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Partner fidelity and environmental filtering preserve stage-specific turtle ant gut symbioses for over 40 million years

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Abstract

Sustaining beneficial gut symbioses presents a major challenge for animals, including holometabolous insects. Social insects may meet such challenges through partner fidelity, aided by behavioral symbiont transfer and transgenerational inheritance through colony founders. We address such potential through colony-wide explorations across 13 eusocial, holometabolous insect species in the ant genus *Cephalotes*. Through amplicon sequencing, we show that previously characterized worker microbiomes are conserved in soldier castes, that adult microbiomes exhibit trends of phylosymbiosis, and that *Cephalotes* cospeciate with their most abundant adult-enriched symbiont. We find, also, that winged queens harbor worker-like microbiomes prior to colony founding, suggesting vertical inheritance as a means of partner fidelity. Whereas some adult-abundant symbionts colonize larvae, larval gut microbiomes are uniquely characterized by environmental bacteria from the Enterobacteriales, Lactobacillales, and Actinobacteria. Distributions across *Cephalotes* larvae suggest more than 40 million years of conserved environmental filtering and, thus, a second sustaining mechanism behind an ancient, developmentally partitioned symbiosis.

KEYWORDS
development, environmental filtering, gut microbiota, partner fidelity, turtle ants

INTRODUCTION

The integral nature of mutualistic symbioses across eukaryotes requires careful regulation of symbiont populations, and a means to prevent the spread of mutualist-exploiting cheaters. Through the partner fidelity, partner choice, and sanction-based mechanisms achieving these ends (Kiers et al., 2003; Nyholm & Mcfall-Ngai, 2004; Sachs et al., 2011; Sachs & Wilcox, 2006; Skelton et al., 2019), a subset of eukaryotes have evolved markedly conserved symbioses (Gaulke et al., 2018; Kiers & Denison, 2008; Werner et al., 2018).

Insects provide especially useful insights into mechanisms promoting beneficial symbiont maintenance and microbiome conservation (Kaltenpoth et al., 2014; Ohbayashi et al., 2020). Partner fidelity is the most widely documented, being evidenced by the many symbionts passaged to offspring through transovarial transfer (Koga et al., 2012; Luan et al., 2016), or a range of "out-of-body" mechanisms (Salem et al., 2015). But many insects acquire symbionts from the environment, retaining specific subsets of the inoculated microbes (Birer et al., 2020; Chandler et al., 2011; Jones et al., 2019; Ravenscraft et al., 2019) due to their physiology and behavior (Engel & Moran, 2013; Tragust et al., 2020), or the actions of their regular symbiont partners (Itoh et al., 2019; Worsley et al., 2021). Among those adopting such environmental filtering mechanisms, some have evolved means for partner choice, selecting beneficial microbial partners amid a broader set of symbiotic suitors (Ohbayashi et al., 2015).

Symbiotic microbes reside in a variety of insect tissues, but gut compartments including the crop, midgut, ileum, and rectum are among the most frequently colonized (Engel & Moran, 2013). Whereas eukaryotic hosts are often touted as stable symbiont habitats, gut microbes encounter changing conditions due to varying diet, physiological stress, the introduction of competitors, and the impacts of insect development (Blum et al., 2013; Broderick et al., 2004; Cariveau et al., 2014; Wang et al., 2021). Promoting shifts in microbiomes across other organisms (Aleman & Valenzano, 2019; Jami et al., 2013; Sudakaran et al., 2012), the numerous changes unfolding across development have heightened effects on gut symbioses in holometabolous insects. This can stem from gut tissue re-organization at metamorphosis (Hammer & Moran, 2019) and loss of symbionts in the voided meconium (Moll et al., 2001). The resulting potential for stage-specific microbiomes is enhanced by the frequent use of differing diets across larval and adult stages. Several studies have, accordingly, reported changes in symbiont composition or the abundances of shared symbionts on the opposite sides of metamorphosis (Hammer et al., 2014; Mason et al., 2019; Shukla et al., 2016; Wong et al., 2011).

Although this symbiotic reset may allow greater flexibility for metamorphosing, microbe-reliant insects (Hammer & Moran, 2019), it could also limit the reaches of partner fidelity, barring mechanisms for persistence or re-acquisition of larva-acquired gut symbionts (Coon et al., 2014; Estes et al., 2009; Johnston & Rolff, 2015). In social insects, several solutions may be at hand, including trophallactic symbiont transfer (Lanan et al., 2016; Martinson et al., 2012; Nalepa et al., 2001) and the persistence of symbionts within-colony-founding individuals (Diouf et al., 2018; Li et al., 2018; Michaud et al., 2020; Mueller et al., 2001).

To comprehend the full relevance of mutualism-stabilizing mechanisms, and the diversity of symbionts among social insects, research is not only required across developmental stages, but also among castes, whose varying contributions to colony founding, foraging, nourishment, and digestion provide added intrigue to their symbiotic study (Erthal et al., 2007; Keller, 1991; Kronauer et al., 2004; Wetterer, 1995). Caste- and development-spanning symbiont studies have been applied to honeybees (Anderson et al., 2018; Kapheim et al., 2015; Martinson et al., 2012; Powell et al., 2018). But similar research on their distant, eusocial cousins—the ants (Hymenoptera: Formicidae)—remains in its infancy (Rubin et al., 2019; Segers et al., 2019).

Numbering in excess of 12,000 species, and weighing in to comprise the world's most abundant insect group, our lack of knowledge on the impacts of development and social behaviors on ants' symbiotic gut microbes is a hefty shortcoming. To address these uncertainties, we focus here on the genus *Cephalotes*. Adult workers of this arboreal, New World ant clade harbor a conserved suite of cuticle-building, amino-acid provisioning, nitrogen-recycling gut bacteria (Duplais et al., 2021; Hu et al., 2018). Many of these symbionts appear ubiquitous, or nearly so, among species of *Cephalotes* and those of the sister genus, *Procryptocerus*, suggesting more than 50 million years of stable symbioses (Hu et al., 2014, 2018; Price et al., 2014; Sanders et al., 2014). Most worker-associated symbionts also belong to host-specific clades (Anderson et al., 2012; Hu et al., 2018; Russell et al., 2009), arguing that conserved partner fidelity—rather than conserved filtering of environmentally acquired bacteria—has driven this stability.

Prior research has suggested that social transmission of gut symbionts has favored this long-term maintenance, as mature *Cephalotes* use oral–anal (also known as proctodeal) trophallaxis to passage symbionts to newly emerged adult siblings (Rodrigues, 2016; Wheeler, 1984; Wilson, 1976). After acquisition, symbionts are sealed into the midgut, ileum, and rectum through the development of a fine filter over the proventriculus (Lanan et al., 2016). Whereas the proventricular filter protects against invasion by exogenous microbes, neither this mechanism, nor trophallactic transmission, can explain *Cephalotes* symbiont specialization without a means for transgenerational inheritance. *Cephalotes* queens may bridge this gap, given their exclusive roles in colony founding.

After dispersal from their parental colonies, and mating on the wing, *Cephalotes* queens—called "gynes" at this stage—establish new colonies in unoccupied tree cavities. After wing loss early on in the new colony's lifespan, a single queen will be the sole provisioner for the first hatched larvae and young adults. We know little about *Cephalotes* queen microbiomes, but the acquisition of specialized, worker-enriched symbionts prior to colony founding would implicate the queen caste as the vehicle of vertical transfer.

As for many ants, we are separately unaware of whether cephalotine larvae harbor dense symbiotic gut communities, and we are similarly ignorant about the sources of any such symbionts. Might specialized bacteria from adults dominate larval gut microbiomes? Given the likely absence of gut microbes in young larvae, pupae, and newly eclosed adults (Roche & Wheeler, 1997; Russell et al., 2009), findings of specialized, adult-enriched symbionts in intermediate- to late-stage larvae would suggest larval acquisition, loss at metamorphosis, and re-acquisition in adulthood. But the differing diets of larvae (Cassill et al., 2005; Erthal et al., 2007), and their lack of a microbe-excluding proventricular filter, suggest that their microbiomes may differ from those of adults, as may the mechanisms by which they are constructed. Accordingly, 16S rRNA amplicon sequencing from numerous workers and a single larva of *Cephalotes rohweri* suggested stage-divergent microbiomes, with little taxonomic overlap (Lanan et al., 2016). Adding to this was a recent shotgun metagenomics study, demonstrating partially divergent larval versus worker microbiomes in *Cephalotes varians* (Bechade et al., 2022). Given the limits of this sampling, the general absence of soldier microbiome surveys, and modest efforts to study queen microbiomes through diagnostic polymerase chain reaction (PCR) and Sanger sequencing (Anderson et al., 2012; Russell et al., 2009), there is a considerable need for a broader investigation.

To achieve a colony-wide view of symbiosis, we studied bacterial symbionts across multiple castes and/or developmental stages from 13 phylogenetically diverse *Cephalotes* species (Figure 1). Using quantitative PCR (qPCR), we first measured bacterial densities across members of four *C. varians* colonies. Adopting standard PCR amplification, we next performed a qualitative assessment of ant–bacteria associations across stages and/or castes for the 12 remaining *Cephalotes* species. With evidence of populous bacterial communities across female castes and most larval stages, we used amplicon sequencing of 16S rRNA to characterize bacterial gut communities. We leveraged our amplicon data to study symbiont operational taxonomic unit (OTU) distributions among larvae, workers, soldiers, and queens, testing capacities for queen-mediated vertical transfer of

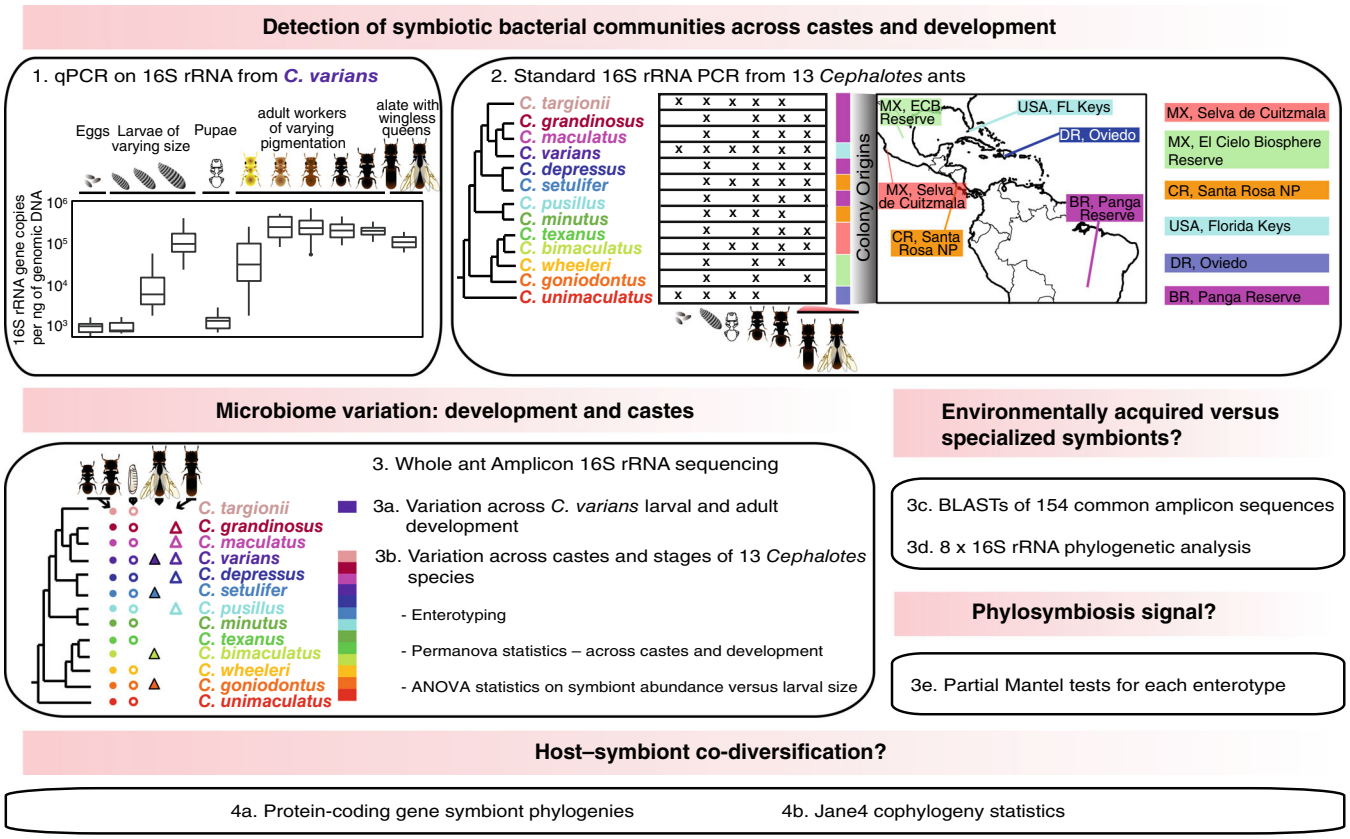


FIGURE 1 Methodologies and research goals of our study. Through extensive qPCR assays on *Cephalotes varians* ants, and standard amplification of bacterial 16S rRNA from 12 congeneric species, we aimed to pinpoint the timing of symbiont acquisition and loss across holometabolous development. BLAST analyses, of 16S rRNA amplicon sequences, subsequently aided in the classification of symbionts as environmentally acquired versus *Cephalotes*-specialized. Using these same amplicon data, we compared microbiomes of winged queens to those of the remaining colony, assessing the capacity for this colony-founding caste to drive vertical symbiont inheritance. We, finally, tested long-term impacts of such partner fidelity through phylosymbiosis and cophylogeny assessments.

seemingly specialized symbionts. Through varied statistical methodologies, we looked for longer term patterns of such partner fidelity, examining relationships among symbiont community similarity and host phylogenetic relatedness. Detecting trends of phylosymbiosis among adults and their gut microbiomes, we then assessed congruence between phylogenies of *Cephalotes* ant hosts and their most abundant adult-enriched symbiont, *Cephaloticoccus* (Verrucomicrobia: Opitutales).

Like adult microbiomes, larval microbiomes were generally conserved across host species. But, in contrast, we saw little evidence of phylosymbiosis between hosts and late-stage larval microbiomes. We, thus, measured relatedness between larval gut symbionts and non-cephalotine bacteria in NCBI, giving insight into their plausible acquisition of gut microbes from the environment. In doing so, we tested the idea that it is not partner fidelity shaping some portions of larval microbiome conservation, but conserved environmental filtering. With the mutualistic nitrogen-recycling services of adult symbionts, and a range of plausible benefits uncovered recently for larval symbionts through metagenome sequencing (Bechade et al., 2022), our findings implicate two distinct mechanisms behind the long-term conservation of symbiotic mutualisms in *Cephalotes*. We discuss these findings in the broader context of mutualisms, social insect symbioses, and the complex trait of colony-level fitness (Segers et al., 2019; Singh & Linksvayer, 2020).

METHODS

Sample collections, DNA extraction, and PCR amplification

Ant samples used in this study were collected between 2012 and 2016 in Brazil, Costa Rica, the Dominican Republic, Mexico, and the USA (Appendix S1: Table S1). Prior to molecular processing, samples were preserved in 100% ethanol at −80°C. Whereas this preservation method appears to impact the relative abundances of some *Cephalotes* symbionts (Sanders et al., 2014), its systematic application here has ideally minimized spurious microbiome signatures.

Collected samples were identified to the species, or near-species, level by Scott Powell and Corrie Moreau using the taxonomic key of De Andrade and Baroni Urbani (1999). Several specimens came from the same colonies as those utilized in the recent genus-wide molecular phylogeny of Price et al. (2022). The 13 targeted species came from 18 colonies, and included *Cephalotes* aff. *bimaculatus*, *Cephalotes depressus*, *Cephalotes grandinosus*, *Cephalotes goniodontus*, *Cephalotes maculatus*, *Cephalotes minutus*, *Cephalotes targionii*, *Cephalotes pusillus*, *Cephalotes setulifer*, *Cephalotes texanus*, *Cephalotes unimaculatus*, *Cephalotes varians*, and *Cephalotes* aff. *wheeleri*. Due in part to the difficulty of full colony nest excavation, we did not achieve systematic sampling of all castes and stages from each species. But, when possible, we extracted DNA from several replicate specimens per category, placing a larger emphasis on replication within *C. varians* due to its greater accessibility.

