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By their own devices: invasive Argentine ants have shifted diet without clear aid from symbiotic microbes

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Abstract

The functions and compositions of symbiotic bacterial communities often correlate with host ecology. Yet cause–effect relationships and the order of symbiont vs. host change remain unclear in the face of ancient symbioses and conserved host ecology. Several groups of ants exemplify this challenge, as their low-nitrogen diets and specialized symbioses appear conserved and ancient. To address whether nitrogen-provisioning symbionts might be important in the early stages of ant trophic shifts, we studied bacteria from the Argentine ant, *Linepithema humile* – an invasive species that has transitioned towards greater consumption of sugar-rich, nitrogen-poor foods in parts of its introduced range. Bacteria were present at low densities in most *L. humile* workers, and among those yielding quality 16S rRNA amplicon sequencing data, we found just three symbionts to be common and dominant. Two, a *Lactobacillus* and an Acetobacteraceae species, were shared between native and introduced populations. The other, a *Rickettsia*, was found only in two introduced supercolonies. Across an eight-year period of trophic reduction in one introduced population, we found no change in symbionts, arguing against a relationship between natural dietary change and microbiome composition. Overall, our findings thus argue against major changes in symbiotic bacteria in association with the invasion and trophic shift of *L. humile*. In addition, genome content from close relatives of the identified symbionts suggests that just one can synthesize most essential amino acids; this bacterium was only modestly abundant in introduced populations, providing little support for a major role of nitrogen-provisioning symbioses in Argentine ant's dietary shift.

Keywords: insects, microbial biology, next-generation sequencing, symbiosis

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Introduction

Symbiotic bacteria have large impacts on the dietary ecology of a broad range of animal species (Tremaroli & Backhed 2012; McFall-Ngai *et al.* 2013). In addition to energy harvesting through the digestion of recalcitrant plant compounds (Van Soest 1994; Karasov & del Rio

2007; Warnecke *et al.* 2007; Hess *et al.* 2011), these microbes can also synthesize and provision essential nutrients that animals cannot make on their own (Douglas 1998; Akman *et al.* 2002; Hansen & Moran 2011). Nitrogen is a limiting nutrient in several diets, including plant tissue, plant sap and nectar, and honeydew from sap-feeding insects (Baker *et al.* 1978; Fischer & Shingleton 2001; Fischer *et al.* 2002). Accordingly, animals that have independently evolved to use nitrogen-poor, carbohydrate-rich diets exhibit functional and,

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sometimes, taxonomic convergence in their symbioses, housing microbes that provision them with essential amino acids (Douglas 1998; Hongoh *et al.* 2008; Muegge *et al.* 2011). Symbiosis has, thus, been proposed as a key innovation for diverse animal taxa specialized on marginal diets. However, the precise mode and timing of symbiont turnover in relation to changing host diet are largely unknown due to the ancient nature of trophic shifts for several of the world's diverse groups of animal herbivores (e.g. ruminants, hemipterans). As a result, it often cannot be determined whether the origins of specialized nutritional symbioses preceded or coincided with host dietary change, playing potentially causative roles, or whether later acquisition of novel symbionts enabled self-sufficient animals with novel feeding habits to become successful over longer timescales. Research in systems with recent dietary shifts can provide insight into the roles of symbiosis in driving, vs. following, trophic change. Importantly, dietary shifts have occurred in several invasive animal species and may be a driver of their success (e.g. see Sol *et al.* 2002; Caut *et al.* 2008; Zhang *et al.* 2010). To date, the roles of symbionts in such trophic shifts and, hence, the spread and damage inflicted by invasive species remain largely unknown.

Among the more damaging invaders are social insects, particularly ants, which can have large effects on the structure and dynamics of invaded communities (Holway *et al.* 2002; Sanders & Suarez 2011). Over 150 ant species have been introduced to new ranges (McGlynn 1999; Rabitsch 2011). Several of these have been identified as invasive species, causing ecological and economic harm in their novel habitats (Holway *et al.* 2002; Sanders & Suarez 2011). Shifts to carbohydrate-rich diets, such as plant nectar and honeydew of sap-feeding insects have been important for multiple ant invasions (Helms & Vinson 2002; Tillberg *et al.* 2007; Kay *et al.* 2010; Green *et al.* 2011; Helms *et al.* 2011; Savage *et al.* 2011; Wilder *et al.* 2011). Yet, while rich in sugars, these diets are poor in essential amino acids (Auclair 1963; Baker *et al.* 1978; Fischer *et al.* 2002), presenting challenges potentially solved by nitrogen-provisioning symbionts. Impressively, ant-specific bacterial symbionts are prevalent – and often ubiquitous – among several ant taxa with long-established nitrogen-poor diets (Cook & Davidson 2006; Russell *et al.* 2009; Anderson *et al.* 2012). The hypothesized roles for these microbes in the evolutionary success of these functionally herbivorous ants (Davidson *et al.* 2003; Russell *et al.* 2009) are at least partially supported through findings of nitrogen recycling and amino acid provisioning by *Blochmannia* endosymbionts of carpenter ants (Feldhaar *et al.* 2007). Yet the necessity of symbionts in the origins of functional herbivory – as

opposed to the eventual success and diversification of established herbivores – has not been established, requiring research on ants with more recently shifted diets.

