact with the tree and create space between the tree and apparatus that reduced the

attraction of non-target species. A fourth treatment category, "ineffective," was designated a posteriori. Colonies in this category did not appreciably partake in the supplemental food treatments; this accounts for differences in sample size among treatment categories, as about half of the *A. cephalotes* supplements were not well utilized. Manipulations were maintained for 2 weeks in July 2011. Additionally, the protocol measuring the prevalence of BLB was repeated at the end of diet manipulation experiment, and the difference between the initial and final prevalence values (ΔBLB) was used to measure how BLB prevalence responded to supplemental food treatment.

How does the distribution of BLB in the environment reflect ambient differences in diet?

Diet was assayed using non-invasive behavioral observations, direct sampling of nectar from foragers, and with stable isotope analyses. Diet observations were conducted in June and July 2012 on a subset of the colonies from the diet manipulation study, and new colonies were added for observations. All behavioral observations of diet were conducted between 2200 and 0500 hours, the peak foraging time of *P. clavata* during the study. Each colony was observed on two separate occasions; at the start of a 30-min observation period, temperature and humidity were recorded, and then the number of foragers returning to the nest was tallied. Whether the ant was bearing a load was recorded, and for those with loads, items carried were classified as prey (ant), prey (non-ant), or nectar. As *P. clavata* carry liquid and solid collections externally in their mandibles, the majority of foraged goods can be classified without interference. A small number of solid items that could not be identified without manipulation were assigned to the category of non-ant prey; this is a reasonable assumption as many potential prey items are cryptic arthropods that upon inspection may not appear to be an insect. After each observation period was complete, nectar was removed from ten opportunistically selected foragers using a clean micropipette, and the sugar concentration of the nectar was measured in the field using a handheld brix refractometer (BTX-1 refractometer, VEE GEE Scientific, Inc., Kirkland, WA, USA). The diet observation protocol was applied to a subset of 23 colonies (stratified across forest age selected haphazardly within each forest age category). Among these colonies, the diet assay was conducted twice, separated by at least 21 days from one another.

The relative dietary sources of N and C were evaluated using stable isotopic analyses using the mean of three right hind tibia and tarsus. Ants were collected in 2011 prior to the diet manipulation study. Stable isotope ratios are represented as $\delta^{15}N$ and $\delta^{13}C$, representing per mil (‰) proportionality of heavy: light isotopes, relative to a universal standard for each. Samples were weighed into tin capsules in an analytical balance to the nearest milligram. Ratios of stable isotopes in the ant tissue were measured with a PDZ Europa 20/−20 isotope ratio mass spectrometer at the UC Davis Stable Isotope Facility. Values of $\delta^{15}N$ and $\delta^{13}C$ were calibrated using values from established laboratory standards, run every 12 samples, and calibrated against NIST Standard Reference Materials. The source of dietary C may be inferred by $\delta^{13}C$, with site-specific information about $\delta^{13}C$ of the dietary sources (Blüthgen et al. 2003). At our particular field site, the $\delta^{13}C$ of nectar is appreciably lower than the $\delta^{13}C$ of all prey of *P. clavata* throughout all forest types (Tillberg and Breed 2004), so a lower $\delta^{13}C$ value is suggestive of a more nectar-biased source of C.

Data were analyzed using JMP versions 9.0 and 10.0 (SAS Institute, Cary, NC, USA). Analyses and model selection procedures are described within the results using Akaike weights to select the most parsimonious model for each analysis.

Results

BLB was found in 14 of the 59 colonies screened. Sequencing from 11 different colonies indicated a single identical phylotype for eight of the colonies (two of which have been deposited into GenBank, T51A1 and PT95A1), two others within 99.8 % similarity (deposited as AntA and Ant13), and one that was within 98 % similarity (deposited as Ant15; Fig. 1). While the frequency varied with environment, many colonies that were close in proximity to one another showed differences in the presence of BLB (Online Appendix A).

Do environmental conditions predict the prevalence of BLB?