Microbiome comparisons among homologous gut regions in larvae and adults are complicated by their divergent morphologies (Weir, 1957). We, thus, adopted an apples-to-apples approach for microbiome study, involving DNA extraction from whole individuals. Prior work on *Cephalotes* has indicated that the vast majorities of adult-associated symbionts colonize the midgut and ileum (Flynn et al., 2021; Kautz et al., 2013). As such, microbial community data derived from whole-body extractions will be largely driven by the bacteria from these gut chambers. Using this approach, we extracted DNA from representative specimens from all available developmental stages, sexes, and castes, including workers, soldiers, queens, males, larvae, pupae, prepupae and eggs, from each targeted colony. Specimens were rinsed in 70% ethanol and then washed three times with molecular-grade water before DNA extraction. Single ants were then ground with a pestle in sterile 1.5-ml tubes after flash-freezing in liquid nitrogen. We extracted DNA from these homogenates with DNeasy Blood and Tissue kits (Qiagen Ltd., Hilden, Germany), following the manufacturer's protocol for Gram-positive bacteria. This method includes lysozyme, an enzyme shown to improve extraction efficiency from some taxa, including not only Gram-positives (e.g., of pertinence here due to larval Lactobacillales in *Cephalotes*; Bechade et al., 2022), but also Gram-negative bacteria, including some Rhizobiales genera (Ketchum et al., 2018).

To detect possible laboratory- or reagent-derived contamination, we included "blank" samples in our DNA extraction batches, following the aforementioned protocol without the addition of ant tissues. To assess the presence or potential absence of bacteria in our studied ants, we amplified the bacterial 16S rRNA gene using universal eubacterial primers 9Fa (5′-GAGTTTGATCITGCTCAG-3′) and 1513R (5′-TACIGITACCTTGTTACGACTT-3′). We also checked for template DNA quality by amplifying part of the ant mitochondrial cytochrome oxidase (*COI*) gene using universal insect primers LCO-1490 (5′-GGTCAACAAA TCATAAAGATATTGG-3′) and BEN (5′-GCTACTACATA ATAKGTATCATG-3′). Further information on PCR primers, cocktail recipes, and cycling conditions can be found in Appendix S1: Table S2, and data on sample quality and bacterial presence can be obtained from Dryad ("Data 1" file deposited in the Dryad Digital Repository; Hu et al., 2022a, 2022b).

Given the general lack of prior study on larval symbionts, we initiated a preliminary localization study using three late-stage *C. varians* larvae. Using freshly collected (i.e., nonpreserved) individuals, and sterilized fine-tipped forceps, we separated the gut wall from the solid mass of gut content in the lumen. DNA was extracted for these separate portions, as above, and the $n = 6$ resulting samples were subjected to amplicon 16S rRNA sequencing, as described below.

Relative age estimates for all larvae and *C. varians* adults

Prior to DNA extraction, all specimens were photographed from the dorsal side by a digital camera connected to a Leica ×80 dissecting microscope under constant lighting conditions. We deposited these photographs in the Dryad repository (Hu et al., 2022a, 2022b). For all larvae, a calibration slide was used to measure length, which we used as an approximation of larval age (Costa & Gomes-Filho, 2002; Zeller & Koella, 2016). In *C. varians* we arbitrarily defined three larval age/length groups for use in statistics. These included a young/small larval group ≤2 mm in length, a middle-aged/medium-sized group 2–3 mm in length, and an older/large group ≥3 mm.

Beyond *C. varians*, we measured larval length from 13 colonies of 11 additional *Cephalotes* species. Using the combined data, we normalized larval length for each specimen, dividing it by the average mesosomal length from workers of the same colony. Normalized measures were subsequently used as cross-species, comparable approximations for relative larval age.

To ascertain the relative ages of adult *C. varians*, we measured cuticular pigmentation, adopting a method developed for leafcutter ants (Armitage & Boomsma, 2010). Photographs of workers, soldiers, and queens were converted to black and white. Dorsal abdominal segments were then used for pigment quantification using ImageJ software. To enable this, we recorded the mean gray value for each specimen, which we calculated by dividing the total gray value of imaged pixels by the total number of pixels in the selected region. For subsequent analyses, designed to compare microbiomes and bacterial titers across age, adult workers of *C. varians* were divided into four groups based on these gray-scale values, with arbitrarily chosen bins of 0–20 (presumed to be the oldest), 20–40, 40–60, and 60–80 (youngest, i.e., callow workers). In the absence of a groundtruthing experiment for *C. varians*, we considered the possibility that adults in the two darkest classes were not substantially different in age, but variable due to other factors affecting pigmentation, using caution in our interpretation of the data. A table of relative age estimates for all larvae and *C. varians* adults can be obtained from Dryad ("Data 2" file deposited in the Dryad Digital Repository; Hu et al., 2022a, 2022b).

Bacterial quantitative PCR

To measure symbiont densities in different developmental stages and castes, we applied quantitative PCR (qPCR) to 151 DNA samples obtained from individuals across four *C. varians* colonies, depositing our data in Dryad ("Data 3" file deposited in the Dryad Digital Repository; Hu et al., 2022a, 2022b). qPCR quantification of bacterial 16S rRNA gene copies was performed with PerfeCTa SYBR Green FastMix (Quanta BioSciences Inc., Gaithersburg, MD, USA) using the primers 515F (5′-GTGCCAGCMGCCG CGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTA AT-3′) (Caporaso et al., 2011). In total, 1 µl of DNA template was added to a cocktail of 10 µl of PerfeCTa SYBR Green FastMix (Quanta BioSciences Inc., Gaithersburg, MD, USA), 0.125 µl of each primer at 20 µM (Invitrogen, Carlsbad, CA, USA), and 8.75 µl of PCR water (Sigma). Reactions were performed on an Mx3005P qPCR System (Agilent Technologies, Santa Clara, CA, USA), using one cycle of 94°C for 10 min, and 35 cycles of 45 s at 94°C, 60 s at 50°C and 60 s at 72°C. Plate reads were performed at the end of each extension step. Melting curve analyses were performed immediately after the last amplification, and included 90 cycles of 0.5°C increments from 50 to 95°C, with 30 s at each temperature. Averages were taken from two technical replicates performed for each ant sample across separate plates. For absolute quantification, each plate included a triplicated 1:10 serial dilution standard curve generated from a linearized plasmid with the 16S rRNA gene of *Escherichia coli* (see Hu et al., 2017 for more detail). We consistently achieved r^2 values of ≥0.999 and reaction efficiencies of ≥94%.

To normalize bacterial density estimates, we divided each 16S rRNA gene copy number average by the total ng of DNA for the given sample, obtained using the Quant-iT dsDNA High Sensitivity Assay kit (Life Technologies, Grand Island, NY, USA) on the GloMax Multi Detection system (Promega, Madison, WI, USA), following the manufacturer's protocol. We used a one-way analysis of variance (ANOVA) to test whether normalized bacterial densities differed across developmental stages and castes, applying Tukey's honestly significant difference (HSD) post hoc tests (Tukey, 1949) to detect pairwise differences among development/caste groups.

Bacterial 16S rRNA amplicon sequencing

DNA extractions from 363 *Cephalotes* ants, three gut content and three gut wall samples from *C. varians* larvae, and

33 blank samples were submitted for amplicon sequencing of the V4 hypervariable region of the bacterial 16S rRNA gene following the protocols of the Earth Microbiome Project (Caporaso et al., 2011). Library preparation and sequencing were performed at the Argonne National Laboratory (Argonne, IL, USA), with paired-end 151-bp sequencing reactions on multiplexed samples across five Illumina MiSeq lanes.

Raw sequence reads were analyzed using mothur v.1.35.1 (Schloss et al., 2009). After read pair assembly, contigs from the five sequencing batches were grouped and further quality filtered by removing contigs outside the 251–255-bp size range, those with ≥10 ambiguous nucleotides, and those with homopolymer tracts ≥8 bp. Contigs from across the 402 sequence libraries were extracted using the get.groups command and concatenated into one FastA and group file. After these quality control steps, 14 *Cephalotes* samples and seven blank libraries with fewer than 3000 reads remaining were eliminated from subsequent analyses.

We used the unique.seqs command to identify unique sequences, reducing the size of the dataset, which was then subjected to a previously published decontamination procedure (Łukasik et al., 2017). Briefly, the maximum relative abundance of each unique sequence was calculated across all blank libraries (value *a*) and, separately, all ant libraries (value *b*). Unique sequences with a ratio of value *a*:value *b* ≥ 0.2 were classified as contaminants and removed from all libraries. Contaminant sequences were most often from Alteromonadales, Pseudomonadales, Bacillales, Lactobacillales, Rhizobiales, Xanthomonadales, Burkholderiales, and Mycobacteriales. The top BLASTn hits of the three most abundant contaminant sequences from bacterial orders commonly found in *Cephalotes* ants were isolated from non-all-not-*Cephalotes* habitats, suggesting that contaminant removal does not affect the integrity of the *Cephalotes* microbiome dataset. Prior work has shown that filtering rare sequences is an effective approach for the removal of sequencing and taxonomic artifacts, and this procedure reduced the complexity of high-throughput sequencing data, with minimal information loss (Cao et al., 2021). Toward these ends, we removed rare unique sequences that did not comprise at least 0.1% of one or more ant sequence libraries. This procedure resulted in the loss of >75% of the starting reads for 28 libraries, which were removed from subsequent analyses. Information on unique sequences removed from those steps can be found in Dryad ("Data 4" deposited in the Dryad Digital Repository (Hu et al., 2022a, 2022b)).

The remaining quality-filtered unique sequences were aligned to the SILVA database (Quast et al., 2013) using the align.seqs command. The commands screen.seqs and filter.seqs were used to ensure that all aligned sequences mapped to the same region. We then performed chimera checking using UCHIME (Edgar et al., 2011), removing all chimeric sequences. Finally, the remaining sequences were classified using the Ribosomal Database Project (RDP) 16S rRNA gene training set (version 16) (Cole et al., 2009). Those classified as mitochondria, chloroplasts, Archaea, or Eukarya were removed using the remove.lineage command.

Unique sequences were then assigned to OTUs at 97%, 98%, and 99% sequence identity levels with the default settings of the opticlust algorithm in mothur. Clustering at 97% identity is standard practice for the field when examining the distribution of bacterial "species." Clustering at 98% and 99% levels enabled finer scale evaluation of symbiont strain distributions, heightening our chances of detecting a cospeciation-driven phylosymbiosis signal (Sanders et al., 2014). Using this output information, we compiled OTU tables containing the numbers of filtered reads assigned to each OTU, taxonomic assignments, and a representative corresponding to the most abundant sequence assigned to each OTU.

Minimum entropy decomposition (oligotyping)

To more precisely characterize bacterial diversity, minimum entropy decomposition (MED) was also performed on our filtered Illumina sequence libraries (Eren et al., 2015). Toward this end, we first formatted the unique sequence count table, fastA file, and taxonomy file obtained just before the aforementioned OTU picking steps, using the "mothur2oligo" tool (available from https://github.com/DenefLab/MicrobeMiseq/tree/master/mothur2oligo). Minimum entropy decomposition analysis was then performed using default parameters. In total, 3744 unique sequences were analyzed, and 1214 outliers exceeding the minimum substantive abundance and maximum variation allowed at each node were removed. The remaining 2530 unique sequences were grouped into 271 MED nodes, or "oligotypes." Following the approach described above for OTUs, we then manually processed output files to create an oligotype OTU table.

Filtering cross-contaminants

Symbiont presence/absence assessments using amplicon sequence data are sensitive to contaminating sequences. For this reason, we identified a minimum abundance threshold for all analyses relying on presence/absence calls. To enable this, we leveraged the inclusion of army ant samples in one of our Illumina MiSeq lanes. Army ants have specialized symbionts that are not found in

Cephalotes, including members of a clade known informally as "Unclassified Firmicutes" (Łukasik et al., 2017). Sequences from this clade were detected at a maximum proportion of 0.002994 within quality-filtered *Cephalotes* libraries from the same lane. We, thus, removed any sequence with a relative abundance of ≤0.002994 in a given sequence library due to its potential as a cross-contaminant. Jaccard distances for 97%, 98%, and 99% OTUs, and for our oligotype clustering results were computed using this filtered dataset.

Assessing beta-diversity across development, colonies, and castes

To determine the similarity of microbial communities across developmental stages, colonies, and castes of our best-sampled species, we computed Bray–Curtis and Jaccard distances on 97% OTU tables for all *C. varians* libraries using the "vegdist" function within the *vegan* package for R v3.6.2 (Oksanen et al., 2007). The Bray–Curtis distance matrix was used for principal coordinates analysis (PCoA) through the execution of the "pco" function within the *labdsv* package for R (Roberts, 2016). We formally compared 97% OTU composition across colonies, stages, and castes using permutational ANOVA tests with 1000 permutations (i.e., Adonis; McArdle & Anderson, 2001; from the *vegan* package in R) on both Bray–Curtis and Jaccard distance matrices. We opted for these specific presence–absence (Jaccard) and abundance (Bray–Curtis) based indices, not only because they reflected different measures of community composition, but because they were also robust to sampling errors (Schroeder & Jenkins, 2018). Explorations of microbiome differences across colonies and developmental stages were also performed using heatmap visualization for microbiomes of the 13 *Cephalotes* species.

Enterotyping and indicator OTU analysis versus caste and stage

To further identify ants with similar bacterial communities, samples from all *Cephalotes* species were clustered into "enterotypes" (Arumugam et al., 2011). Briefly, the 97% OTU table was converted to a biom-format OTU table using QIMME version 1.9.1 (Caporaso et al., 2010) for computation of the weighted UniFrac distance metric according to the relative abundance of the top 39 identified OTUs. All ant libraries were clustered using the partitioning around medoids (PAM) clustering algorithm (Kaufman & Rousseeuw, 1990). The optimal number of clusters in our dataset was identified by the Calinski–Harabasz (CH) index (Appendix S1: Table S3). We then performed the between-class analysis using "ade4" in R, plotting the results with the "s.class()" function in the *adegraphics njm* package. We identified indicator 97% OTUs, for which indicator values were >0.25, with $p < 0.05$, using the "indval" function in the package *labdsv* in R (Appendix S1: Table S4) (Roberts, 2016). To gain further insight into the enterotype distributions across different developmental stages or castes, we plotted enterotype frequencies across larvae of varying age/body length. We, further, assessed enterotype assignments for the microbial communities of each sampled worker, soldier, winged and wingless queen.

Microbiome similarity versus host phylogeny and geography

To ascertain whether host relatedness recapitulates symbiont community similarity, we performed a phylosymbiosis assessment, conducting partial Mantel tests in the *vegan* package in R. To account for confounding effects of geography, between-sample geographic distances were computed based on GPS coordinates for each sample (Appendix S1: Table S1) using the AMNH geographic distance calculator tool (Ersts, 2013). An average of geographic distances was calculated if there was more than one colony included for a specific ant species. Host phylogenetic distances were calculated from a time-calibrated *Cephalotes* phylogeny (Price et al., 2016), using the cophenetic function within the *ape* package, implemented in R. Mantel tests were then implemented using the *vegan* package in R, testing for significant associations between bacterial community dissimilarity, host phylogenetic distance, and geographic distance. Separate Mantel tests were run for each enterotype. For our measures of community similarity/beta-diversity, we utilized both Bray–Curtis and Jaccard distances, calculated from oligotype data, or with 99%, 98%, or 97% OTUs. We took the average of such dissimilarities among replicate sequence libraries from *Cephalotes* species pairs, to reduce values to single datapoints, doing this separately within each enterotype-specific analysis.

To visualize the impacts of host phylogeny, we used the *labdsv* package in R (Roberts, 2016) to perform PCoAs separately for each enterotype. Bray–Curtis distance measures, computed from oligotype distributions, were used as inputs for these analyses, and for each of our three plots we included only those sequence libraries assigned to the focal enterotype. Individual datapoints were then assigned colors corresponding to previously defined host species groups (Sanders et al., 2014).

Testing for cospeciation between *Cephalotes* and *Cephaloticoccus* symbionts

Building on our above phylosymbiosis analysis, we measured congruency between the *Cephalotes* ant phylogeny and that of their most dominant symbiont, species from the *Cephaloticoccus* genus (Opitutales). To improve phylogenetic resolution beyond levels provided by 16S rRNA segments, we mined shotgun metagenomic data from *Cephalotes* on the IMG website (https://img.jgi.doe.gov/), retrieving sequences for five genes—*uvrB*, *23S rRNA, dnaA*, *recA*, and *rpoB*—that are widely conserved across bacterial clades (He et al., 2017; Pilhofer et al., 2008; Spring et al., 2016). To achieve this, we BLAST'd homologues for each gene from two *Cephaloticoccus* species (IMG Project IDs: Gp0154034; Gp0110136) against 18 *Cephalotes* metagenomes in the IMG database (Hu et al., 2018). Hits exceeding 30% query coverage were downloaded if they exceeded % identity thresholds matching natural breakpoints in the data. Below these thresholds, BLAST hits appeared much less similar to queries and were almost always from scaffolds classifying to other taxa (i.e., not Verrucomicrobia or Opitutales). The thresholds chosen were: 83% for *uvrB* and *recA*; 87% for *dnaA*; 88% for *rpoB*; and 98% for 23S rRNA. For each metagenome, similar depths of sequence coverage from the chosen genes' containing scaffolds and the typical presence of just one dominant *Cephaloticoccus* per host based on our observations (Hu et al., 2018; but see Hu et al., 2014), suggested that the chosen genes originated from a single symbiont genome.