To address these shortcomings, we focus here on the invasive Argentine ant, *Linepithema humile*. Native to South America, *L. humile* is one of the most widely distributed introduced animal species, with a presence in portions of North America, Asia, Africa, Australia and Europe, and on many oceanic islands (Suarez *et al.* 2001; Wetterer *et al.* 2009). Within introduced regions, Argentine ant colonies belong to geographically expansive supercolonies, defined by high genetic similarity and a lack of intraspecific aggression among workers over large scales (Tsutsui *et al.* 2000; Giraud *et al.* 2002). Reduced population-level aggression brought about through fusion into supercolonies has seemingly lessened intraspecific competition, helping *L. humile* to dominate habitats within their introduced range (Holway *et al.* 1998; LeBrun *et al.* 2007; Suarez *et al.* 2008). In addition to this mechanism, their heightened use of carbohydrate-rich diets, like insect honeydew and plant nectar, has been integral to the success of *L. humile* as an invader (Suarez *et al.* 1998; Tillberg *et al.* 2007; Kay *et al.* 2010; Hanna *et al.* 2015). Much like red imported fire ants (Wilder *et al.* 2011), current evidence points to a drop in trophic position for *L. humile*, at least within some populations. Indeed, the carnivorous diet typical of native populations from Argentina has been lost from introduced populations in California, whose isotope signatures suggest trophic overlap with omnivores and herbivores (Tillberg *et al.* 2007). Field experiments support the importance of this dietary shift in the Argentine ant's invasive success, as carbohydrate supplementation fuels colony growth, worker activity and aggression, thus enhancing competitive performance (Grover *et al.* 2007).

While the Argentine ant is a scavenging predator in both its native and introduced range, honeydew and nectar appear to provide major inputs to the nitrogen budgets of introduced populations (Tillberg *et al.* 2007). But given the rarity of essential amino acids in these foods (Auclair 1963; Baker *et al.* 1978; Fischer *et al.* 2002), and the numerous precedents from other animals with extensive use of nitrogen-poor diets (Douglas 1998; Brune & Dietrich 2015), we hypothesized a role for nitrogen-provisioning symbionts in the success and altered diet of the Argentine ant. Support for this hypothesis would implicate bacteria in the early stages of major trophic shifts, potentially explaining macroevolutionary correlations between symbiosis and nitrogen-poor diets (Russell *et al.* 2009; Anderson *et al.* 2012).

To study the bacterial associates of Argentine ants, we used quantitative PCR and Illumina MiSeq amplicon

sequencing of the bacterial 16S rRNA gene. Comparisons between ants from Argentina (native range) and California (introduced range) allowed us to measure whether microbiota have clearly changed during or since their invasion into this region in 1905 (Woodworth 1908). We more directly tested the relationship between ecology and symbiosis through a study of bacterial communities across a real-time trophic shift. Specifically, Argentine ants invaded Rice Canyon, California, in the mid-1990s, dropping in trophic level during the subsequent eight years (Tillberg *et al.* 2007). Through studies of symbionts from worker ants sampled across this duration we addressed the potential for trophic shifts to drive changes in symbiotic microbes over a short, yet multiyear time span. To examine shorter-term symbiont responses, we used laboratory-raised *L. humile* reared on carbohydrate-rich or protein-rich diets, assessing symbiont community alterations across 20- to 40-day periods. Finally, to infer the nitrogen-provisioning capacities for the identified symbionts of Argentine ants, we examined the genomic coding capacities of close relatives of the dominant *L. humile* symbionts, addressing their potential for nitrogen fixation, nitrogen recycling and amino acid biosynthesis. Our results provide insight into the biology of species invasions, while yielding one of the first glimpses into the timing of symbiont turnover in association with major ecological shifts in their host animals.

Materials and methods

Sample collection and dietary experiments

Linepithema humile samples were collected in both native and introduced ranges (Table S1, Supporting information) through the use of pitfall traps containing propylene glycol, or through aspiration followed by immediate preservation in 95–100% ethanol. Just over half of the field-collected samples were kept in ethanol at room temperature prior to processing, while the remainder were either processed within 3 weeks of collection or frozen in ethanol at -20°C for longer-term storage. Native range sampling across dozens of colonies took place between 1997 and 2003 (samples collected by authors AVS and DAH) and again in 2009 (samples collected by author EL). In introduced populations, Argentine ants exist in widespread behaviourally, chemically and genetically defined ‘supercolonies’ that have likely originated from separate introductions from the native range (Brandt *et al.* 2009). Collections from the United States in both 2011 and 2014 (DH) targeted four known supercolonies in California (CA), including the main or ‘Large’ California (L) supercolony, the Sweetwater (SW) supercolony, the Lake Hodges (LH)

supercolony and the Lake Skinner (LS) supercolony (see Tsutsui *et al.* 2003b for details and locations of supercolonies). In addition, ants representing the Large supercolony were repeatedly sampled from Rice Canyon, CA, between 1996 and 2003 (collected by AVS). These latter samples were from the same collections used for the study by Tillberg and colleagues (Tillberg *et al.* 2007), who reported a decrease in trophic position of *L. humile* across this short timescale due, likely, to an increased use of nitrogen-poor liquid foods.

To directly assess the impact of nutrient ratios on bacteria of *L. humile*, we used data from a prior experiment on two ant colonies (Table S1, Supporting information) collected near San Diego, CA in July 2011. Ants from each colony were reared in the laboratory for 1–6 weeks on an *ab libitum* diet of fruit flies and Bhaktar diet. Colony fragments were then assigned to a diet with either a 1:6 or 6:1 protein:carbohydrate ratio (at 100 g/L density; see Table S2, Supporting information for details). Adult workers were preserved in 100% ethanol immediately prior to the experiment (day 0) or at either 20 or 40 days after experimental initiation. Samples were then stored at -80°C until processing for DNA extraction.

DNA extraction and PCR amplification

DNA extractions targeted 479 field-caught ants, including: 162 adult workers and five queens from Argentina; 89 workers, 24 larvae, 16 pupae and 23 queens collected across four CA supercolonies sampled in 2011 and 2014; and 160 workers from the Large supercolony in Rice Canyon, CA, sampled in 1996, 1998, 2002 and 2003. In addition, we extracted DNA from 72 adult workers derived from the aforementioned dietary experiment.