We evaluated competing models using multiple environmental factors, ordinal logistic regression, to identify the most parsimonious predictors of the prevalence of BLB. One model incorporated factors varying at larger scales: soil type, forest age, mean temperature, and maximum temperature. The second model incorporated the local abundance of resources, including the presence of EFN-bearing trees at the nest site, whether primary nest tree bears EFNs, and the distances to nests and trails of the most common prey, *A. cephalotes*. The third model incorporated structural factors that may affect foraging efficiency and access to resources, including canopy cover, route connectivity, DBH of the primary nest tree, presence of rough bark at the site, which favors foraging by *P. clavata* without competition from smaller ants. In total, three factors were identified to have significant effects; these were considered individually in the model selection procedure. Complete environmental information for a few colonies was not available (because of datalogger failure or lack of

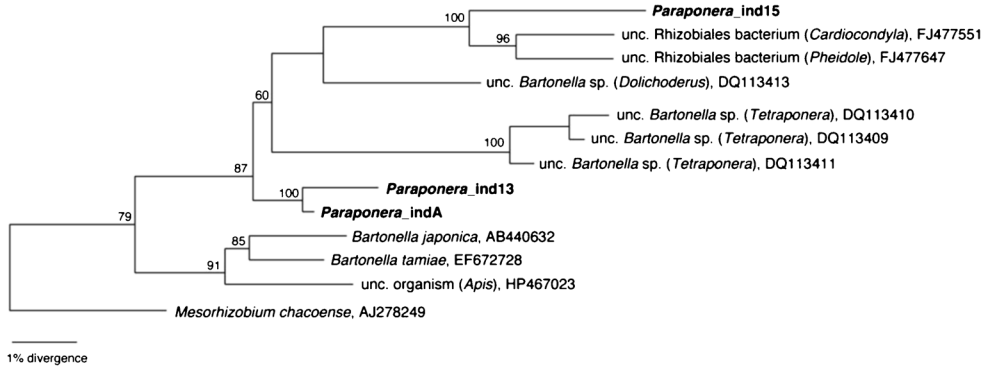

Fig. 1 Phylogenetic position of the BLB in this study, relative to other closely related Rhizobiales in GenBank, based on sequence divergence within the 16S rRNA gene. Additional sequences were obtained from GenBank and compiled and aligned with our 16S rRNA sequences using the ARB-automated alignment tool with subsequent manual refinements. For near full-length representatives and closest relatives, neighbor-joining analysis was conducted with Olsen distance correction. Numbers next to nodes correspond to bootstrap values >70 based on and 5,000 replicates. *unc.* uncultured

EFN information about certain rare canopy tree species), and in these model selection analyses, these colonies were excluded so that competing models using the remaining 57 colonies could be fully compared with one another. While multiple models were statistically significant, the most parsimonious model to account for the occurrence of BLB was forest age (Table 1). Among our field locations, BLB was less prevalent in late secondary forests and more prevalent in early secondary forests (Fig. 2). We separately tested whether BLB was associated with *P. macroloba*, the dominant tree at the site. Of six sites with *P. macroloba*, three (50 %) scored positive for BLB. Of 54 sites without *P. macroloba*, 18 (33 %) contained BLB in any sampling event; this was not a significant difference (χ^2_1=0.63; p=0.43).

How does diet affect the prevalence of BLB?

The change in BLB prevalence after supplemental food treatments was evaluated as ΔBLB, representing the difference in prevalence between the post-treatment and pre-treatment

Table 1 Selection of competing ordinal logistic models to account for the prevalence of BLB within colonies of *Paraponera clavata*. Among 59 colonies screened for BLB, 57 colonies were used in this analysis

Variable	Effect likelihood X^2	Whole model X^2	DF	p value	AICc	Akaike weight
Model 1: large-scale environment		4.60	6	0.013	78.103	0.055
Forest age	15.38		4	0.0015		
Soil Type	3.08		1	0.079		
Temperature (mean)	0.03		1	0.851		
Temperature (max)	0.04		1	0.860		
Model 2: resource environment		5.44	4	0.245	83.499	0.004
Distance to *A. cephalotes* trail	0.31		1	0.573		
Distance to *A. cephalotes* nest	1.30		1	0.255		
EFN-bearing primary tree	2.81		1	0.094		
EFN trees in nest site	4.68		1	0.031		
Model 3: structural environment		12.16	4	0.0162	76.772	0.107
Canopy cover	1.05		1	0.306		
DBH of primary nest tree	0.07		1	0.783		
Rough bark at nest site	5.43		1	0.020		
Route connectivity	6.06		1	0.014		
Individual factors as separate models						
Forest age		*12.97*	*3*	*0.005*	*73.461*	*0.562*
EFN trees in nest site		1.02	1	0.312	80.688	0.015
Rough bark at nest site		3.90	1	0.048	77.804	0.064
Route connectivity		6.11	1	0.014	75.601	0.193