Using the top two non-*Cephalotes* derived hits from NCBI as outgroups, we aligned sequences in SeaView (Gouy et al., 2010), using the alignment for preliminary maximum likelihood analyses with RAxML on the Cipres Web portal (Miller et al., 2011). Replicate sequences from single host species grouped into monophyletic clades and typically comprised different portions of the focal gene. We retained only the longest sequence in such instances, re-running RAxML analysis with 1000 bootstrap replicates on these pruned datasets. Of the resulting trees, we kept the two with the highest average bootstrap support at each node, that is, the *rpoB* and *uvrB* genes. Applying default parameters and event costs, we compared the topologies of these gene trees to that of a *Cephalotes* host tree (Price et al., 2016), using the cophylogeny software Jane4, and applying 1000 randomized tip mapping permutations to evaluate statistical significance.

Origins of adult and larval-enriched symbionts: BLASTn and phylogenetics

Prior studies on adult microbiomes have shown that approximately 95% of 16S rRNA sequences from *Cephalotes* workers came from seemingly specialized lineages, with long branch separation from free-living relatives and membership exclusive to bacteria from *Cephalotes* and its sister genus *Procryptocerus* (Hu et al., 2014, 2018). To determine whether bacteria from our broader sampling of castes and developmental stages belonged to such lineages, we performed BLASTn searches against the NCBI nr database. As query sequences we used the most abundant, "representative," 16S rRNA sequence from 154 97% OTUs. These OTUs comprised the entirety of those either exceeding the contamination-robust relative abundance threshold of 0.002994 in at least two sequence libraries, or those exceeding at least 0.05 in one library. When top hits: (1) came from another cephalotine ant (i.e., of the *Procryptocerus* or *Cephalotes* genus), (2) exhibited >90% sequence identity versus our query sequence, and (3) had been defined previously as a member of a cephalotine-specific clade, we considered the query OTU to be a specialized symbiont. Those not meeting these criteria were considered, at this stage, to be nonspecialized. We gave additional scrutiny to several of these bacteria ("Symbiont Phylogenetics" file deposited in the Dryad Digital Repository; Hu et al., 2022a, 2022b), performing phylogenetic analyses on newly identified 16S rRNA sequences to identify relatives from NCBI. In a small number of cases, these assessments reversed our BLAST-based conclusions.

We, finally, computed average and median relative abundance values for each of the 154 aforementioned OTUs within queens (winged vs. wingless), soldiers, workers, or larvae, for each colony. Summing the fractions of all *Cephalotes*-restricted OTUs, we ascertained whether the specialized fraction of the microbiome varied across caste or stage.

RESULTS

Symbiont densities vary across *C. varians* development: qPCR

In our first assessment of microbiome variation across *Cephalotes* ants, we utilized 16S rRNA-based qPCR to measure bacterial titer in four *C. varians* colonies. Our two-way ANOVA detected no significant effect of colony on normalized bacterial density ($F(3) = 1.122$, $R^2 = 0.003$, $p = 0.344$; Appendix S1: Table S5). But this same ANOVA model revealed a significant effect of developmental stage/caste designations ($F(11) = 95.08$, $R^2 = 0.876$, $p < 2 \times 10^{-16}$), suggesting variation in symbiont density between some combination of eggs, differentially aged larvae, prepupae, pupae, soldiers, queens, or adult workers.

Using Tukey's post hoc HSD tests we ascertained that eggs and young larvae (size <2 mm) had the lowest numbers of bacteria across the four *C. varians* colonies (Figure 2a), with average 16S rRNA gene copy numbers, per ng DNA, of 4.34×10^3 and 5.08×10^3. Unnormalized qPCR measures were comparable between eggs and our blank, negative control samples (8.63×10^2 vs. 5.00×10^2) suggesting that *C. varians* ants are nearly sterile upon hatching. The first detectable change in bacterial titer unfolded as larvae transitioned from small (<2 mm) to intermediate size (2–3 mm), with normalized 16S rRNA copy numbers increasing to 2.82×10^4 copies/ng DNA ($p_{<2 \text{ vs. } 2-3} = 0.040$; Appendix S1: Table S5). Normalized titers increased again as larvae aged further, reaching 2.50×10^5 copies/ng DNA for individuals >3 mm in length ($p_{2-3 \text{ vs. } >3} < 0.001$), before falling drastically at the prepupal stage to 3.17×10^3 copies/ng DNA ($p_{>3 \text{ vs. prepupae}} < 0.001$). There was no detectable change in titer into the pupal stage, with 16S rRNA averages at 2.49×10^3 copies/ng DNA ($p_{\text{pre-pupae vs. pupae}} = 0.999$). Densities in newly eclosed, callow workers (pixel value ≥60) then rose to 1.31×10^5 copies/ng DNA ($p_{\text{pupae vs. adult-pixel}>60} < 0.001$), despite high variability among individuals (Figure 2a). Adult workers at the next age class (pixel value = 40–60) showed an additional, but smaller increase in bacterial titer, with 5.45×10^5 16S rRNA copies/ng DNA ($p_{\text{adult-pixel}\geq 60 \text{ vs. adult-pixel-40–60}} < 0.001$). Plausibly older workers, with darker pigmentation (pixel classes 0–20, 20–40), however, showed no evidence for higher titers. Further, all post-callow adult stages exhibited greater consistency in 16 rRNA copy number when compared with callows (Figure 2a; Appendix S1: Table S5), suggesting stable and consistent titers.

The most darkly pigmented workers (pixel value = 0–20) showed pigmentation overlap with one alate queen, one wingless queen, and 10 soldiers ("Data 1" file deposited in the Dryad Digital Repository; Hu et al., 2022a, 2022b) suggesting roughly similar age. Comparisons of bacterial titers among these castes suggested little difference in symbiont quantities, with 16S rRNA copy numbers/ng DNA equaling 3.57×10^5 (alate) and 1.14×10^5 (wingless) for the single queens, and averages of 4.03×10^5 and 4.18×10^5 for soldiers and workers. Accordingly, *p*-values from pairwise Tukey HSD comparisons were nonsignificant, ranging from 0.997 to 1.0 (Appendix S1: Table S5).

The presence of populous symbiont communities varies across *Cephalotes* development: Standard PCR

After discovering the above-described developmental time course for symbiont proliferation, and ascertaining that all female castes harbor symbionts during adulthood, we used standard PCR amplification with universal 16S rRNA gene primers as a crude approximation for the presence of modestly abundant symbiont communities. Building off prior work showing that weak-to-no amplification with such primers corresponded to low qPCR measures of symbiont titer (Hu et al., 2017), we targeted 522 ants spanning castes and stages of all 13 studied *Cephalotes* species (Figure 2b). Eggs from three species ($n = 13$ samples) failed to amplify with universal bacterial PCR primers. But amplification did occur for 20.6% ($n = 34$) of young larvae (normalized size <2) from across four *Cephalotes* species. In contrast, 74.6% ($n = 47$) of middle-aged larvae from 11 species (normalized size = 2–3) yielded 16S rRNA PCR amplification, as did 94.5% ($n = 73$) of older larvae from 12 cephalotine species (normalized size >3). All 37 prepupae, sampled across seven *Cephalotes* species, failed to amplify with universal bacterial primers, whereas amplification was seen for only 1/57 pupae obtained from three host species. At the adult stage, all wingless queens ($n = 18$, from six species), alate queens ($n = 7$, from four species), and soldiers ($n = 57$, from 11 species) yielded 16S rRNA PCR amplification, as did 177/178 workers (from 13 species). These trends, thus, mirrored our quantitative estimates from *C. varians*. With the vast majority of subsequently sequenced samples harboring similar, stage-specific bacterial communities (below), they are consistent with a process of early gut symbiont acquisition, loss prior to pupation, and re-acquisition in adulthood.

Whole ant community composition across castes and stages of *C. varians*

Having assessed within-colony and across-development symbiont dynamics with the above data, we next set out to characterize the composition of symbiotic bacterial communities. We began with a study of *C. varians*, subjecting 145 samples with PCR-detectable bacterial communities to amplicon sequencing of the bacterial 16S rRNA gene using Illumina MiSeq technology. We removed one library with <1000 reads, and focused on the remaining 144. After removing contaminants, chimeras, and nonbacterial sequences, 1,597,177 quality-controlled sequences remained for further analysis, an average of 11,092 sequences per library. Unique sequence, OTU, and oligotype tables from these analyses can be found in Dryad ("Data 5–9" file deposited in the Dryad Digital Repository; Hu et al., 2022a, 2022b).

Permutational multivariate ANOVA statistics indicated that some combinations of 97% OTU composition and/or relative abundances differed significantly across *C. varians* development (Figure 3; Appendix S1: Table S6; Bray–Curtis: $F(1) = 129.617$, $R^2 = 0.415$, $p = 0.001$;

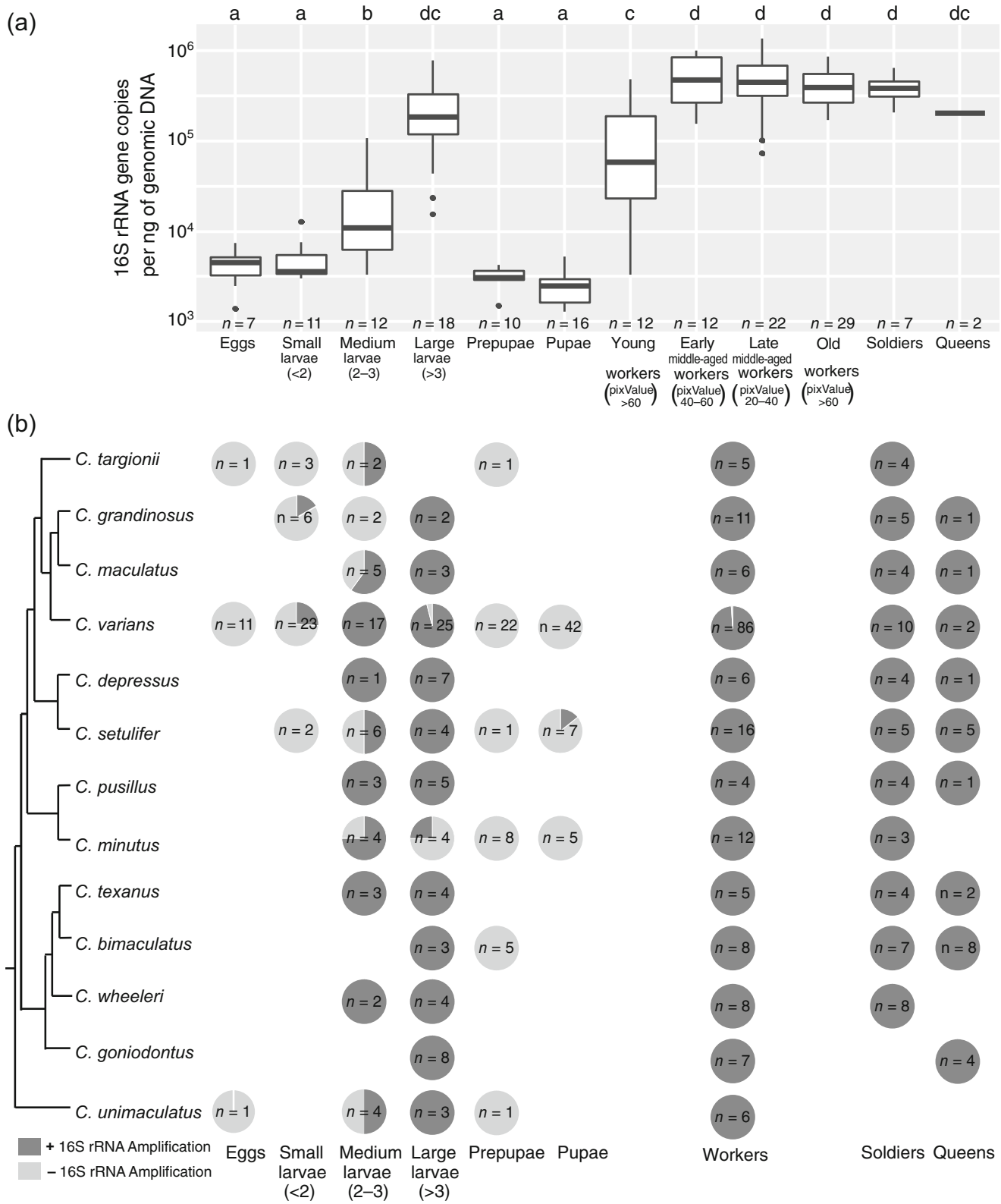


FIGURE 2 Bacterial presence and abundance across developmental stages and castes of *Cephalotes* ants. (a) Results of qPCR assays. Stem and whisker plots illustrate medians, quartiles, and outliers of 16S rRNA copy number/ng genomic DNA for each stage/caste. Letters above the graph reveal results of Tukey HSD comparisons, with different letters revealing castes/stages with differing quantities of bacteria. Sample sizes indicated at bottom of graph. (b) Relatedness among the 13 studied *Cephalotes* species (Price et al., 2016), and our overall results of standard PCR with universal bacterial 16S rRNA primers indicating whether individuals from particular castes/stages had detectable titers of bacteria (dark gray) or no detectable quantities (light gray). Results shown in the form of pie charts indicate the proportions of each caste/stage amplifying, with sample sizes inside each pie chart. Alate and wingless queen data are pooled here.

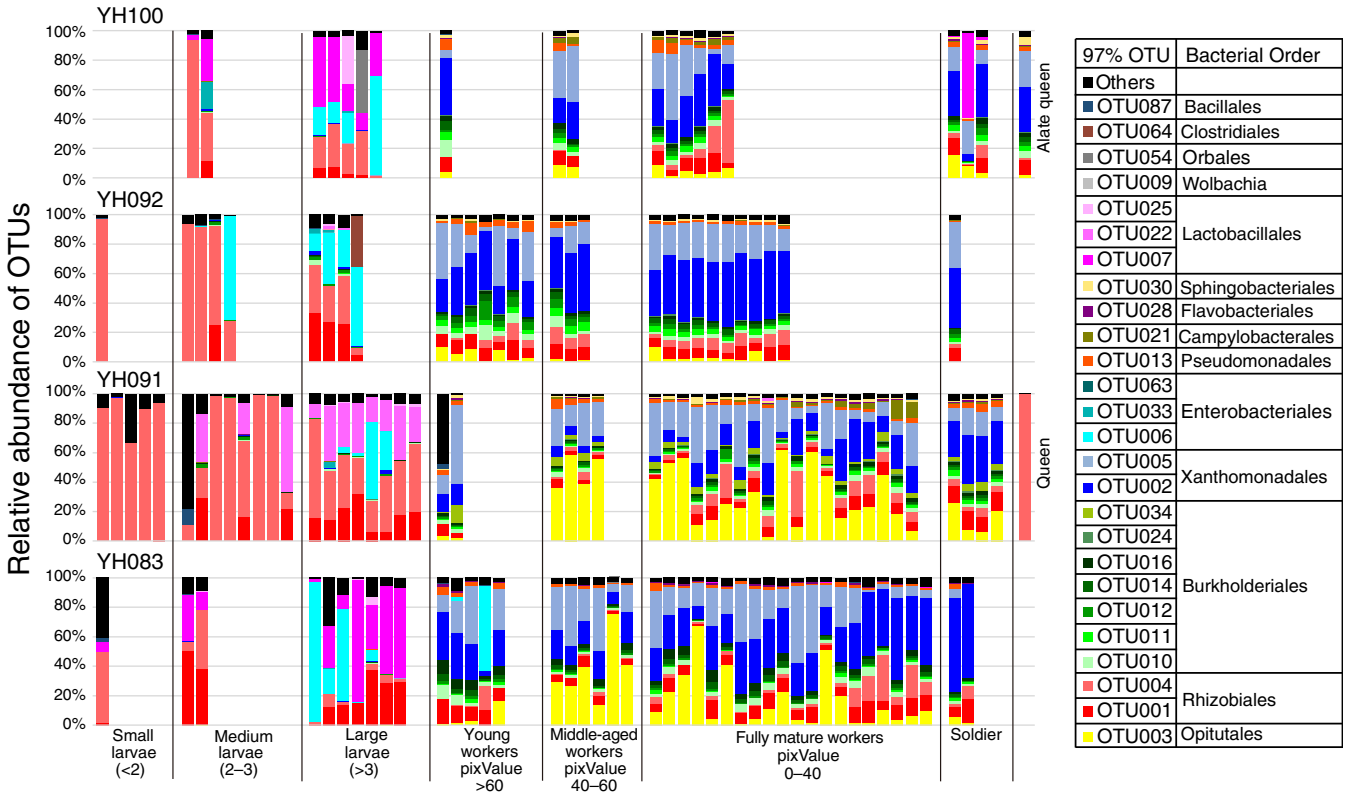


FIGURE 3 Bacterial composition among developmental stages and castes of *Cephalotes varians*. All data were obtained via Illumina amplicon sequencing of the 16S rRNA gene from individual ants. Taxonomic composition of each individual sequence library from *C. varians*, across four colonies (YH100, YH091, YH092, YH083). Stacked bar graphs illustrate bacterial composition at the 97% operational taxonomic unit (OTU) level, with similarly colored OTUs hailing from the same orders.