Argentine ant workers, queens and brood were rinsed in 70% ethanol and sterile deionized water before DNA extraction. Whole ant tissues from single ants were then placed into sterile 1.5-mL tubes and ground with sterile pestles after freezing in liquid nitrogen. Extractions then proceeded using the DNeasy Blood & Tissue kit (Qiagen Ltd.) according to the manufacturer’s protocol for gram-positive bacteria. To facilitate detection of possible laboratory- or reagent-derived contaminants, we also performed several ‘blank’ extractions, following the same protocols in all regards except for the addition of ants.

To check the quality of DNA of the Argentine ant samples, we amplified the ant nuclear gene, *abdA*, using primers AbdA-F1 (5'-AGICCGACGGGTTCGAGTC-3') and AbdA-R1 (5'-GCIGCGGAIACGTAIGGATACA-3'). These primers had been designed to target a conserved region of hymenopteran *abdA* sequences obtained from

NCBI's GenBank database. PCRs were set up in 10 µL volumes, as described further in Table S3 (Supporting information). To assess the presence of bacteria in our studied ants, we amplified the bacterial 16S rRNA gene with universal eubacterial primers 9Fa (5'-GAGTTT GATCITIGCTCAG-3') and 1513R (5'-TACIGITACCT TGTTACGACTT-3') (see Table S3, Supporting information for PCR primers, reaction recipes and cycling conditions).

In spite of amplification with ant *abdA* primers, a number of our extractions failed to yield strong amplification signal with universal 16S rRNA primers for bacteria. In addition, several yielded very faint amplification that was often comparable to levels seen in negative controls. To initiate an investigation into the quantities of bacteria within Argentine ants, we quantified PCR product band intensity using the histogram feature in Adobe Photoshop (CS3). Each band on a gel image was covered by a fixed box with the same size throughout the analysis using the marquee tool in Adobe Photoshop. To obtain a normalized measure of band intensity, the mean intensity of each band was divided by the average of intensity value of the 2-kb band from three replicate DNA ladder runs on the same gel (Table S1, Supporting information).

Bacterial quantitative PCR

While the above band intensity approach gave qualitative insights into the potential issues of low bacterial titre, follow-up quantitative PCR (qPCR) experiments were also employed to more carefully investigate this matter. To measure symbiont densities in natural and introduced habitats, we selected 32 native *L. humile* workers (EL; 2009 collections) from Argentina, 14 introduced *L. humile* workers collected from Rice Canyon, CA, prior to the trophic shift (AVS collections from 1996 to 1998) and 15 *L. humile* workers from this same population after the trophic shift (AVS collections from 2002 to 2003). For each of the two colonies in the dietary experiment, we also included three workers from day 0, just prior to the experimental start date, along with four to six each from the 1:6 and 6:1 protein:carbohydrate diets ($n = 3$ from each time point per treatment per colony, with an exception of the day 40, P:C 6:1 treatment for one colony, where $n = 1$). All of the chosen templates had quality DNA for PCR, based on *abdA* amplification; selection of workers for qPCR was otherwise random. To provide perspective on bacterial density estimates in Argentine ants, we also included 15 *Cephalotes varians* samples from a single colony (collected by author YH) in Big Pine Key, Florida, USA (N 24°38.383', W 81°20.467'). DNA from single *C. varians*

workers was extracted in the same fashion as that for *L. humile*.

Copy numbers of bacterial 16S rRNA genes were estimated using qPCR with PerfeCTa SYBR Green FastMix (Quanta BioSciences Inc., Gaithersburg, MD, USA) on an Mx3005P qPCR System (Agilent Technologies, Santa Clara, CA, USA), and qPCRs were performed as described further in Table S3 (Supporting information). Two or three independent replicates were performed for each ant sample across separate plates. Each plate included triplicated standards at multiple concentrations, prepared using serial dilutions of linearized plasmid with the 16S rRNA gene of *Escherichia coli*. Further detail on plasmid generation and construction of the standard curve for qPCR protocols can be found in Appendix S1.

Data from qPCR were processed using MXPRO-MX3005P software v. 4.10 (Stratagene, La Jolla, CA, USA). Melting curves of all samples were manually examined, and samples with melt peaks outside the 82–88 °C range were excluded from further analyses. This temperature range encompassed the expected melting temperatures for the 16S rRNA amplicon across a range of bacteria with varying %GC. We therefore reasoned that products melting below or above this range were likely impacted by primer dimers or nonspecific genomic amplification.

To standardize our estimates of 16S rRNA gene copy number (averaged across the three technical replicates per sample), total DNA concentrations of all samples used for qPCR were quantified 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) for high-throughput DNA quantification. For subsequent graphing and statistics, we used the number of bacterial 16S rRNA gene copies divided by total DNA quantity (in ng) as our standardized measure of bacterial density. We used two-tailed, unpaired *t*-tests to compare standardized density measures between native and introduced populations and across the two dietary treatments.

16S rRNA amplicon Illumina sequencing and quality control protocols

A total of 131 *L. humile* ant samples with relative 16S rRNA PCR band intensity scores of ≥ 0.5 were submitted for amplicon sequencing of the 16S rRNA gene V4 region using primers 515F (5'-GTGCCAGCMGCCGCG GTAA-3') and 806R (5'-GGACTACHVGGGTWTCTA AT-3'). Each sample, and hence each sequence library, corresponded to a DNA extraction from one adult or juvenile ant ($n = 110$ adult workers, $n = 7$ larvae, $n = 6$ pupae and $n = 8$ adult queens), allowing us to study

interindividual variability in ant-associated microbiota. Library preparation and sample barcoding were performed by Argonne National Laboratory, where samples were subsequently multiplexed and paired-end-sequenced on an Illumina MiSeq platform.