Italics indicate the most parsimonious model

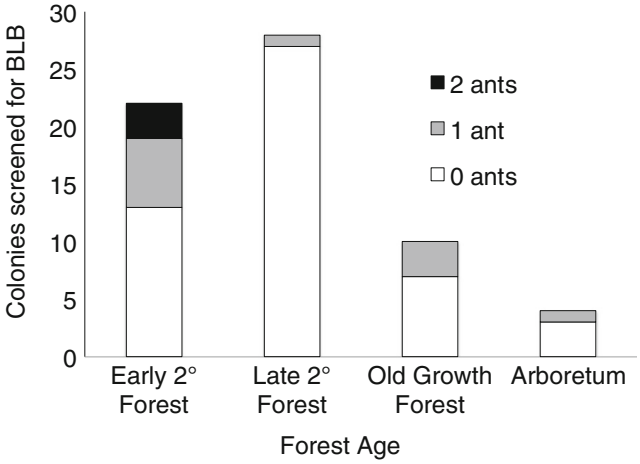

Fig. 2 The distribution of BLB among colonies of *P. clavata* across a variety of forest ages. For each colony, three individuals were screened for the presence of BLB; no colonies had all three ants scoring positive for BLB

colony screenings. A generalized linear regression was used to evaluate the effect of treatment on ΔBLB, with a Poisson distribution and log link function and with treatment category as the predictor variable. There was a significant effect of sucrose solution treatments enhancing the prevalence of BLB over the course of the 2-week manipulation, but no effect of supplemental prey (GLM χ^2_3=10.53; p=0.0146; Fig. 3).

How does the distribution of BLB in the environment reflect ambient differences in diet?

We compared how the dietary sources of C and N varied with the environment and the prevalence of BLB using stable isotopes. We used the same modeling approach used for

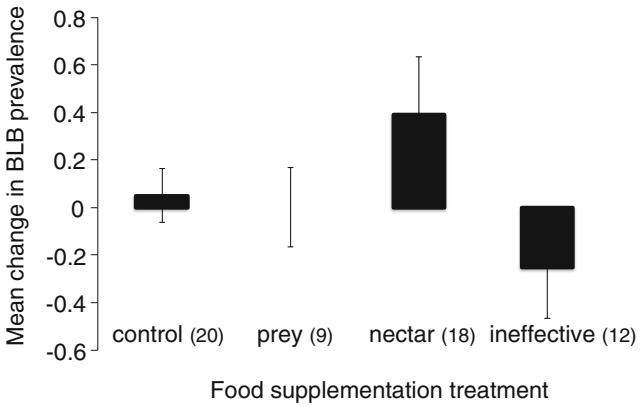

Fig. 3 The effect of treatments on the prevalence of BLB within colonies of *P. clavata*. The mean change in the number of ants that scored positive for BLB among three individuals per colony immediately before and immediately after treatment. Ineffective treatments represent those which supplemental prey or nectar was presented but was not appreciably collected by the colony under treatment. Statistics are in Table 2. *Error bars* represent standard error; sample sizes are in *parenthesis*. The bar is absent from the prey category as the value was precisely zero, with SE as indicated

BLB prevalence, but because the response variables (δ^{15}N and δ^{13}C) are continuous, we used two generalized linear models, and we added BLB prevalence as a factor. Properties of the structural environment were the most parsimonious predictors of both δ^{13}C and δ^{15}N of *P. clavata* colonies, with some additional support for forest age as a predictor of δ^{13}C (Tables 2 and 3). Although diet altered the presence of BLB in the manipulation, the presence of BLB was not predictive of δ^{13}C or δ^{15}N.