Jaccard: $F(1) = 89.087$, $R^2 = 0.342$, $p = 0.001$), and also among colonies (Bray–Curtis: $F(3) = 7.813$, $R^2 = 0.075$, $p = 0.001$; Jaccard: $F(3) = 6.329$, $R^2 = 0.073$, $p = 0.001$). Across *C. varians* larvae, six dominant 97% OTUs, including Rhizobiales (OTU001 and OTU004), Lactobacillales (OTU007, OTU022, and OTU025), and Enterobacteriales (OTU006), accounted for an average of 87.1% of the reads. But, in very young larvae (normalized length < 2), Rhizobiales OTU004 was the only dominant bacterium. Another type of Rhizobiales, from OTU001, became common subsequently, beginning in middle-aged larvae (normalized length = 2–3) and continuing into the oldest larval stages (normalized length > 3). Lactobacillales OTUs 007, 022, and 025, as well as Enterobacteriales OTU006, were the most abundant taxa in the oldest larvae, and were also common in some larvae from the middle age class.

With the exception of one mature queen, the microbiomes of *C. varians* adults differed from those of larvae (e.g., Figure 3). To begin, Lactobacillales and Enterobacteriales were extremely rare in workers, soldiers, and one premated alate queen. Rhizobiales were ubiquitous, in contrast, but only modestly abundant. Adults, instead, harbored OTUs found rarely, or much less abundantly, in larvae, including highly abundant OTUs from the orders Opitutales (OTU003) and Xanthomonadales (OTU002 and OTU005), seven OTUs from the Burkholderiales, and individual OTUs from the orders Pseudomonadales, Flavobacteriales, Campylobacterales, and Sphingobacteriales. The dominant OTUs from these orders accounted for an average of 93.95% of the sequence reads in each library.

We ran permutational multivariate ANOVA analyses, using a 97% OTU table, on age-matched, mature workers and soldiers (pixel value < 20). Such assessments revealed no microbiome differences among these castes for either Bray–Curtis or Jaccard dissimilarity metrics (Appendix S1: Table S6; Bray–Curtis: $F(1) = 2.376$, $R^2 = 0.043$, $p = 0.074$; Jaccard: $F(1) = 2.023$, $R^2 = 0.029$, $p = 0.107$). Our remaining caste sampling, that is, across queens, lacked sufficient replication for statistics. But anecdotally, a virgin winged queen (colony YH100) harbored a similar gut bacterial community in comparison to its sibling workers and soldiers, whereas the previously mentioned mature, wingless queen (colony YH091) was dominated by Rhizobiales OTU004, similar to young larvae from the same colony (Figure 3).

PCoA on the full 97% OTU table reinforced the above results (Appendix S1: Figure S1), separating gut

communities of larvae versus workers and soldiers, along the first PCoA axis (46.43%). Only two adults harbored microbiomes clustering with those of larvae, including a worker dominated by Lactobacillales OTU007, and the aforementioned mature/wingless queen. Matching the compositional patterns from Figure 3, the symbiotic community of the winged (a.k.a. alate) queen clustered along the first axis with those from workers and in all but one soldier.

Gut localization of larvae-associated symbionts in *C. varians*

Having identified larval symbionts through whole-body DNA extractions, we set out to better localize these microbes within hosts, focusing on three amplicon sequencing libraries from gut wall tissue, and three from gut content, obtained from three late-stage *C. varians* larvae. Comparisons with whole-body, late-stage larval sequence libraries revealed similarity (Appendix S1: Figures S2 vs. S3), with a high abundance of Rhizobiales OTUs 001 and 004, Lactobacillales OTU007, and—in one case—Enterobacteriales OTU006. Lactobacillales OTU25 and OTUs from adult-enriched symbionts in the Xanthomonadales and Burkholderiales were present at lower titers. Sequences from additional OTUs made up <10% of each library. OTU004 (Rhizobiales) was consistently more abundant in all three gut tissue versus gut content comparisons. The remaining, aforementioned OTUs showed similar relative abundances across the two sample types. Given the small number of dissected samples, these trends require further study. But the resemblance between larval gut versus whole-body sequence libraries suggested that most of the bacteria from our main larval dataset were, indeed, gut inhabitants.

Whole ant microbiome composition across castes and stages of 13 *Cephalotes* species: 97% OTU distributions

We examined whether patterns of community structure found in *C. varians* were consistent across 12 additional *Cephalotes* species. In total, 209 further 16S rRNA sequence libraries were generated toward this end, with an average of 8714 sequences per library after quality and contaminant filtering. Our remaining analyses included 192 of these quality-controlled libraries (i.e., all but those from 16 callow adults and 1 pupa). Focusing on 97% OTU clusters, a permutational multivariate ANOVA revealed significant differences in the bacterial communities between larvae and adults for all *Cephalotes* species, except *C. targionii* (Appendix S1: Table S7; Adonis permANOVA tests: $p < 0.05$). This was true for both Bray–Curtis and Jaccard measures of beta-diversity.

To characterize taxa driving differences across development, we constructed a heatmap showing the average relative abundance of 80 of the most common 97% OTUs for each developmental stage and caste per colony (Figure 4; see legend for inclusion criteria). As seen for *C. varians*, larval samples of different *Cephalotes* species harbored partially distinct symbiotic communities compared with adults, with most adult-enriched symbionts exhibiting rarity, or low-abundance, in larvae. Using a relative abundance threshold of 0.002994 to ascertain presence/absence, and focusing on OTUs found in at least 30% of all sampled adult workers (Appendix S1: Figure S3), we found that the most depleted OTUs came from the Opitutales (OTU003, 98.1% of workers vs. 8.7% of larvae), Comamonadaceae (OTU020, 74.0% of workers vs. 8.7% of larvae), Xanthomonadales (OTU005, 70.8% of workers vs. 1.9% of larvae), Pseudomonadales (OTU013, 69.5% of workers vs. 4.9% of larvae), Flavobacteriales (OTU028, 46.8% of workers vs. 1.0% of larvae), Campylobacterales (OTU021, 40.3% of workers vs. 3.9% of larvae), and Sphingobacteriales (OTU030, 33.1% of workers vs. 0% of larvae). Showing more intermediate patterns were Alcaligenaceae OTUs 010 (93.5% of workers vs. 35.0% of larvae), 012 (97.4% of workers vs. 40.8% of larvae), and 016 (83.8% of workers vs. 19.4% of larvae), plus Xanthomonadales OTU002 (74.0% of workers vs. 17.5% of larvae). We deposited the top-ranked 97% OTUs by caste and stage across all *Cephalotes* ants in Dryad ("Data 10" file deposited in the Dryad Digital Repository; Hu et al., 2022a, 2022b).

Using the same abundance threshold for presence/absence assignment, and focusing on OTUs found in at least 10% of larval samples (Appendix S1: Figure S3), OTUs enriched in larvae included Lactobacillales OTUs 007 (3.2% of workers vs. 36.9% of larvae), 022 (2.6% of workers vs. 28.2% of larvae), 025 (0% of workers vs. 35.9% of larvae), 082 (0% of workers vs. 11.7% of larvae), and 086 (0% of workers vs. 10.7% of larvae). Enterobacteriales OTU006 (1.3% of workers vs. 45.8% of larvae) and OTU033 (0% of adults vs. 13.6% of larvae) showed a similar trend, as did Actinobacteria OTUs 062 (Actinomycetales; 0.6% of workers vs. 10.7% of larvae) and 080 (Bifidobacteriales; 0% of workers vs. 16.5% of larvae).

Distinct from these patterns were trends for Rhizobiales symbionts, which were common in both adults and larvae. OTU001 was found, for instance, in 100% of workers and 79.6% of larvae. OTU004 was in 76.6% of workers and 83.5% of larvae. Although less prevalent, OTUs 017 (15.6% of workers vs. 19.4% of larvae) and 053 (11.0% of workers vs. 17.5% of larvae) showed similar patterns.

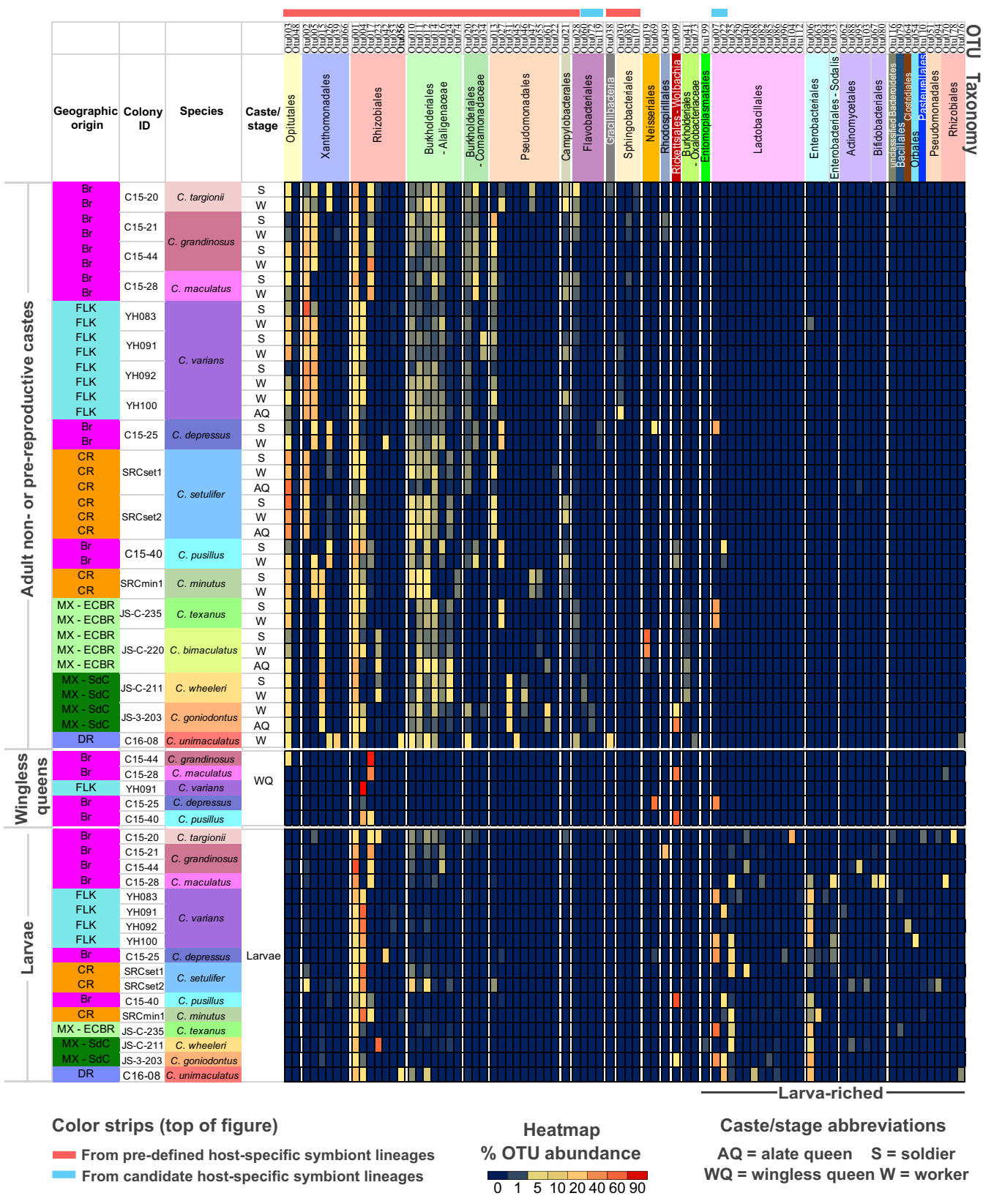


FIGURE 4 Relative abundance of common bacterial 97% operational taxonomic units (OTUs) across castes and developmental stages, with an expanded view of bacteria at least occasionally abundant in larvae. This heatmap illustrates averages for each developmental stage or caste (worker, soldier, alate queen, wingless queen, or larva) within each studied colony. Shown here is a subset of the 154 OTUs that exceeded 0.002994 relative abundance in at least two sequence libraries, or at least 0.05 in one. Of the 80 such OTUs illustrated here, all either exhibited a pooled average of at least 0.01 across the individuals of at least one caste or stage in a given colony or exceeded 0.002994 average relative abundance in a caste or stage across two or more colonies.

Maximum and average relative abundance of OTUs across development revealed qualitatively similar trends, indicating that adult-enriched symbionts with low to intermediate prevalence across larvae are usually found at low relative abundance within these immatures (Appendix S1: Figure S3; Data 10 deposited in the Dryad Digital Repository; Hu et al., 2022a, 2022b). Two Alcaligenaceae OTUs, 010 and 012, provided interesting exceptions, with maximum relative abundances in larvae (0.309 and 0.157) either exceeding or approaching those in workers (0.121 and 0.188). Rhizobiales, with their dual-stage prevalence, showed similar patterns, with greater average and maximum relative abundance in larvae versus adults, as exemplified by OTUs 001, 004, and 053, but not OTU017. Larvae-enriched symbionts were, conversely, most often rare in adults when detected, with the exceptions of OTU007 (Lactobacillales) and OTU006 (Enterobacteriales), which both reached sporadically high maximum relative abundances in workers (0.506 and 0.578).

Many of the above general trends of larval versus adult differences—gleaned from adult workers—were recapitulated when comparing alate queens and soldiers against larval immatures (Figures 3 and 4; Appendix S1: Table S5). But, whereas these adult castes harbored fairly similar microbiomes, there were some key exceptions. In *C. depressus* and *C. pusillus*, for instance, Lactobacillales were seemingly enriched in soldiers relative to workers. Furthermore, in the former species a rare bacterium from the Neisseriales was also common in soldiers, but not workers. In contrast, Campylobacterales appeared more abundant in workers than soldiers in five out of seven colonies harboring this microbe, suggesting interesting avenues for future study.

When expanding our adult caste comparisons to include five wingless, mature queens sampled from five *Cephalotes* species, we saw greater microbiome divergence (Figure 4). With little resemblance to those of other adult counterparts, symbiotic communities of some such queens instead resembled those of young larvae, with dominance by Rhizobiales OTUs 004 and 017. Mature queen microbiomes also had lower diversity, with an apparent absence of many of the adult-enriched OTUs present in their mature offspring. Representing a slight exception to the Rhizobiales-enrichment trend was a queen from *C. depressus*, possessing such bacteria at lower titers, but showing enrichment, instead, for the same Neisseriales and Lactobacillales found in abundance within their soldiers (see above). The mature queens from *C. pusillus* and *C. maculatus* colonies uniquely harbored *Wolbachia*, in addition to Rhizobiales. This was a conspicuous pattern for the latter given this symbiont's apparent absence from the queen's offspring (Figure 4).

Whole ant microbiome composition across castes and stages of 13 *Cephalotes* species: Oligotype distributions

To understand strain-level diversity across developmental stages and castes, we looked at 16S rRNA oligotype distribution within the 40 focal symbiont OTUs with relative abundance >0.05 in at least one sample (Appendix S1: Figure S4). Although slowly evolving genes like 16S rRNA can obscure fine-scale strain resolution, we detected several distinct oligotypes within the most dominant OTUs. Similar to our 97% OTU result, permutational multivariate ANOVA revealed that larvae from 11 species harbor significantly different bacterial communities versus adults at this narrower clustering width (Appendix S1: Table S7; Adonis permANOVA tests: $p < 0.05$).