Raw reads were initially analysed using MOTHUR v.1.33.3 (Schloss *et al.* 2009) and the MOTHUR MISEQ SOP (Kozich *et al.* 2013) was followed unless otherwise noted. After read pair assembly, we removed contigs falling outside the 251–255 bp range along with those possessing ambiguous nucleotides in primer or barcode regions. Sequences with homopolymer tracts exceeding 10 bp were also removed. A total of 2 830 719 contigs from across our 131 samples were then extracted using the `get.groups` command. The commands `unique.seqs` and `count.seqs` were used to identify unique sequences and their frequencies within each sample. Each unique sequence was aligned to the SILVA-compatible alignment database using the `align.seqs` command. The aligned sequences were trimmed to the same alignment coordinates by the command `filter.seqs`. Chimera checking was then performed using UCHIME (Edgar *et al.* 2011). For this procedure, we used our trimmed unique sequences to create a *de novo* reference sequence database that was used for chimera detection. After removing chimeric sequences, the remaining sequences were classified against the Ribosomal Database Project (RDP) 16S rRNA gene training set (version 10) with a threshold of 80% bootstrap confidence using a naïve Bayesian classifier (Wang *et al.* 2007). At this stage, sequences classifying to mitochondrial, chloroplast, Archaea and Eukarya were removed using the command `remove.lineage`.

Our assessment of unique sequences revealed some overlap between those abundant in blanks and those with some abundance in ant samples, especially those deemed to have low quantities of bacteria (Fig. S1B, Supporting information). A similar phenomenon has been reported previously for samples with low starting quantities of bacterial DNA (Salter *et al.* 2014), arguing that contaminant removal should be part of the analysis pipelines for such amplicon sequencing data sets (Galan *et al.* 2016). To remove these contaminant sequences, we developed a protocol based on filtration of abundant unique sequences found in the sequence libraries from our ant-free, 'blank' samples. After implementing the quality control protocol described in the above paragraph, we used the postfiltration mothur-generated count table to calculate the relative abundance of each unique sequence in every quality-controlled library. One option for contaminant removal would involve removing all sequences found in blanks from ant-associated libraries. However, reads from specialized core bacterial symbionts of *Cephalotes* ants (processed in the

same Illumina MiSeq lane) were detected in blank libraries [e.g. reads belonging to *Cephalotococcus* sp. from the Opitutales (Lin *et al.* 2016) made up an average of 0.087% sequences in blank libraries]. Similarly, sequences found to dominate libraries from Argentine ants were also found in blank samples, making up between 0.001% and 1.78% of the filtered sequence libraries. Such 'cross-contamination' has been documented previously and has several known causes (Kircher *et al.* 2012; Quail *et al.* 2014; Galan *et al.* 2016). Regardless of its cause, it presents a challenge to straightforward sequence filtration.

To avoid removal of biologically relevant sequences, while still removing contaminants, we employed an alternative approach, assigning sequences as contaminants if they showed high abundance in blank libraries and low abundance in the majority of ant-associated libraries. Specifically, for each unique sequence, we extracted two values including the maximum relative abundance in all six blank samples (value a) and the maximum relative abundance in all 131 ant libraries (value b). Any unique sequence with a ratio of value a: value b exceeding or equal to 0.1 was classified as a contaminant and removed from the data set. All remaining unique sequences with maximum relative abundances <0.2% across all ant libraries were also excluded, to aid in the elimination of 'satellite' sequences ultimately originating from contaminants. This procedure also had the effect of removing erroneous sequences derived from true-ant associates. Most of the sequences in this ≤0.2% abundance class were represented by just one or a few sequence reads in a small number of samples, consistent with their origination through PCR or sequencing error. Finally, after these quality control steps, libraries with fewer than 3000 reads remaining were eliminated from subsequent analyses.

Following contamination removal, the 1272 remaining unique sequences (see details in Table S4, Supporting information) from across our Argentine ant samples were assigned to operational taxonomic units (OTUs) at 97% sequence similarity using the nearest neighbour option. A final OTU table (see details in Table S5, Supporting information) was generated describing information on abundance and taxonomy of bacteria from all ants in our study. This table was converted to a biom-format OTU table in QIIME version 1.6.0 (Caporaso *et al.* 2010). It was then used for subsequent QIIME-based analyses.

Upon inspection of taxonomic composition of the filtered ant-associated libraries, we found one community type to be dominated by a mix of bacteria. These were unlike other sequence libraries, which were dominated by single bacterial taxa. In addition, the presence of

these mixed community types showed a strong correlation with relative 16S rRNA band intensities: all were found exclusively in ants with <0.77 relative 16S rRNA band intensity scores, while ants scoring above this value possessed communities dominated mostly by single taxa (Fig. S1A, Supporting information). A closer inspection revealed that the dominant members of these 'ant-associated' communities (again, those with <0.77 relative band intensity) showed very similar taxonomic profiles to those seen in blank libraries (Fig. S1A, Supporting information). As contaminants are known to dominate sequence libraries obtained from samples with low microbial biomass (Salter *et al.* 2014), we found it most prudent to eliminate all sequence libraries from ants with 16S rRNA band intensity scores <0.77 even though many of their putative contaminant sequences had survived our initial contaminant filtration step.