Behavioral observations of diet and measurements of nectar concentrations from 30 colonies were evaluated to further articulate the relationships among diet, isotopic ratios, forest structure, forest age, and BLB prevalence. The mean frequency of foragers returning with nectar droplets in their mandibles was 67.8 % (±12.7 SD), with 11.7 % (±6.6) returning with a prey item, and the remaining 20.4 % (±11.7) returning with no load. Two colonies, which collected more prey than nectar, were extreme outliers preventing the normalization of distributions and were excluded from subsequent analyses. The mean sugar concentration of the nectar collected by each colony was 13.7 % (±2.9) equating to 0.4 M (assuming sucrose equivalency). We tested whether the three environmental parameters associated with isotopic dietary indicators (forest age, route connectivity, and canopy cover) were also predictive of the frequency of nectar as a food item (the fraction of workers returning with nectar) and the sugar levels in nectar using a two separate generalized linear models. There was no relationship between these parameters and the frequency of nectar collection among returning foragers (GLM χ^2_6= 8.629; p=0.196). The concentration of nectar did vary predictably with forest type (Table 4, Fig. 4a). We tested for a relationship between isotopic ratios of C and N with nectar concentration using simple linear regression. There was no relationship between the δ^{15}N of *P. clavata* and sugar concentration (r^2=0.004, $F_{1,18}$=0.07, p=0.79), but the δ^{13}C of *P. clavata* colonies was closely predicted by sugar concentration ($F_{1,18}$= 30.3, p<0.0001; Fig. 4b).

Discussion

We have documented that the distribution of the Rhizobiales *Bartonella*-like bacterium (BLB) is nonrandom, reflecting the diet and environment of the host. The prevalence of BLB in *P. clavata* responds to a change in diet and is predicted by environmental factors. When fed supplementary sugar, BLB became more prevalent within colonies, relative to control colonies and those given supplemental protein. Prior to diet supplementation, BLB was more prevalent in colonies located in young secondary forest and nearly absent from older secondary forests. These young secondary forests differ from

Table 2 Selection of generalized linear models evaluating the $\delta^{15}N$ of *P. clavata* colonies

Variable	Effect X^2	Whole model X^2	DF	*p* value	AICc	Akaike weight
Model 1: large-scale environment		10.38	6	0.109	125.38	<0.001
Forest age	4.19		3	0.242		
Soil type	0.10		1	0.747		
Temperature (mean)	0.98		1	0.323		
Temperature (max)	4.81		1	0.028		
Model 2: resource environment		9.43	6	0.151	126.34	<0.001
Distance to *A. cephalotes* trail	2.12		1	0.146		
Distance to *A. cephalotes* nest	0.15		1	0.696		
EFN-bearing primary tree	1.07		1	0.301		
EFN trees in nest site	0.04		1	0.846		
Prevalence of BLB	5.13		2	0.077		
Model 3: Structural environment		19.96	4	0.0005	110.49	0.423
Canopy cover	12.15		1	0.0005		
DBH of primary nest tree	0.06		1	0.804		
Rough bark at nest site	0.03		1	0.854		
Route connectivity	6.62		1	0.010		
Individual factors as separate models						
Temperature (max)		1.58	1	0.209	121.64	0.0016
Canopy cover		*13.22*	*1*	*0.0003*	*110.00*	*0.540*
Route connectivity		7.70	1	0.0055	115.51	0.034

Italics indicate the most parsimonious model

Table 3 Selection of generalized linear models evaluating the $\delta^{13}C$ of *P. clavata* colonies

Variable	Effect X^2	Whole model X^2	DF	*p* value	AICc	Akaike weight
Model 1: large-scale environment		23.67	6	0.0006	140.04	0.028
Forest age	21.20		3	<0.0001		
Soil type	0.03		1	0.854		
Temperature (mean)	2.16		1	0.142		
Temperature (max)	2.39		1	0.122		
Model 2: resource environment		8.50	6	0.204	155.21	<0.001
Distance to *A. cephalotes* trail	0.18		1	0.675		
Distance to *A. cephalotes* nest	1.20		1	0.272		
EFN-bearing primary tree	0.07		1	0.793		
EFN trees in nest site	0.04		1	0.835		
Prevalence of BLB	4.32		2	0.116		
Model 3: Structural environment		*23.87*	*4*	*<0.0001*	*134.53*	*0.438*
Canopy cover	*3.35*		*1*	*0.067*		
DBH of primary nest tree	*0.25*		*1*	*0.620*		
Rough bark at nest site	*4.22*		*1*	*0.040*		
Route connectivity	*19.30*		*1*	*<0.0001*		
Individual factors as separate models						
Forest age		20.65	3	<0.0001	135.24	0.307
Rough bark at nest site		3.17	1	0.075	147.99	<0.001
Route connectivity		15.32	1	<0.0001	135.84	0.227

Italics indicate the most parsimonious model

Table 4 A generalized linear model evaluating the predictors of mean nectar sugar concentration collected by foragers of *P. clavata*. Twenty colonies, with full data for all variables, were included in this model

Variable	X^2	DF	*p* value
Whole model	18.31	4	0.0011
Canopy cover	0.70	1	0.402
Forest age	9.61	2	0.0082
Route connectivity	2.09	1	0.148

older forests in their access to canopy resources as well as in the concentration of nectar. This understanding of the ecological adaptation in a non-obligate bacterial associate, in a naturally occurring omnivorous population in the field, may be informative for models regarding the evolution of nutritional symbioses.