Despite these differences, we found identical oligotypes across siblings from these stages for a handful of trans-developmentally shared OTUs, including those from the Rhizobiales (OTUs 001, 004, 017, 023, 042, and 056). Sibling workers, soldiers, and winged queens from the same colony similarly shared oligotypes, suggesting a homogenizing effect of colony on microbiome composition. Microbiomes of wingless queens remained nondiverse at the oligotype level. Those sharing 97% OTUs with colony-mates shared oligotypes with them, as well, with very few exceptions.

Phylogenetic affinities of abundant bacterial species from *Cephalotes* larvae

To initiate a study on the evolutionary origins of larva-enriched symbionts, we used maximum likelihood analysis for phylogenetic inference on abundant sequences from the Rhizobiales (Analysis no. 1), Enterobacteriales (Analysis no. 2), and Lactobacillales (Analysis no. 3). Our Rhizobiales phylogeny indicated that 16S rRNA gene sequences from such microbes grouped into two monophyletic, *Cephalotes* ant-specific lineages (Figure 5a). Symbiont strains from Rhizobiales OTU001 formed a distinct clade, which was sister to a "crown group" clade comprised primarily of symbionts from other ants. Nested within this crown group was a second lineage comprised exclusively of *Cephalotes* associates, including OTUs 004, 017, 023, 042, and 056.

Our maximum likelihood phylogeny of Lactobacillales bacteria (Figure 5b) revealed that 16S rRNA gene sequences from the more common OTU007 (sister to *Lactobacillus homohiochii* and *L. fructivorans*) and OTU022 (sister to two uncultured bacteria) formed separate, well supported *Cephalotes*-specific clades, removed from free-living bacteria by modestly long branches. Although generally enriched in

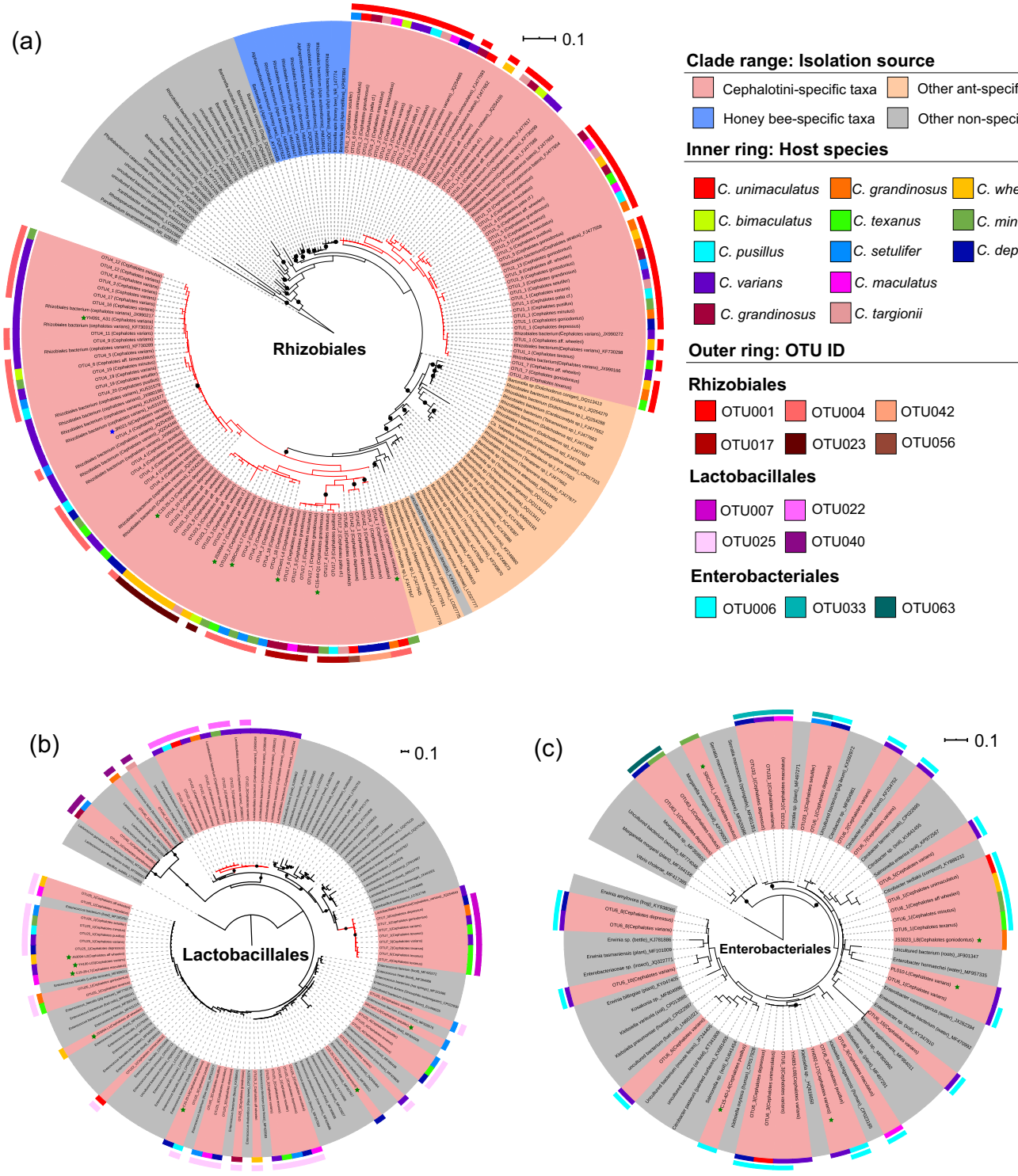


FIGURE 5 (a–c) Maximum likelihood phylogenies of three taxa found in abundance in larvae across the *Cephalotes* genus. All phylogenies were inferred from alignments of 16S rRNA sequences. Those from amplicon sequencing in this study are indicated with colored shading in the outer color strips, which indicate bacterial taxonomic assignments. Those produced from our Sanger sequencing are highlighted with stars (culture independent sequence from ant = green; sequence from cultured symbiont isolate = blue). The remainder were identified through BLASTn searches against the NCBI nr database. Names of sequences from cephalotine ants are enclosed in pink shading. Those from other ants are in orange. Those from other hosts or habitats are in gray. The interior color strip identifies the cephalotine host species. OTU, operational taxonomic unit.

larvae, 23 of 24 adult ants harboring Lactobacillales possessed a bacterium from one of these plausibly specialized OTUs. Symbiotic strains from OTU025 Lactobacillales, also common in larvae, but seemingly absent from adults, did not form a host-specific clade, instead showing close relatedness to free-living *Enterococcus*.

In contrast with the patterns for Rhizobiales and Lactobacillales, 16S rRNA gene sequences from three Enterobacteriales OTUs, dominant in some *Cephalotes* larvae did not form any host-specific clades, grouping instead with a range of free-living bacteria from this order (Figure 5c). In particular, sequences from OTU006 showed relatedness to *Erwinia*, *Enterobacter*, *Salmonella*, *Pantoea*, and *Citrobacter*. OTU033 showed phylogenetic affinity to *Serratia*. The more distantly related OTU063 was related to *Morganella*.

Identifying host-specialized versus environmentally acquired bacteria: BLAST and phylogenetic analyses

The above results suggest that cephalotine microbiomes consist of both specialists and environmentally acquired bacteria. To expand on this assessment, we performed a series of BLAST and phylogenetic analyses on 154 common OTUs. Although 103 97% OTUs appeared unrelated to previously defined specialists, 51 yielded a top hit to a bacterium from a known cephalotine-specific clade. Across these instances the average sequence identity was 98.0%.

We further scrutinized the BLAST results for the 67 most abundant putative nonspecialists (out of $n = 103$), through maximum likelihood phylogenetic analysis ("Symbiotic Phylogenetics" file deposited in Dryad; Hu et al., 2022a, 2022b). Through this method, we overturned BLASTn-based conclusions of nonspecialization for only five OTUs, revealing robustness for most of our conclusions, that is, that putative nonspecialists are often closely related to free-living bacteria (e.g., Figure 5b,c). Assignments to cephalotine-specialized lineages versus nonspecialized lineages for the chosen OTUs are deposited in Dryad ("Data 11" file deposited in the Dryad Digital Repository; Hu et al., 2022a, 2022b).

Proportions of adult versus larval microbiomes comprised of host-specialist bacteria

Using the combined set of BLAST and phylogenetic results, we computed the proportions of individual ant microbiomes comprised of specialized bacteria, across stages and castes for our 18 studied colonies (Appendix S1: Figure S5). In adults, microbiomes of pre- and non-reproductive castes contained similarly high fractions of bacteria from cephalotine-specialized clades, that is, those found exclusively in *Cephalotes* and/or *Procryptocerus* ants. Specifically, the median summed relative abundances for such specialized bacteria were 0.984, 0.983, and 0.987 across alate queens, soldiers, and workers. The averages for alate queens were slightly lower compared with the other two castes (0.869 AQ, 0.940 S, and 0.956 W) due to the high abundance of nonspecialized *Wolbachia* in those of *C. goniodontus*.

In contrast with these patterns, symbionts from cephalotine-specialist clades comprised a median of 0.469 of mature/wingless queens' microbiomes (average = 0.661). These lower fractions were driven by an abundance of *Wolbachia* or Neisseriales in a subset of these $n = 5$ samples, and by the paucity or absence of specialized symbiont OTUs in the Xanthomonadales, Burkholderiales, Pseudomonadales (i.e., the genus *Ventosimonas*), Campylobacterales, Flavobacteriales, Gracillibacteria, and Sphingobacteriales. Along similar lines, *Cephaloticoccus* symbionts from the Opitutales—typically the most dominant microbes across adults—were abundant in just one mature queen. Indeed, in focusing on the non-Rhizobiales portion of wingless queens' specialized core microbiomes, we saw that *Cephaloticoccus*, and the few other, remaining specialists made up a median of 0 and an average of 0.109 of their microbiomes. Proportions comprised by these non-Rhizobiales specialized bacteria were, contrastingly, high for winged queens, soldiers, and workers, with respective averages equaling 0.756, 0.717, and 0.719. It is interesting to note that most Rhizobiales found in mature/wingless queens hailed from only one of the two deep-branching cephalotine-specific lineages in this order (Figure 5), with an absence of OTU001, found in all other castes and stages of every colony in our study (Figure 4).

Bearing some resemblance to wingless queens, the proportions of the larval microbiome made up of bacteria from cephalotine-specialist lineages were considerably lower than those for alate queens, soldiers, and workers, with a median and average of 0.700 and 0.675. Removal of Rhizobiales from consideration pushed these values down to 0.162 and 0.206. Additional removal of Lactobacillales OTUs 007 and 022 yielded median and average values of 0.023 and 0.060 for the specialized fraction of the larval microbiome, suggesting the rarity of other core, adult-enriched specialist symbionts.

Of notable exception were five colonies in which the remaining specialized, adult-enriched symbionts comprised >0.070 of the symbiotic community. In these cases we found a modest abundance of specialized Burkholderiales OTUs from the Alcaligenaceae, which comprised between

0.061 and 0.279 of the total microbiome. Specialized Xanthomonadales were generally rare, although they did constitute an average relative abundance of 0.065 in one colony from *C. setulifer*.

Nonspecialized portions of the larval microbiome were most often made up of Lactobacillales (from beyond OTUs 007 and 022), Enterobacteriales, and sporadically abundant bacteria, including those from the orders Actinomycetales and Bifidobacteriales in the Actinobacteria (Figure 4). Bacteria from this phylum made up an average of 0.019 of the larval microbiome, reaching a maximum average of 0.121 in larvae of *C. maculatus*.

Whole ant microbiome composition across castes and stages of 13 *Cephalotes* species: Enterotyping analysis inferred from 97% OTU composition

With our results suggesting impacts of development and caste on microbiome composition, we aimed to further characterize symbiotic bacteria of *Cephalotes* ants through enterotyping analysis on 97% OTU data from our 336 whole ant Illumina sequencing libraries. Calinski–Harabasz values reached a peak of 0.829 at $n = 3$ clusters, compared with CH values of 0.662 and 0.781 under models with $n = 2$ and $n = 4$ (Appendix S1: Table S3), suggesting the presence of three community types or "enterotypes" (Figure 6a). Enterotype 1 consisted of microbiomes from workers, soldiers, and 14 out of 16 alate queens (Figure 6b). Indicator OTU analyses revealed that this cluster was dominated by 97% OTUs from the Opitutales ($n = 1$ OTU), Burkholderiales ($n = 6$ OTUs), Xanthomonadales ($n = 2$ OTUs), Flavobacteriales ($n = 1$ OTU), Campylobacterales ($n = 1$ OTU), and Sphingobacteriales ($n = 1$ OTU), with the latter taxon being concentrated in *C. varians* (Figure 6c; Appendix S1: Table S4).

Enterotype 2 included microbiomes of larvae, four out of five mature/wingless queens, and two out of 16 alate queens, in addition to very small numbers of workers and soldiers (Figure 6b). Characteristic of this cluster were Rhizobiales OTUs 004 and 017 (Figure 6c). Microbiomes assigned to the third enterotype were mostly from larvae. However, one came from a wingless queen, whereas a few belonged to soldiers and workers. Rhizobiales OTU001 was an indicator OTU for this enterotype, as was Enterobacteriales OTU006, along with three Lactobacillales OTUs (Figure 6c; Appendix S1: Table S4). Estimating larval developmental stage through body length measures, we noticed that Enterotype 3 was dominated by microbiomes of older (i.e., larger) larvae, whereas the mature/wingless queen-including Enterotype 2 was primarily comprised of young larval microbiomes (Figure 6b).

Correlations between host phylogeny and cephalotine microbiomes differ across enterotypes

PCoA plots (Figure 7) suggested that host phylogeny plays a significant role in predicting community structure for ants hosting Enterotype 1-clustering microbiomes, but not clearly those clustering with Enterotypes 2 or 3. To formally test whether cephalotine microbiome composition is predicted by host evolutionary history, we investigated the impact of the *Cephalotes* phylogeny and geographic location on community similarity using partial Mantel tests. Bray–Curtis and Jaccard index dissimilarity matrices computed for Enterotype 1-classifying samples, were significantly correlated with the host phylogenetic distance matrix, at 97%, 98%, and 99% OTU clustering widths, and at the oligotype level, after controlling for geographic distance ($p \leq 0.001$; Table 1).

After controlling for phylogeny, geographic distance was found to significantly correlate with microbiome dissimilarity, for the majority of clustering widths, for both Bray–Curtis and Jaccard approaches ($p < 0.05$; Table 1). But r-squared values were lower for geography than phylogeny, suggesting a larger effect of the latter for Enterotype 1.

For Enterotype 2, host phylogenetic distance was significantly correlated with Jaccard dissimilarity after controlling for geographic distance, at all sequence clustering widths ($p < 0.05$; Table 1). These correlations were not, however, significant at any level of sequence clustering when using the Bray–Curtis dissimilarity matrix. Average r-squared values for the effect of phylogeny were slightly lower, for Jaccard-based analyses on Enterotype 2 (0.405) compared with those on Enterotype 1 (0.654). For this second enterotype, correlations between community dissimilarity and phylogeny-controlled geographic distances were only significant at the 98% clustering level.

For Enterotype 3, host phylogenetic distance and community dissimilarity were not significantly correlated after accounting for geographic distance, except for a significant positive correlation for the Jaccard distance computed at the 99% OTU and oligotype levels. Average r-squared values, however, were again comparatively low versus those from Enterotype 1 (0.374 vs. 0.646), when averaged across these significant clustering widths. In addition, the correlations between geographic distance and community dissimilarities for Enterotype 3 were only significant at the oligotype level for Bray–Curtis measures (Table 1).