Assessment of beta diversity

Using the remaining quality-controlled libraries, we aimed to compare bacterial communities across *L. humile* supercolonies, geographic regions, trophic shifts and dietary treatments. Towards these ends we used the QIIME pipeline to compute the Bray–Curtis dissimilarity index between all pairs of libraries, using 97% OTUs as the taxonomic units for these calculations. Prior to these calculations, sequence libraries were rarefied to 3000 reads – a quantity just below the 3024 reads in the smallest quality-controlled/filtered sequence library. The resulting Bray–Curtis distance matrix was used for principal coordinates analyses (PCoA) in QIIME 1.6.0 and PCoA plots were visualized using R v1.33.3 (www.r-project.org). Using this same distance matrix, we assessed the significance of microbiome divergence using permutational ANOVA tests with 1000 permutations, through execution of the Adonis function (McArdle & Anderson 2001) within the Vegan package, implemented in R (Oksanen *et al.* 2015). ANOVA tests (implemented in QIIME 1.8.0) were used to identify OTUs with differing relative abundance among different supercolonies and dietary treatments – the two explanatory variables with significant effects on whole community similarity.

Enterotyping

To identify ant samples harbouring similar microbial community structures, we used the methods described by Arumugam *et al.* (2011) to test for the presence of 'enterotypes' in our ant samples. Briefly, we applied the partitioning around medoids (PAM) clustering algorithm to the 97% OTU-level abundances of all ant

samples with the Jensen–Shannon divergence distance matrix. The Calinski–Harabasz (CH) index was calculated using the clusterSim package in R to determine the optimal number of clusters in our data sets (Table S6, Supporting information). Then, we performed between-class analysis (BCA), plotting the results using the ade4 package in R. Indicator OTUs driving the differentiation of enterotypes were identified using the indval function in the labdsv package in R (Table S7, Supporting information).

Phylogenetic analysis of dominant bacterial OTUs

We performed a phylogenetic analysis to establish the evolutionary relationships among the dominant bacterial OTUs found in *L. humile*. Our analyses included representative sequences for the four enterotype indicator OTUs: OTU0002 (*Lactobacillus* sp. from the Lactobacillales), OTU0003 (Acetobacteraceae sp. from the Rhodospirillales), OTU0004 (*Enterobacteriales* sp.) and OTU0007 (*Rickettsia*). We also included representatives from the remaining four OTUs that made 60% or more of the sequences from at least one quality-controlled sequence library: OTU0001 (*Pseudomonadales* sp.; two libraries), OTU0011 (*Wolbachia* sp.; one library), OTU0017 (*Entomoplasmatales* sp.; one library) and OTU0034 (*Bacillus* sp.; one library). Finally included were six to fifteen of the top BLASTn hits for each representative sequence. The rationale for BLASTn hit inclusion was based on two goals: first, to obtain a good representation of close relatives, and second, to minimize redundancy. To meet this latter criterion, we chose just one representative hit in cases where several of the closest relatives came from a single research study. In addition to these sequences, we also downloaded those from known symbionts in bacterial taxa related to those in our study. Our phylogenetic analyses also included full-length sequences from each of the four enterotype-characterizing indicator OTUs. To obtain these, we identified samples dominated by one of these four bacterial symbionts, specifically those in which the OTU made up ≥90% relative abundance. PCRs using universal bacterial primers 9Fa and 1513R were performed on the relevant DNA samples, and we performed subsequent Sanger sequencing using these same primers. The resulting products were clean sequences, likely from a single dominant symbiont. Each corresponded to the expected indicator OTU.

The above sequences were aligned using the Ribosomal Database Project Sequence aligner (Cole *et al.* 2009). A maximum-likelihood phylogeny was then constructed using RAXML-HPC BlackBox with default parameters on the CIPRES Science Gateway web portal (Miller *et al.*

2010). Optimal trees for our analyses were visualized using the Interactive Tree of Life Website (Letunic & Bork 2007).

Analysis of metabolic potential

To gain insight into the nitrogen-provisioning potential of the most common symbionts of Argentine ants, we examined the completeness of several nitrogen metabolism pathways across genomes of related bacteria, focusing on nitrogen fixation, nitrogen recycling and amino acid biosynthesis. Generally speaking, genomes were included in the analysis if their encoded 16S rRNA gene(s) showed 100% identity to any of the three dominant bacteria in our study. Beyond these representatives, we also included genomes from the IMG/M database for additional species (1–2 strains each) and genera (1–2 species each) identified as the closest relatives of the dominant *L. humile* associates as ascertained through 16S rRNA BLAST searches. In summary, our assessments of likely coding capacity for the *Lactobacillus* symbiont included eight genomes from close relatives in this genus (GOLD Analysis Project IDs Ga0024727, Ga0011978, Ga0036890, Ga0029141, Ga0029146, Ga0029167, Ga0009394 and Ga0015084). Analyses on the dominant Acetobacteraceae, *Asaia*-like symbiont included 11 genomes from various genera within the Acetobacteraceae family (GOLD Analysis Project IDs Ga0030134, Ga0009744, Ga0000371, Ga0055316, Ga0053738, Ga0056169, Ga0055370, Ga0009692, Ga0020725, Ga0049120, Ga0059181). Finally, inferences made for the *Rickettsia* symbiont involved 12 genomes from the *Rickettsia* genus (GOLD Analysis Project IDs Ga0009536, Ga0030139, Ga0030147, Ga0030144, Ga0009547, Ga0009349, Ga0021218, Ga0017030, Ga0009523, Ga0030146, Ga0030140, Ga0009535). For all analysed genomes, we reported on the presence or absence of genes in the focal metabolic pathways using the IMG/M-ER pipeline.

To aid in our interpretation of likely metabolic capacities, we performed a phylogenetic analysis of the dominant symbionts of *L. humile* and the bacteria used for genome analyses. Full-length 16S rRNA gene sequences were extracted from the aforementioned genomes and were individually uploaded to the Ribosomal Database Project website for sequence alignment (Cole *et al.* 2009) along with the nearly full-length 16S rRNA Sanger sequences from the three dominant symbionts in our study. The three taxon-specific alignments were then uploaded to the CIPRES web portal for maximum-likelihood phylogenetic analyses using the default parameters of the RAXML-HPC BLACKBOX tool (version 8.2.4) (Stamatakis 2006). We combined the resulting trees with our nitrogen metabolism gene presence/absence data to

infer likely nitrogen-provisioning capacities of the identified symbionts.