The change in the presence of BLB in this ecological study recapitulates the evolutionary patterns of Rhizobiales seen in cross-species comparisons of omnivorous and herbivorous ants. The prevalence of the N-cycling microbe increased in response to an experimental introduction of sucrose, and the evolutionary studies indicated that species with more carbohydrate-rich diets were more likely to be associated with obligate N-cycling microbes (Russell et al. 2009; Anderson et al. 2012). Increased occurrence of the bacterium when foraging for more carbohydrates suggests a possible adaptation indicated by diet-dependent plasticity.

Caution is required when interpreting the effect of manipulations on the ecology of an organism that is effectively invisible to investigators in the field and, for all we know, may be widespread throughout the forest outside hosts. Nevertheless, we show that this particular microbe belongs to a lineage of known ant associates, and when both observational and manipulative results show consonant findings, there is increased support for the argument that BLB and *P. clavata* share a nutritionally functional relationship.

I is unclear whether the distribution patterns that we have found merely reflect the relative availability in the environment or an adaptive response on the part of the hosts. Sugar supplementation increased the prevalence of BLB in colonies over the timescale of 2 weeks. It is not clear whether *P. clavata* actively regulates the presence of BLB or if the BLB is opportunistically taking advantage of increased sugar resources inside the colony. This should not preclude an attempt to evaluate whether there is support for a potential reciprocal role between BLB and *P. clavata*. If BLB is a mutualist, for example, in N cycling, then we would expect colonies with high sugar in their diets to not exhibit signs of N limitation. Conversely, if BLB is an opportunist taking advantage of high sugar inputs in order to increase activity levels within colonies, then the benefits of increased sugar in *P. clavata* colonies should be absent or modest.

The structure of the forest itself affects the sources of C and N used to build tissue in *P. clavata*, whereas the concentration of nectar in the diet closely predicts the source of C but not the source of N. This does not contradict the possibility that BLB may facilitate the acquisition of nitrogen in *P. clavata*, as this indicates that the ultimate source of C varies with its concentration. This experiment was not designed to test for a specific functional role of BLB, but rather to test for factors that shape its distribution. Our results point clearly towards forest age, the diet of the host and the distribution of sugar in the environment, and this should inform investigations into the physiological ecology of this association.

The physical structure of the forest influences how *P. clavata* workers access resources in the rainforest canopy. More than 90 % of the biomass of large-stature forests is made up of trees with diameters greater than 10 cm, but this is not the case in young secondary forests (Clark et al. 2001). Forest age was found to be a clear predictor of BLB prevalence, though forest age is confounded with other aspects of forest structure, such as EFNs (Bentley 1976). As forests mature, the routes to canopy diminish, and also the canopy itself closes and becomes more

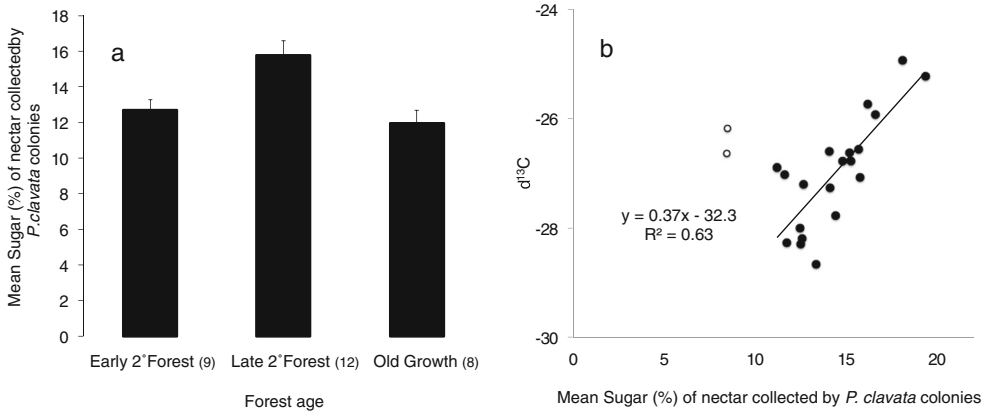

Fig. 4 Predictors of sugar concentration in the nectar collected by *P. clavata* foragers. *Panel a* indicates differences in nectar among forest age; *error bars* represent SE and sample sizes are indicated in *parentheses*. In *panel b*, positive relationship between $\delta^{13}C$ and nectar sugar is presented. *Open circles* indicate two outliers excluded from the analysis; these two colonies forage less frequently for sugar as well has having low sugar concentrations. Statistics are in Table 4