We repeated partial Mantel analyses for Enterotype 3 samples with only specialized, adult-enriched bacteria or with nonspecialized bacteria likely acquired from the environment. Focusing on 99% OTU and oligotype

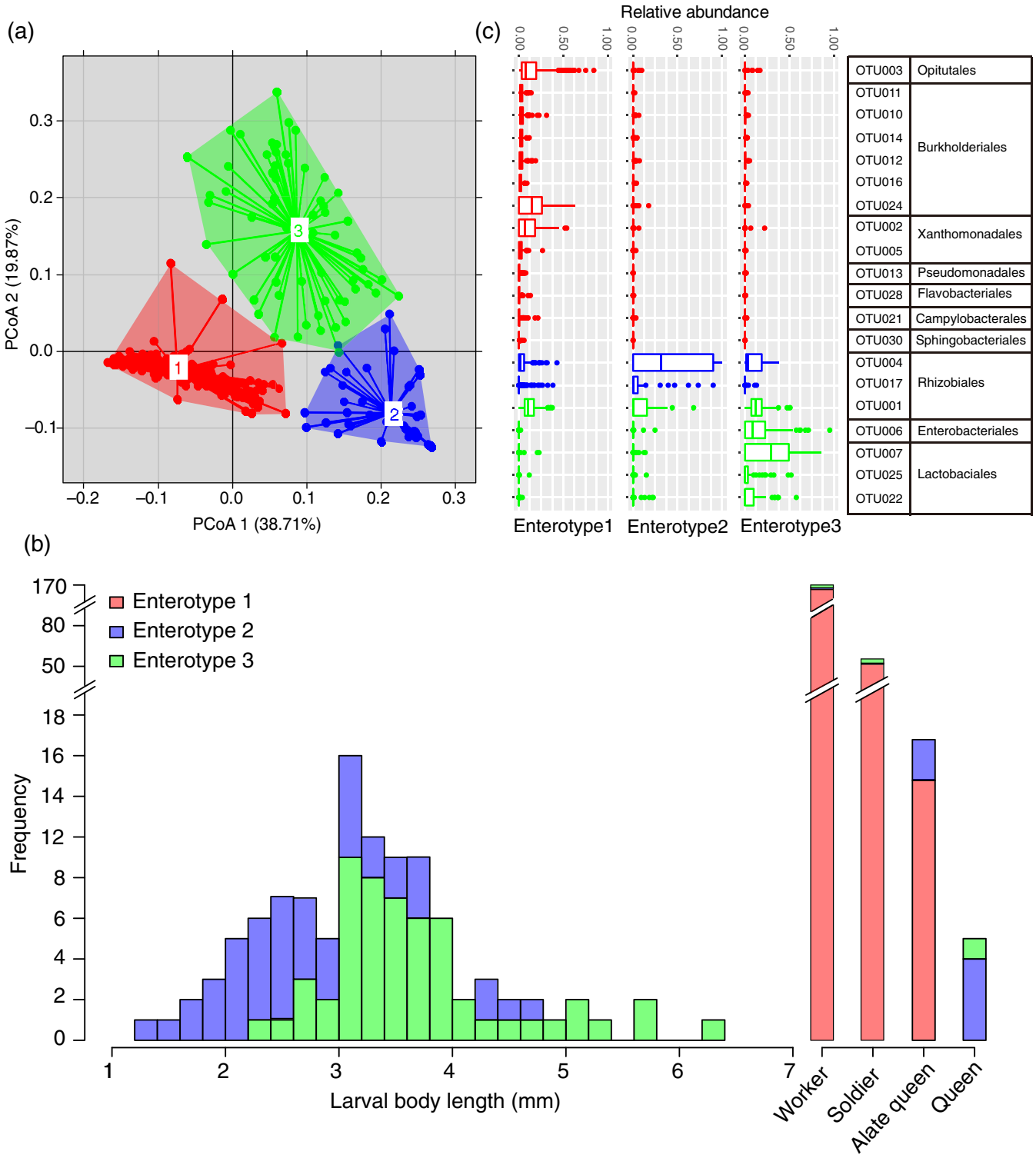


FIGURE 6 Enterotyping analysis categorizes microbiomes from 13 *Cephalotes* species into three clusters. Sequence libraries clustered at 97% operational taxonomic unit (OTU) levels, were used to compute weighted UniFrac values. Using the resulting matrix, libraries were clustered with the partitioning around medoids (PAM) algorithm, and the Calinski–Harabasz (CH) index was used to identify the optimal number of clusters as $n = 3$. (a) Between-class analysis, performed with the package *ade4* in R, was used for this first plot, which shows similarities of sequence libraries from Enterotypes 1 versus 2 versus 3 across two PCoA axes. (b) Microbiomes from larvae of varying size (normalized sizes on *x*-axis) binned, differentially, into Enterotypes 2 versus 3. Most adult castes binned to Enterotype 1, with the exception of mature (wingless) queens. (c) Stem and whisker plots illustrate medians, quartiles, and outliers for each 97% OTU found significant in our indicator OTU analyses. Relative abundances of these OTUs, therefore, vary significantly across enterotypes.

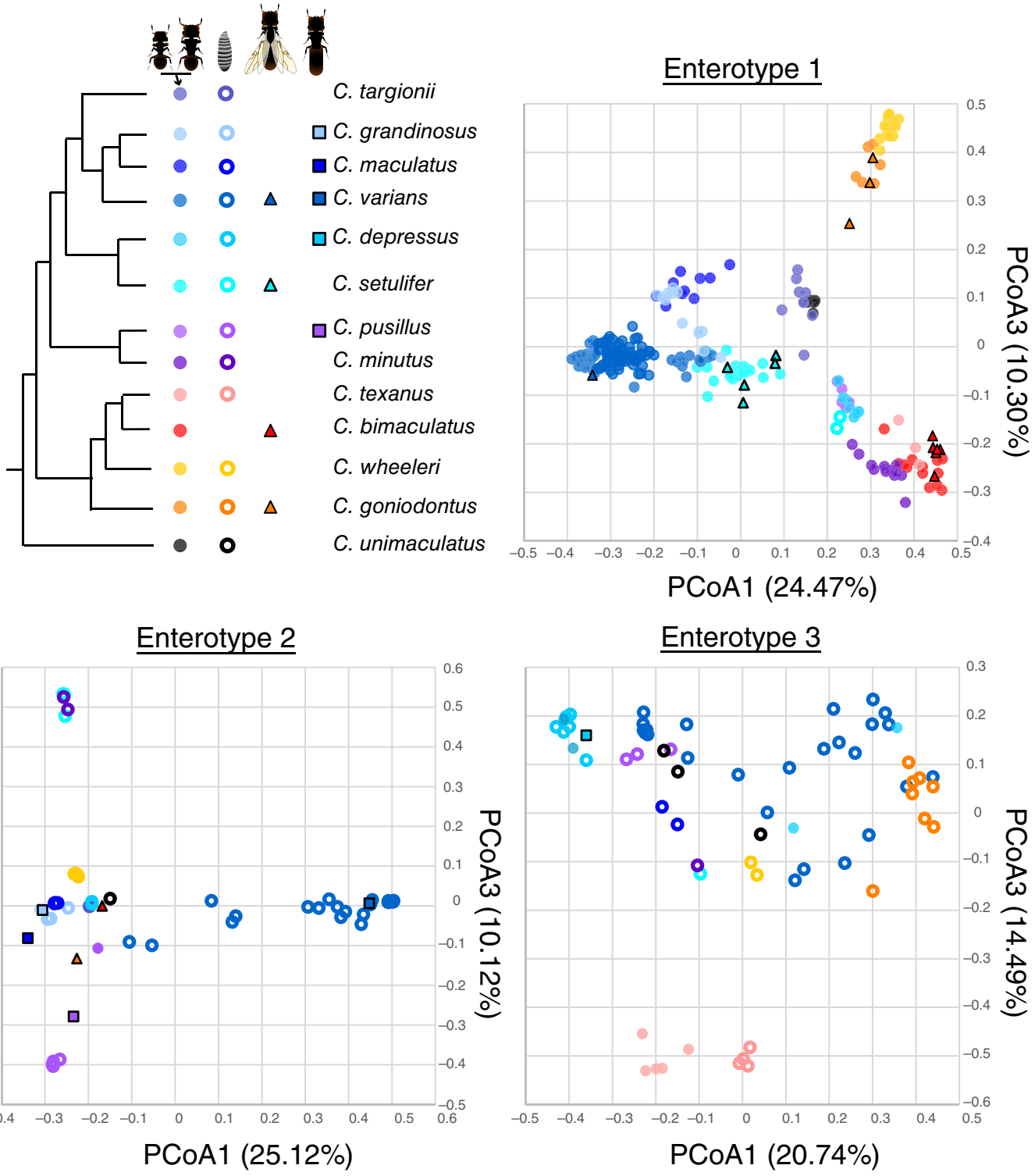


FIGURE 7 PCoA plots on microbiomes from each enterotype. Data were generated from Bray–Curtis dissimilarity measures computed on our oligotype table, with separate plots and analyses for the libraries assigning to each enterotype. Phylogeny at the left shows host relatedness, and connects different colors and symbols to varying *Cephalotes* species, castes, and developmental stages. Clustering by host relatedness is evident in the top left panel, for the first enterotype, a pattern consistent with findings from our partial Mantel test results.

levels, and beta-diversity metrics computed with the Jaccard index, we found that phylogenetic distance remained a significant predictor of microbiome dissimilarity when only specialized bacteria were included. When only nonspecialists were included prior to Jaccard distance matrix calculation, phylogeny was

TABLE 1 Results of partial Mantel tests showing the influence of host phylogeny and geography on microbiome similarity for each enterotype.

			97% OTU		98% OTU		99% OTU		Oligotype	
Enterotype ID	**Effect**	**Controlling for**	**Partial R^2**	***p* value**	**Partial R^2**	***p* value**	**Partial R^2**	***p* value**	**Partial R^2**	***p* value**
Based on Bray– Curtis distance matrix										
Enterotype 1	Host phylogeny	Geography	0.457	*0.001*	0.515	*0.001*	0.496	*0.001*	0.519	*0.001*
	Geography	Host phylogeny	0.297	*0.015*	0.447	*0.003*	0.284	*0.010*	0.157	0.141
Enterotype 2	Host phylogeny	Geography	0.079	0.537	0.196	0.143	0.111	0.353	0.130	0.081
	Geography	Host phylogeny	0.132	0.220	0.429	*0.005*	0.170	0.151	0.176	0.15
Enterotype 3	Host phylogeny	Geography	0.017	0.563	0.026	0.858	0.068	0.660	0.045	0.776
	Geography	Host phylogeny	−0.129	0.200	−0.034	0.816	−0.066	0.688	0.338	*0.025*
Based on Jaccard distance matrix										
Enterotype 1	Host phylogeny	Geography	0.646	*0.001*	0.676	*0.001*	0.656	*0.001*	0.636	*0.001*
	Geography	Host phylogeny	0.477	*0.005*	0.547	*0.001*	0.452	*0.003*	0.363	*0.008*
Enterotype 2	Host phylogeny	Geography	0.363	*0.004*	0.434	*0.004*	0.562	*0.001*	0.262	*0.039*
	Geography	Host phylogeny	0.033	0.801	0.252	*0.035*	0.048	0.690	0.131	0.328
Enterotype 3	Host phylogeny	Geography	−0.006	0.959	0.193	0.225	0.375	0.021	0.372	*0.020*
	Geography	Host phylogeny	0.204	0.190	0.222	0.176	0.301	0.051	0.257	0.106

Note: Italic font indicates *p*-values less than 0.05.
Abbreviation: OTU, operational taxonomic unit.

not significant (Appendix S1: Table S8). These results suggested a strong effect of host phylogeny on microbiomes made up of specialized *Cephalotes* symbionts (Enterotypes 1 and 2), but not on those with substantial fractions of free-living bacteria (Enterotype 3).

Cophylogeny analysis: *Cephalotes* species versus *Cephaloticoccus* symbionts

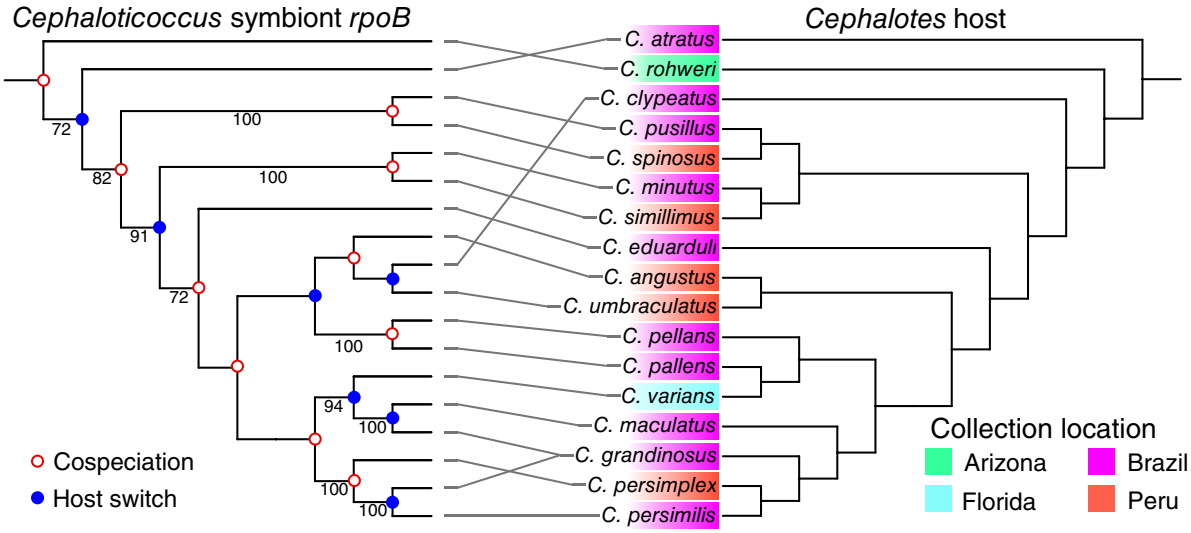
To assess whether phylosymbiosis signals could extend from cospeciation between turtle ants and their symbionts, we began an exploration of host–symbiont phylogenetic congruence (Figure 8). Using sequence data from a prior metagenomic study (Hu et al., 2018), we inferred phylogenies of adult-enriched *Cephaloticoccus* (Opitutales) symbionts from separate analyses of two nonlaterally transferred protein-coding genes. Bootstrap support values for the nodes in each phylogeny were generally high, exceeding 80 for 10/16 internal nodes of the *uvrB* tree and for 9/16 internal nodes of the *rpoB* tree.

We compared these two phylogenies against a host *Cephalotes* phylogeny (Price et al., 2016) using Jane4. Both symbiont gene trees exhibited more congruence with the host phylogeny than expected by chance (Figure 8). Event-based mapping estimated 10 cospeciation events compared with seven host switches for the *rpoB* versus host tree comparison. Additionally, the overall "cost" of our inferred *rpoB* tree—18—was substantially lower than all costs estimated for 1000 randomized datasets (average randomized cost = 27.82). Results were similar for the *uvrB* tree, with nine cospeciation events compared with seven host-switching events, and an actual cost of 19 compared with an average of 27.74 for randomized datasets. Among the branching patterns consistent with cospeciation events, we saw several suggesting that phylogenetic congruency was not a function of geography. Symbionts from the closely related *C. pusillus* (Brazil) and *Cephalotes spinosus* (Peru), for instance, grouped together with high bootstrap support (*uvrB* = 95; *rpoB* = 100), as did symbionts from relatives *C. minutus* (Brazil) and *Cephalotes simillimus* (Peru) (*uvrB* = 80; *rpoB* = 100). Thus, whereas the symbionts used for this analysis came from ants in a small number of geographic locales, host evolutionary history appears to be a geographically robust predictor of symbiont gene tree topology for at least one cephalotine-specific, adult-enriched symbiont.

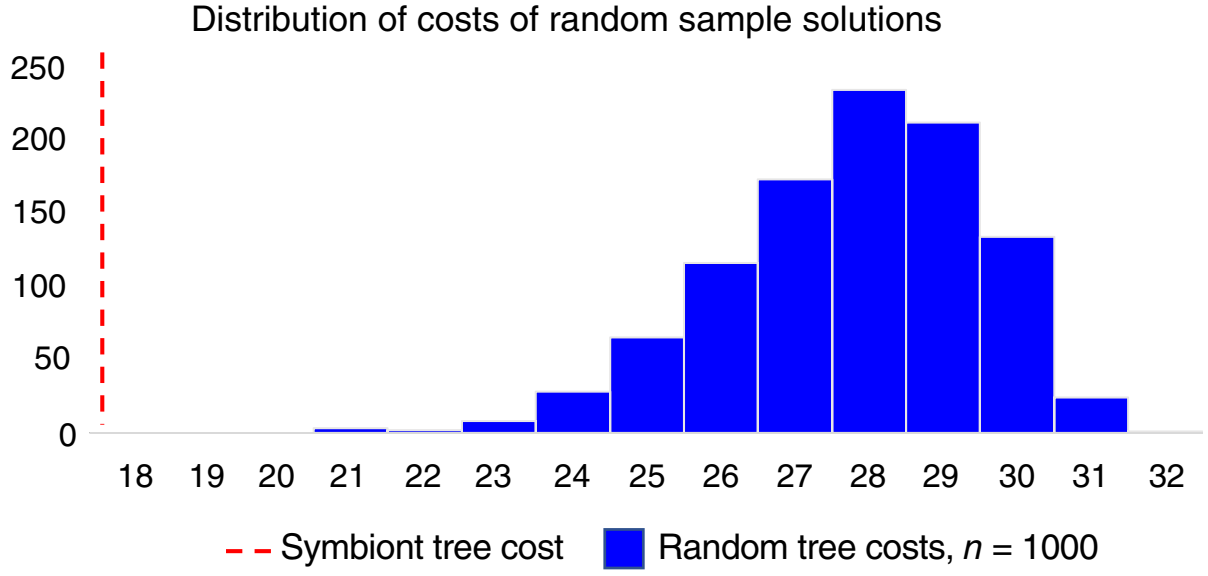
DISCUSSION

Motivated by prior findings of microbiome shifts across holometabolous insect development (Hammer & Moran, 2019); by discoveries and expectations that some social insect castes do, or should, harbor microbes with divergent functions (Sinotte et al., 2020); by the recent discoveries of the actual and potential functions of turtle ant

(a)



(b)



(c)

Gene	No. Cospeciations	No. Host Switches	Cospeciations : Host switches	Cost of symbiont gene tree	Average cost of random trees	SD of random trees	Effect size	% of random tree costs ≥ true tree
rpoB	10	7	1.4	18	27.82	1.75	5.6	0%
*uvrB**	9	7	1.3	19	27.76	1.6	5.4	0.1%

FIGURE 8 Cophylogeny analyses for *Cephaloticoccus* symbionts. (a) Tanglegram of rpoB symbiont gene tree (from *Cephaloticoccus* symbionts extracted from metagenomes of Hu et al., 2018) versus *Cephalotes* host tree (from Price et al., 2016). For the symbiont gene tree, we list all bootstrap values >70. Cospeciation and host-switching events are indicated at each symbiont node and were inferred based on the top solution from Jane4. (b) Cost histogram from 1000 randomized tip mapping permutations in Jane4. A lower cost indicates fewer inferred host-switching events, and therefore stronger phylogenetic congruence. (c) Table of Jane4 analysis statistics.

gut microbiomes (Béchade et al., 2021; Duplais et al., 2021; Hu et al., 2018; Wertz & Béchade, 2020); and by our understanding of mechanisms promoting beneficial symbioses (Ohbayashi et al., 2020), we set out to establish a comprehensive, colony-level view of the turtle ant microbiome across the *Cephalotes* phylogeny. Expanding upon foundational work on these social, holometabolous insects, our study provides several insights with broader relevance for understanding the microbiomes of animals.