Results

Community composition and phylogenetic placement of Argentine ant-associated microbiota

Bacterial 16S rRNA gene and ant *abdA* gene PCR screens were performed on DNA samples from 411 field-caught *Linepithema humile* workers (Table S1, Supporting information). Among these samples, 328 workers yielded strong positive amplification for the ant *abdA* gene, suggesting they were of suitable quality for PCR. In spite of this, 64.9% failed to amplify with universal bacterial 16S rRNA primers. Patterns of 16S rRNA failure for quality samples showed little correlation with the duration of preservation. For example, we observed 16S rRNA amplification failure for 39.5% of quality worker, larva, pupa and queen samples processed within 11–19 days of collection (CA 2014). In contrast, workers from our oldest collection (Rice Canyon, 1996 & 1998) yielded the lowest rate of amplification failure, at 22% (Table S8, Supporting information). A more detailed consideration of sample quality and potential degradation is presented in the Supplementary Materials (Appendix S2; Table S8, Supporting information). But in short, patterns of 16S rRNA amplification failure lead us to conclude that sample degradation is in no way a major driver of our analyses on *abdA* positive (i.e. quality) samples. Our frequent failure to amplify 16S rRNA from Argentine ants appears, instead, to be part of an emerging phenomenon across the ants (Rubin *et al.* 2014; Russell *et al.* 2017), which may frequently harbour low-density bacterial communities.

Our qPCR analysis suggested that 16S rRNA band intensity scores can be used as rough approximations for relative bacterial density in ant-associated samples (Fig. S2, Supporting information; linear regression analysis, $F = 44.93$, $P = 5.49 \times 10^{-8}$). Using this approach, we found that sequence libraries from ants with low bacterial 16S rRNA band intensities (below the arbitrary value of 0.77) were dominated by contaminants found in abundance in 'blank' libraries (Fig. S1, Supporting information). For samples included in qPCR analysis, it was also evident that some of those with the lowest estimates of 16S rRNA copy number were indeed those with serious contamination at the sequencing stage (Fig. S2, Supporting information). In contrast, samples with higher estimated bacterial loads almost always had sequence libraries dominated by identical bacterial strains from one of three groups: *Rickettsia*, an *Asaia*-like acetic acid bacterium from the Rhodospirillales, or a

Lactobacillus florum-like bacterium from the Lactobacillales. As discussed further below, the former two bacteria exhibited close relationships to known insect-associated symbionts, while the latter was related to sugar-loving symbionts from *Drosophila* guts and, even more so, to bacteria from floral nectar, a common food of Argentine ants.

After removal of contaminant sequences and contaminant-dominated libraries, Illumina sequencing data for 51 field-caught workers (out of 328 specimens deemed suitable for PCR) were analysed to study bacterial communities across geographic, temporal and supercolony scales (Fig. 1A). Results of enterotyping revealed the presence of just four community types ('enterotypes') across field-caught ants, each characterized by a single indicator OTU (Fig. S5B, Supporting information). These patterns match those visualized in Fig. 1A, helping to illustrate the simple and fairly stereotyped nature of Argentine ant bacterial communities.

The dominant bacterial symbionts from introduced Argentine ant populations were also dominant in the native range of this host species (Fig. 1A). Most prominent were OTU0002, a species from the genus *Lactobacillus* (>80% Ribosomal Database Project Classifier bootstrap support; order Lactobacillales), and OTU0003, the *Asaia*-like bacterium, which classified to the family Acetobacteraceae (with >80% RDP Classifier bootstrap support; order Rhodospirillales). While domination by the *Lactobacillus* symbiont was more common, both OTUs had widespread representation, dominating communities of: (i) 10 of 13 workers from Argentina; (ii) all workers from the invasive Large supercolony; and (iii) the two Lake Hodges supercolony workers from 2011 yielding quality amplicon sequence libraries. In contrast, the four Lake Skinner supercolony workers with successfully sampled microbiota were enriched for OTU0007, from the genus *Rickettsia* (with >80% bootstrap support). This bacterium was also abundant in the