distant while the concentration of sucrose increases in older secondary forest. While we did not find a specific effect of EFN-bearing plants on the prevalence of BLB, colonies forage substantial distances in the canopy, and the access to the canopy itself may be a more important regulator of diet than the species composition of the trees near the nests of *P. clavata*. Forest structure does matter for foraging, as *P. clavata* workers do not forage on narrow-stemmed plants unless baited (Yanoviak et al. 2012). We remain unsure what properties of young forests influence the prevalence of BLB—whether it is the ambient availability of the microbe itself or the environmental conditions that alter the benefits of association with the ant host. In concert, our findings suggest that the latter scenario plays a role. We have no data to evaluate the former, and in such system with current technology, both expensive and creative approaches would be required to develop a clearer understanding.

In the present study, we were not able to evaluate the specific effect of unmanipulated diet on the prevalence of BLB because of the relatively reduced sample size in the diet observations, as well as the fact that BLB is uncommon outside early secondary forests. We would expect that the concentration of nectar would be positively associated with BLB, which would further reinforce our suggestion that BLB facilitates the acquisition of N in *P. clavata*.

The heterogeneity of facultative BLB in *P. clavata* follows the pattern of BLB association among ants with low-nitrogen diets established by Russell et al. (2009). Further study is required to determine whether the predictive environmental factors act independently or if they reflect association with a single common predictor. Investigating the differences between early secondary and late secondary forests may provide insight into variable BLB presence. Likewise, establishing the functional relationship of the BLB with *P. clavata* will help to define the environmental factors important in determining the presence of BLB.

As dietary carbohydrates increase BLB prevalence, what affects the origin of C that ants use to build tissue? The tight relationship between $\delta^{13}C$ and nectar concentration requires an explanation even if we have incomplete information about the environmental distribution of $\delta^{13}C$. Two competing explanations are presented. First, different plant species can demonstrate remarkably different $\delta^{13}C$ values in nectar in part reflecting different photosynthetic mechanisms (Wolf and Hatch 2011). The $\delta^{13}C$ of carbon fixed under higher light or water stress is higher (Farquhar et al. 1989). The differences among the ants may merely reflect differences in the physiology of their nectar sources.

The alternative explanation for the relationships between $\delta^{13}C$ and nectar concentration is an actual change in resource use by ants that tracks nectar concentrations. At our field site, relative to prey, nectar has a markedly lower $\delta^{13}C$ (Tillberg and Breed 2004). We then may conclude that colonies foraging on low concentration sugar use more nectar-based C to build tissue than colonies that forage on high concentration nectar. The tissues we sampled, the legs of adults, presumably are principally comprised of C allocated to larvae during development. Colonies collecting higher concentration nectar should have more energy to forage for prey and will be able to produce larvae that are fed mostly prey in their diets. Conversely, colonies collecting lower concentration nectar will have less energy to forage for prey and may allocate more carbohydrates derived from nectar to feed to larvae. Further investigation should be able to resolve how the carbon source varies so closely with nectar concentration. Regardless, it appears that colonies will only focus on collecting nectar if concentrations remain above 10 %. Among the colonies in the diet study, all but two retrieved nectar that contained >10 % sugar content. The two colonies that brought back <10 % nectar were also those that had the smallest fraction of nectar loads among returning foragers (34 and 39 %, with *z*-scores less than −2). These two colonies were the outliers in Fig. 4b, suggesting that these colonies were not foraging for nectar because it did not provide adequate rewards.

In summary, we have made progress in understanding the field ecology of facultative nutritional symbioses by understanding how the provision of energy and the structure of the environments affect the prevalence of the association. This is a new step in the future challenges in understanding how facultative nutritional symbioses may lead to the evolution of obligate relationships and in the basic ecology of facultative associations.

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Data accessibility Sequences from five ants are deposited into GenBank, with the following accession numbers: KC478384, KC478385, KC478386, KC478387, and KC478388.

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