Through our sequencing, we observed that workers and soldiers harbored similar microbiomes, with a few exceptions, and that worker microbiomes may stabilize during early adulthood (Figure 3). We also found that microbiomes of unmated, alate queens resembled those of workers and soldiers, with the vast majority of their microbes coming from cephalotine-specific clades (Figure 4). Differing from prior work in social hymenopterans like honeybees (Anderson et al., 2018; Kapheim et al., 2015; Powell et al., 2018), this discovery suggests that such "gynes" acquire the parent colony's microbiome prior to dispersal and mating, enabling transgenerational symbiont passage and partner fidelity (Figure 9). But intriguingly, mature, wingless queens from established colonies possessed simple microbiomes, often enriched for Rhizobiales OTUs with abundance in young larvae. The two discoveries suggested a process of microbiome succession unfolding after colony founding (Figure 9).

As for adults, we found larval microbiomes to be highly conserved across 40 million years of *Cephalotes* evolution. But larval and adult microbiomes differed in several regards. Whereas both stages begin—at egg hatching and eclosion—with individuals harboring very few bacteria (Figure 2), the Rhizobiales symbionts dominating the guts of young larvae comprised just one of the almost 12 adult-enriched symbiont clades found in most adults past the early callow stage (Figures 3 and 4). As larvae grow, however, their microbiomes undergo a stereotyped pattern of community succession, as inferred from our discovery that late-stage (i.e., large) larvae harbor a mixture of adult-enriched specialists and bacteria of likely environmental origin (Figures 3, 5, and 7).

Our statistical analyses indicated that the similarity of the adult microbiome among *Cephalotes* species (Figure 6) recapitulates host relatedness (Table 1), with at least one symbiont showing partial cospeciation with its cephalotine hosts (Figure 8). Host phylogeny does a poorer job of predicting microbiome similarity in older larvae (Table 1), reflecting the abundance of particular, free-living bacterial taxa found throughout the genus. We conclude that the highly conserved symbiont compositions of cephalotine ants have been governed by distinct mechanisms—partner fidelity and environmental filtering—with differing relative impacts across development.

Conspicuous symbioses in ants, symbiont conservation, and partner fidelity

Whereas symbiosis is an important part of eukaryotic biology (Parfrey et al., 2018), we have intriguingly learned much about its significance from studying "exceptions to the rules," including cases in which symbionts are harbored at low densities (Hammer et al., 2017) or those in which they have been lost, or replaced, after ancient associations (Sudakaran et al., 2017). Emerging from such work is the discovery that highly integrated and ancient symbioses can be hard to escape (Bennett & Moran, 2015; Meseguer et al., 2017), but that the origins of novel resource acquisition or defensive strategies (Fernandez-Marin et al., 2015; Werner et al., 2018), or the acquisition of key genes via lateral gene transfer (Wybouw et al., 2016), can prevent or eliminate the need for symbionts.

In light of this, and the findings that microbes are harbored at low densities across many ants (Hu et al., 2017; Moreau, 2020; Russell et al., 2017; Sanders et al., 2017), the cephalotine-gut microbe symbiosis appears fairly conspicuous. With the *Cephalotes* genus dating in excess of 46 million years, the conservation of symbionts in adults and larvae is noteworthy, all the more so in the face of habitat and caste differentiation exhibited across this clade (Price et al., 2016). The presence of highly abundant gut bacteria is an enriched property for tree-dwelling, low trophic-level ants, like *Cephalotes* (Sanders et al., 2017). The internal housing of specialized, symbiotic bacteria in such ants is likely to be a derived state (Russell et al., 2017; but see Jackson et al., 2020). Like *Cephalotes*, other ants at low trophic levels feed on combinations of spores, pollen, vertebrate excreta, insect honeydew, extrafloral nectar, and plant wound secretions (Davidson et al., 2003; Russell et al., 2009, 2017). Symbiosis within some of these groups appears ancient, as exemplified by the Camponotini and their ~40 million year partnership with midgut-associated, intracellular *Blochmannia* symbionts (Wernegreen et al., 2009).

Extracellular gut microbiomes can show levels of conservation approaching, or exceeding, those of cephalotine ants in other insect systems. This is true, to a degree, for termites. Whereas the diversity of termite gut microbiomes has complicated coevolutionary assessments, it is now known that associations between these social, hemimetabolous insects and several of their bacterial or protist gut symbionts stretch back in excess of 150 million years (Brune & Dietrich, 2015; Ohkuma et al., 2009). Coming from less diverse bee gut microbiomes are several core symbiont specialists, including *Gilliamella* and *Snodgrassella*, which are argued to have been acquired by social, corbiculate bees nearly 80 million years before

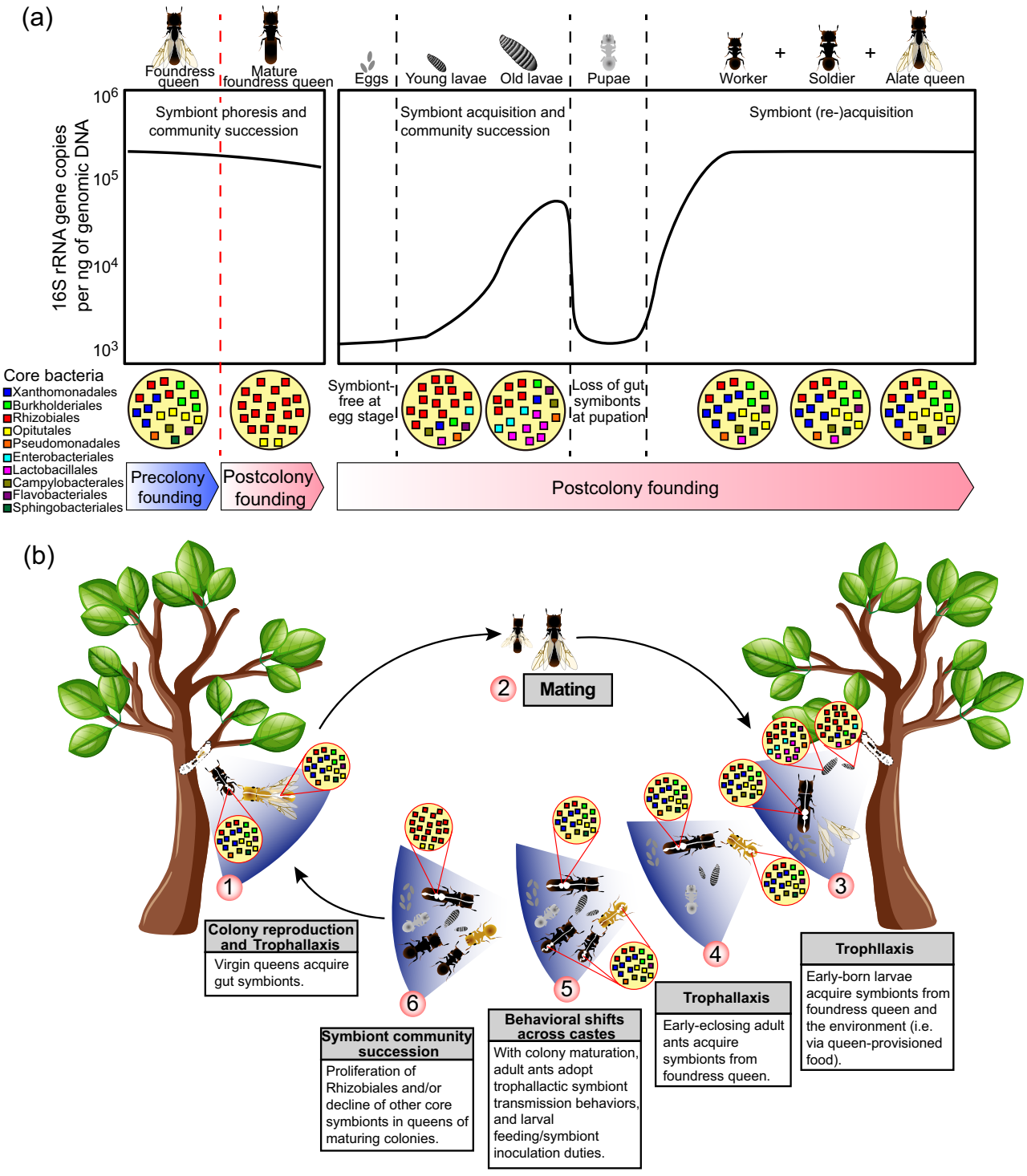


FIGURE 9 Summary figure, illustrating the mechanisms of microbiome acquisition, community succession, and the impacts of caste and development. (a) Symbiont dynamics over the lifespan of an individual al ant across its development with the adult-stage symbiont transition proposed in queens at left, and the typical progression for all ants, regardless of caste, on the right. (b) Hypothesized life cycle of the microbiome in relation to the *Cephalotes* ant colony life cycle. Process begins with production of winged (alate) queens, which mate on the wing with males, and found new colonies, often individually. The presence of worker- and soldier-like microbiome, known to be transmitted through oral–anal trophallaxis (and "walled-in" by a proventricular filter) in alate queens implies that they bring the specialist microbiomes with them upon mating and new colony founding, which would indicate a route for vertical symbiont transfer. Young queens would then need to be the inoculation sources for the specialist microbiomes (e.g., Rhizobiales in larvae; and the diverse range of operational taxonomic units in adults) in early-born larvae and adults. As colonies mature, workers and soldiers would take on trophallactic inoculation roles. Queens are then hypothesized to undergo a microbiome succession toward a lower diversity, often Rhizobiales-enriched community. Bacteria from noncephalotine-specialized taxa are typically very closely related to free-living microbes. It is proposed that these are selectively acquired, and retained, by larvae from food.

present (Kwong et al., 2017). These two examples revealed that the age of the *Cephalotes* microbiome association is by no means unprecedented, nor is our discovery that symbionts from this group may cospeciate with their hosts (Figure 8). Hinted at through indirect measures in a prior *Cephalotes* study (Sanders et al., 2014), partial cospeciation has also been evidenced for the aforementioned symbioses in bees (Koch et al., 2013) and termites (Noda et al., 2018).

The known means of social symbiont transmission and cross-generation inheritance in these above systems (Diouf et al., 2018; Koch et al., 2013; Martinson et al., 2012; Powell et al., 2014; Salem et al., 2015), argue, in part, for microbiome conservation through partner fidelity (Kaltenpoth et al., 2014). Key to the former in termites is oral–anal trophallaxis, also commonly referred to as proctodeal trophallaxis. Used as a means to facilitate nitrogen recycling in some insects (Machida et al., 2001), drinking of anal secretions from other members of the colony facilitates the movement of microbes within a social insect colony, as argued for termites, ants, and some bees (Cook & Davidson, 2006; McMahan, 1969; Powell et al., 2014). The precedent for this behavior in cephalotine ants (Lanan et al., 2016; Wheeler, 1984; Wilson, 1976); our findings of the core worker microbiome in winged, premated queens suggest a means by which parent *Cephalotes* colonies pass their microbes to the next generation (Figure 9). Over long timespans, such vertical transmission should select for beneficial microbes (Sachs et al., 2011).

The conserved vertical transfer has unfolded for numerous, transovarially transferred intracellular symbionts (Moran et al., 2008). It has also occurred for some extracellular symbiotic associates (Hosokawa et al., 2006; Kaltenpoth et al., 2014; Reis et al., 2020), including farmed symbionts not housed within or on the host body. Perhaps the most famous in this category is the attine ants and their cultivated fungi. Like *Cephalotes*, new attine ant colonies are founded by queens, after mating flights, without assistance from other castes. Ensuring the propagation of their externally digestive fungal cultivars, these queens transport a fungal inoculum in a cephalic cavity known as the infrabuccal pocket (Mueller et al., 2001). *Acropyga* ants provide yet another instance in which dispersing queens transmit symbiotic associates, exhibiting separately evolved mechanisms for vertical transmission of trophobiotic scale insects (Blaimer et al., 2016; LaPolla, 2005).

Whereas such transgenerational transfer promotes partner fidelity, it is not a one-way ticket to cospeciation. This is evidenced by the attine–fungus association, in which incongruent phylogenies suggest a combination of host switching and novel cultivar domestication (Mehdiabadi et al., 2012; Mikheyev et al., 2010). In parallel with this finding, cospeciation between *Cephaloticoccus* symbionts and *Cephalotes* hosts appears somewhat imperfect (Figure 8). For the remaining adult-enriched symbionts, inferences on the rates of host switching, loss, duplication, and cospeciation await rigorous phylogenetic analysis.

Impacts of environmental filtering on microbiomes of *Cephalotes* and beyond

Whereas nonnegligible portions of the *Cephalotes* larval microbiome owe their conservation to partner fidelity, a second mechanism—environmental filtering—has plausibly driven long-term associations with Enterobacteriales, some Lactobacillales, and Actinobacteria found in later-stage larvae. We propose that some component of the *Cephalotes* larval gut physiology, or perhaps members of their gut microbiome (Scheuring & Yu, 2012), could favor the persistence of particular microbes acquired from the environment. Enterobacteriales and Lactobacillales microbes have been found previously on pollen, including the pollen collected and stored by hymenopterans (Graystock et al., 2017), providing a plausible source for acquisition by cephalotine. We note, further, that tendencies of the larval gut to support the growth of some adult-enriched bacteria (e.g., Rhizobiales, Alcaligenaceae, Xanthomonadales), but not others (e.g., Campylobacterales, *Cephaloticoccus* from the Opitutales), suggest that filtering mechanisms shape more than just the environmentally acquired inoculum encountered by larvae.

Beyond *Cephalotes*, environmental filtering plays a common role in gut community assembly across insects (Chandler et al., 2011; Kennedy et al., 2020; Ravenscraft et al., 2019). It has, indeed, promoted long-term homogenization in the guts of some such animals, including those of the genus *Drosophila*, whose symbioses exhibit no apparent phylosymbiosis signal (Martinson et al., 2017; Wong et al., 2013). Widespread associations with related environmental bacteria from the Enterobacteriaceae, Orbales, Acetobacteraceae, and Lactobacillales among distantly related *Drosophila* species, reveal similar trends to those seen for the cephalotine larvae in our study (Chandler et al., 2011; Martinson et al., 2017).

Beyond *Drosophila*, some studies have revealed even higher specificity of environmentally derived symbioses, with insects showing capacities to preferentially retain beneficial symbionts. Evidence for this phenomenon, termed partner choice, has been obtained for the bean bug *Riptortus pedestris*, and is achieved due to evolved anatomical gut features, the corkscrew motility of their preferred *Burkholderia* symbionts, and the heightened competitive abilities of these widespread, beneficial

partners (Ohbayashi et al., 2020). Partner choice is also evident within the beewolf–antennal *Streptomyces* symbiosis, acting alongside partner fidelity mechanisms in the maintenance of an ancient, defensive symbiosis (Kaltenpoth et al., 2014). Whether *Cephalotes* ants have evolved precise means to select beneficial symbionts for growth in the larval gut is not yet established, although it remains an intriguing prospect.