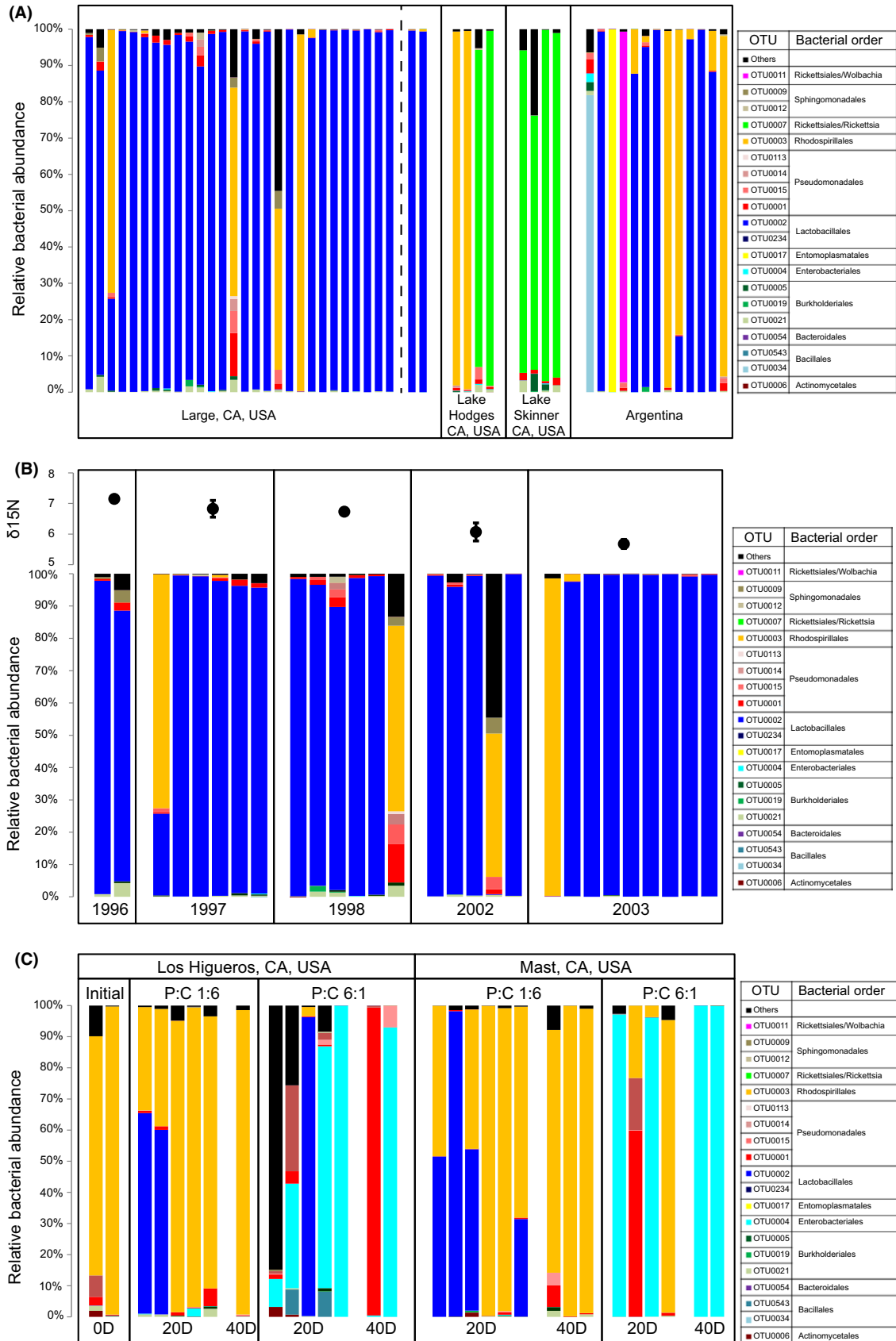
two successfully sampled worker-associated communities from the Lake Hodges supercolony in 2014, but it was not found in ants collected from native Argentina.

In sampling other castes and developmental stages, we found a similar tendency for common amplification failure using universal bacterial 16S rRNA primers on queens (20/28 quality samples amplifying), larvae (15/24 quality samples amplifying) and pupae (7/16 quality samples amplifying). Of the two larvae and one pupa sampled with amplicon sequencing from Lake Hodges (2014), the dominant bacteria were different from those found commonly in adult workers (Fig. S3, Supporting information). In contrast, the *Rickettsia* symbiont (OTU0007) dominated the bacterial communities from larvae ($n = 3$), pupae ($n = 2$) and queens ($n = 3$) of the Lake Skinner supercolony (2014), revealing strong homogeneity across castes and developmental stages for this social group (Fig. S3, Supporting information).

Maximum-likelihood phylogenetic analyses of 16S rRNA genes revealed that *L. humile* symbionts from OTU0003 and OTU0007 were closely related to previously identified symbiotic bacteria (Fig. 2). Specifically, bacteria from OTU0003 clustered with Acetobacteraceae found previously in other ants, including several *Pseudomyrmex* species along with *Cephalotes varians*. Bacteria from OTU0007 were closely related to endosymbionts in other arthropods, exhibiting 100% 16S rRNA identity to heritable *Rickettsia bellii* symbionts of ticks (Stothard & Fuerst 1995) and weevils (Lu *et al.* 2014), and to an uncharacterized associate of bees (Gerth *et al.* 2015). In contrast, bacteria from OTU0002 were most closely related to free-living environmental bacteria, sharing an identical 16S rRNA sequence with *Lactobacillus florum* strains from flowers (Vásquez *et al.* 2012), wine (NCBI accession no. JN819210) and clean-room facilities (Venkateswaran *et al.* 2003).

When focusing on finer scale taxonomic patterns within the three dominant OTUs, we found just a single

Fig. 1 Abundance of bacterial species and taxa across *Linepithema humile*. Bar graphs for each library show the percentage of quality filtered Illumina sequence reads classified to various 97% OTUs. Rare bacterial OTUs (found in under 40% of libraries and with a maximum relative abundance <8% across all libraries) were combined into a single category ('Others'). (A) Bacterial communities of *L. humile* in both native and introduced populations. Note, the dashed line within the Large supercolony portion separates Rice Canyon samples from those sampled in later years (2011) across different locations of this supercolony's range. (B) A portion of data from the Large supercolony in panel A is shown again here to highlight Large supercolony bacterial communities specifically from the Rice Canyon (CA, USA) population studied in a time-series analysis of trophic level (Tillberg *et al.* 2007). Bacterial communities from workers are shown in relation to the time since invasion of this site (1996) and their trophic position over time. Stable isotope data illustrated in the top part of this panel (from Tillberg *et al.* 2007) show the approximate trophic position of *L. humile* in various years. Decreasing $\delta^{15}\text{N}$ values for worker ants over time reflect a shift towards greater use of nitrogen-poor liquid foods. Years of sampling from 1996 to 2003 are abbreviated at the bottom of the panel. (C) Bacterial communities of laboratory-reared Argentine ants fed diets with differing ratios of carbohydrate ('C') and protein ('P'). The time of sampling in relation to the start of the experiment is indicated at the bottom of the panel (0D = before administering experimental diets; 20D and 40D = 20 or 40 days after the switch to the given diets). At the top of the bar graphs in panel (C) we indicate the particular diet as well as the colony ID indicated by site of collection.