Development shapes holometabolous insect microbiomes

Symbiotic gut communities of larvae and adults are expected to differ for most holometabolous insects (Hammer & Moran, 2019). This stems not only from their distinct ecology, but also from the blank slate gut environments that often occur upon egg hatching and pupal eclosion (Moll et al., 2001; Roche & Wheeler, 1997; Wang & Rozen, 2017). Opposing forces, however, may partially homogenize microbiomes harbored on opposite sides of metamorphosis, including maternal symbiont deposition upon eggs in their prehatched stage; larval development in habitats seeded by adult microbes; social transfer; developmental persistence of symbiont-housing tissues and their contained symbionts; symbiont migration and tissue recolonization within pupating hosts; and insect consumption of symbiont-housing body contents shed before pupation (Estes et al., 2009; Pais et al., 2018; Salem et al., 2015; Stoll et al., 2010; Wang & Rozen, 2017).

Whereas our prior findings suggested that turtle ant adults and larvae share some specialized symbionts (Russell et al., 2009), we show here that the microbiomes from these cephalotine stages exhibit only partial overlap. In particular, after the removal of likely cross-contaminants, we estimate that an average of 31.5 bacterial 97% OTUs are housed, per colony, when assessed among workers and larvae for the 12 *Cephalotes* species censused at both stages. Among these, 10.8 OTUs are shared; 7.9 are found at detectable levels only in workers; whereas the remaining 12.8 OTUs are confined to larvae. A table of shared 97% OTUs among workers and larvae is available in Dryad ("Data 12" file deposited in the Dryad Digital Repository; Hu et al., 2022a, 2022b).

This discovery resembles findings from other holometabolous insects, including the emerald ash borer. In this beetle, 13 of 20 bacteria detected in at least two larval and/or adult samples were shared across these stages, which differentially feed on the cambium and phloem, versus leaves, of ash trees (Vasanthakumar et al., 2008). Like the emerald ash borer, substantial proportions of larval midgut bacteria are harbored in the guts of adult forest cockchafers, pest scarab beetles with distinct root- and leaf-feeding habits as larvae and adults (Arias-Cordero et al., 2012). Fitting a similar mold is the butterfly *Heliconius erato*. Amplicon sequencing of the 16S rRNA gene from this species showed that three of the top 10 bacterial phylotypes were shared across adults and larvae, despite adult consumption of pollen and nectar compared with the leaf-feeding habits of larvae (Hammer et al., 2014).

Contrasting with these findings are discoveries from *Drosophila melanogaster*, in which five bacterial species comprising ≥90% of most sampled microbiomes were each shared across adults and larvae in one laboratory-based study (Wong et al., 2011). It has been traditionally thought that gut microbes of *Drosophila* do not establish residency and that they are, instead, transient members of the fly diet that are (re-)seeded in food, through defecation (Broderick et al., 2014; Douglas, 2018). Work here, however, suggests that gut bacteria closely associate with cephalotine larval gut tissue (Appendix S1: Figure S2), as shown previously for adults (Roche & Wheeler, 1997). Due to their blind gut morphology and the resulting lack of defecation, *Cephalotes* larvae may further engage in sustained relationships with even lumen-dwelling bacteria, unlike adult or larval *Drosophila*. Lacking the proventricular filter of cephalotine adults (Lanan et al., 2016), larval guts may more regularly encounter environmental bacteria, contributing to the free-living bacterial enrichment in immatures observed throughout the *Cephalotes* genus.

Dissociated mutualism: Symbionts may benefit relatives of their current hosts

An exception to the earlier mentioned transient symbioses between gut bacteria and *Drosophila* is a newly identified bacterium, *Acetobacter thailandicus*, which can stably colonize the crop of adult *D. melanogaster*. This microbe was found not to increase adult fitness, but the fitness of larvae, which acquired the microbe after adult inoculation into the surrounding food media (Pais et al., 2018). This finding supports a broader trend in which holometabolous insect adults carry and vector microbes of benefit to their young offspring, despite a possible lack of direct benefits to the adults, themselves (Kaltenpoth et al., 2005; Shukla et al., 2018). In the *Cephalotes* system, it is certainly possible that a subset of the specialized symbionts found in alate queens, soldiers, or workers assist larvae rather than their carriers. Benefits to larvae may unfold through the inoculation of symbionts with helpful activity in larval guts or through trophallactic feeding of symbiont-derived metabolites produced in adult "nutrient factories" (Ankrah et al., 2018).

Among other social insects, there are likely to be numerous opportunities for cross-stage symbiont exchange given

the close proximity of larvae and adults, and the capacities for trophallactic inoculations. Accordingly, the partial overlap between adult and larval gut microbiomes has been reported for honeybees and bumblebees (Mohr & Tebbe, 2006), with an occasional presence of adult-enriched symbionts, like *Gilliamella* and *Snodgrassella*, in larvae (Hroncova et al., 2015; Martinson et al., 2012; Tarpy et al., 2015; Vojvodic et al., 2013). Most consistent among investigations on these bees, however, are observations that larval bacterial loads are lower than those of adults, with some evidence for an increase in community size with larval age (Hroncova et al., 2015; Martinson et al., 2012; Tarpy et al., 2015). Evidence also suggests community succession in larval gut communities with age (Hroncova et al., 2015; Vojvodic et al., 2013), providing an additional similarity to our present *Cephalotes* findings (Figures 2 and 3).

Among other bacteria in honeybees, several investigations have uncovered an abundance of the adult-associated "Alpha 2.2" symbiont in larvae (Martinson et al., 2012). Formally named *Parasaccharibacter apium* (Corby-Harris et al., 2014), this bacterium is dominant among the cultivable fraction of gut bacteria from young larvae (Vojvodic et al., 2013). Whereas rare in workers (Kapheim et al., 2015; Tarpy et al., 2015), *P. apium* has been found in conditioned and stored pollen (Martinson et al., 2012), royal jelly, and tissues of nurse bees that would presumably facilitate its trophallactic transfer to larvae. Given that some *P. apium* strains boost larval fitness (Corby-Harris et al., 2014), inoculation could be key to larval development and, therefore, the overall fitness of the colony.

The impacts of caste on (holometabolous) social insect microbiomes

Distributional trends of *P. apium* comprise part of a broader pattern of caste-correlated microbiome differentiation (Anderson et al., 2018; Jones et al., 2018; Kesnerova et al., 2020), a conclusion reinforced by their observed abundance in mature honeybee queens (Kapheim et al., 2015; Tarpy et al., 2015). Caste differences have been demonstrated or predicted in other social insect systems, although this topic remains somewhat unexplored (Sinotte et al., 2020). We observed subtle symbiont differences among cephalotine workers and soldiers, including tendencies toward specialist Campylobacterales enrichment in workers, and episodic enrichment of Neisseriales and Lactobacillales OTUs in soldiers. Clear worker versus soldier differences were absent from most species, however, suggesting a need for closer study. Notwithstanding the below-discussed microbiome divergence in mature queens, the absence of large, consistent microbiome differences among other adult castes of *Cephalotes* raises interesting questions.

Cephalotes soldiers are linked to colony defense and specialization in nesting niches. Underlying these phenomena is the finely selected trait of soldier head width, which governs the use of nest cavities with specific entrance diameters (Powell, 2008; Powell et al., 2020). Whereas these roles do not create a priori expectations of intercaste microbiome differences, further study on the roles of soldiers, versus workers, in symbiont transfer (Lanan et al., 2016; Rodrigues, 2016) or nutrient transfer within the colony will shed light on the castes' roles in maintaining symbiosis, distributing symbiont-derived nutrients, or benefiting directly from their own housed symbionts. Metatranscriptomic or metabolomic studies would enable complementary lines of inquiry into realized, in vivo symbiont function across castes. When coupled with symbiont genomics, the latter methodology has demonstrated gut symbiont-driven metabolism of dietary, pollen-derived nutrients in honeybees (Kesnerova et al., 2017). These capacities are intriguing given the slight microbiome differences between honeybee foragers and nurses (Kesnerova et al., 2020), and the tendencies of the former to consume less pollen than the latter (Anderson et al., 2014).

Symbiont function and implications for variable symbioses within colonies

Gut microbiomes of insects can improve host fitness through a variety of mechanisms, ranging from detoxification (Ceja-Navarro et al., 2015; Mason et al., 2014), to defense (Koch & Schmid-Hempel, 2011), and to a series of nutrient-themed functions. These latter benefits include increased digestive efficiency (Brune, 2014; Ebert et al., 2020; Engel et al., 2012; Salem et al., 2020; Zheng et al., 2017), improved nitrogen economies through the fixation or recycling of nitrogen (Bar-Shmuel et al., 2020; Hansen et al., 2020; Ulyshen, 2015), and the provisioning of essential, limiting nutrients like amino acids and B vitamins (Ayayee et al., 2014; Salem et al., 2014; Vogel & Coon, 2020).

In *Cephalotes* ants, members of the core, specialized adult microbiome—including cospeciating *Cephaloticoccus* symbionts—have been shown to recycle waste nitrogen. Workers obtain large quantities of this recycled, upgraded element in the form of amino acids (Hu et al., 2018). Through metagenomic sequencing, an even broader suite of functions has been proposed for adult-enriched, specialist bacteria, and environmental bacteria enriched in larvae (Béchade et al., 2021). Among functions common in adults' symbionts were the biosynthetic capacities for most B vitamins and catabolic capacities to break down

recalcitrant plant cell wall fibers, including xylan and some forms of pectin. Among larvae, environmentally derived Enterobacteriales and, more ambiguously originating Lactobacillales, encode similar nitrogen-recycling, B-vitamin synthesizing, and xylan-catabolizing functions. Potentially unique among larval symbionts may be cellulose degradation and polygalacturonase activity catabolizing the backbones of the most common pectins found in pollen-heavy, *Cephalotes* diets.

The open digestive systems of larvae implicate them as the solid food-digesting caste (Eisner, 1957; Erthal et al., 2007). Plant cell wall-degrading activities of larval gut symbionts could, thus, free up usable carbon for the colony. Such access could have large impacts on the activity, competitive abilities, and growth rates of *Cephalotes* colonies, having profound significance when sugar-rich foods, like honeydew or plant nectar, are in short supply (Grover et al., 2007; Wittman et al., 2018). With specialized Rhizobiales and other larval gut bacteria encoding fermentative capacities (Béchade et al., 2021), short-chain fatty acids emerge as candidate sources of ant energy, a possibility worth exploring through future study.

The nitrogen-recycling and amino-acid biosynthetic capacities of larval symbionts could be of further importance in sustaining colony fitness. With ant larvae requiring more protein than adults (Cassill et al., 2005; Csata & Dussutour, 2019; Sorensen & Vinson, 1981), the question arises as to why worker-associated symbionts invest in nitrogen-recycling and amino-acid synthesis (Hu et al., 2018). Whereas we cannot rule out the impacts on queen reproduction or direct aspects of worker fitness, it is certainly possible that these functions provide a greater direct benefit to larvae. Prior studies, for example, have suggested that storage proteins can accumulate in ant workers and that these protein levels are depleted in the presence of larvae (Dussutour & Simpson, 2009; Wheeler & Martinez, 1995). Through trophallaxis, and regulated worker physiology, it is thus conceivable that a fraction of symbiont-fueled nitrogen metabolism in workers (Hu et al., 2018) supports the larval, or queen egg-laying, nitrogen economy. Whether the digestive and fermentative abilities of larval symbionts are of symmetrical use to the energy budgets of adult workers (Grover et al., 2007) is also an intriguing possibility.

Of further intrigue are the low-diversity microbiomes of mature turtle ant queens. In a recent study, symbionts of *Temnothorax nylanderi* queens were found to impact egg production (Segers et al., 2019). Might mature queens' gut symbionts directly sustain reproduction in *Cephalotes*? In addressing this, it is of interest to note that a bacterium with close relatedness to mature queen-enriched Rhizobiales symbionts has had its genome sequenced (Hu et al., 2018). Interestingly, this strain—JR021-5—lacks the ability to recycle nitrogen and to synthesize most amino acids, as does a close young larva-enriched relative sampled recently through metagenomics (Béchade et al., 2021). The absence of extensive nitrogen metabolism does not rule out a supportive role for queen reproduction. But the high nitrogen requirements documented for female insect reproduction (Rivera-Pérez et al., 2017), at least question this possibility.

The impacts of Rhizobiales symbionts on turtle ant queens could alternatively be realized through protective, rather than nutritive or energetic, functions. In *Temnothorax* ants, patterns of queen gene expression shift from a program of antipathogen/parasite defensive function toward greater investment in antioxidant activity, suggesting two protective functions that symbionts could fulfill (Negroni et al., 2019). Oxidative damage is of further importance to aging queens of honeybees, with evidence for a series of evolved host- and, possibly, microbe-mediated mechanisms to mitigate such damage (Anderson et al., 2018). Might Rhizobiales protect queens from senescence-inducing oxidative damage? Such a possibility is not far fetched, as at least some turtle ant Rhizobiales encode proteins for the synthesis of aryl polyenes (Chanson et al., 2021), carotenoid-like molecules protecting against oxidative damage (Schoner et al., 2016).

Heightened immune services could also benefit long-lived ant queens inhabiting pathogen-rich habitats (Baer et al., 2006; Galvez & Chapuisat, 2014). Indeed, the higher pathogen resistance in *Lasius* and *Formica* queens, after mating, suggests an eventual upregulation of protective mechanisms during the process of queen maturation and aging (Galvez & Chapuisat, 2014). Building off these ideas, enrichment of the earlier described *Parasaccharibacter apium* in mature honeybee queens is an intriguing pattern; and findings that this symbiont improves colony-level resistance against microsporidian parasites are consistent with symbiont-invested protection of reproductive social insect castes (Corby-Harris et al., 2016). Strong parallels between the distributions of *P. apium* and Rhizobiales (JR021-5)—across larvae, queens, and the crops of nonreproductive adults (Flynn et al., 2021; Rodrigues, 2016)—hint at similar defensive roles for *Cephalotes*-associated symbionts. This, in turn, raises the question of whether selection for symbiont-conferred defense is ultimately responsible for community succession in mature queens, after passing on colony nourishment and symbiont transferring duties to their early-born offspring (Figure 9). A similar potential for symbiont defense in young larvae, possibly too small to support the colony's nutritive needs, is of further interest in explaining larval microbiome succession, and in the broader quest for an integrated, colony-level view of symbiosis and social insect fitness.

CONCLUSIONS

Through characterizing gut microbiomes across life stages and castes from 13 species of *Cephalotes*, we conclude that colony-founding, alate queens ("gynes") enable the transgenerational transfer of specialized, adult-enriched symbionts. This partner fidelity mechanism couples the histories of ants and gut symbionts, and is a likely driver of *Cephalotes*-microbe cospeciation reported, for the first time, in our study. *Cephalotes* larvae acquire their gut symbionts from both adult-enriched and environmental microbiomes. The conserved suite of larva-associated free-living bacteria suggests the conservation of habitat filtration mechanisms for more than 40 million years of cephalotine evolution. In summary, like other social insects, we find that social symbiont transfer and symbiont phoresis with colony-founding castes enable the conservation of beneficial microbiomes, circumventing the limits imposed by metamorphosis. But the preserved abilities to acquire similar environmental microbes require further mechanistic study, as do hypothesized impacts of gut symbionts on colony-level fitness.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data (Hu et al., 2022a, 2022b) are available in Dryad at https://doi.org/10.5061/dryad.kwh70rz5d and in Zenodo at https://doi.org/10.5281/zenodo.7055485. All 16S amplicon sequencing data have been deposited in the GenBank Short Read Archive under BioProject PRJNA767930 at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA767930/. The 16S rRNA sequences produced from our Sanger sequencing and highlighted with green stars in Figure 5 are available from the GenBank database with accession numbers OK348261–OK348280. *Cephalotes* Metagenomes (Project IDs: Gp0095985, Gp0095983, Gp0125961, Gp0125962, Gp0125963, Gp0125964, Gp0125967, Gp0125968, Gp0125969, Gp0125970, Gp0126569, Gp0126571, Gp0126580, Gp0126572, Gp0126573, Gp0126574, Gp0126575, Gp0126577) and two *Cephaloticoccus* genomes (Project IDs: Gp0154034, Gp0110136) were downloaded from the IMG website (https://img.jgi.doe.gov/) by searching the project IDs.

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Additional supporting information can be found online in the Supporting Information section at the end of this article.

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