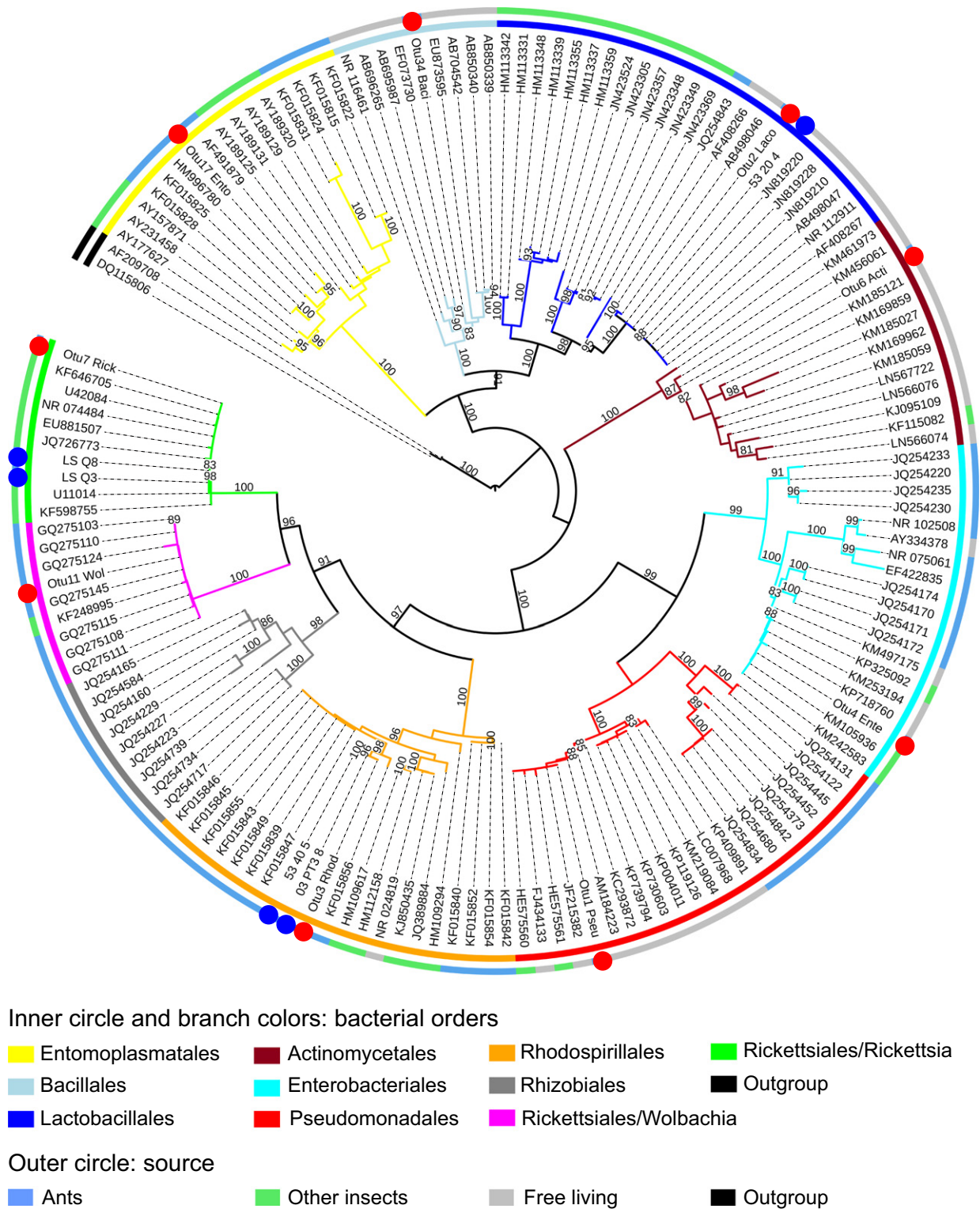


Fig. 2 Phylogenetic tree of dominant bacteria from *Linepithema humile* and their closest GenBank relatives. Depicted on this maximum-likelihood phylogeny are representatives from all dominant OTUs in Argentine ants exceeding 60% relative abundance in at least one ant sample. Top BLASTn hits are also included, as are representative bacteria from various animal hosts in these same lineages. The inner circle and branch colours illustrate bacterial taxonomy while the outer circle shows the habitats from which representative bacteria were isolated. Red and blue circles along the outer circle indicate OTUs identified by Illumina sequencing and Sanger sequencing in this study, respectively. The colour scheme here is consistent with those in Figs 1 and 3.

common 16S rRNA genotype for each symbiont, suggesting limited strain diversity within and across natural populations (Fig. 3A). Interestingly, identical unique sequences for the *Lactobacillus* (Lactobacillales) and *Asaia*-like (Rhodospirillales) OTUs were found in ants from both native and introduced ranges. Combined, these findings suggest that just a few bacterial strains dominate the microbiota of Argentine ant workers in the southwestern United States and that two of the three dominant symbiont strains possess a transcontinental distribution. Furthermore, workers from the same supercolonies showed a tendency towards domination by the same symbiont strain, raising interesting questions about the mechanisms of symbiont acquisition.

Unlike these common associates, three other species dominated communities of just one Argentina-derived worker each, including OTU0034 (order Bacillales, genus *Oceanobacillus*), OTU0011 (order Rickettsiales, genus *Wolbachia*) and OTU0017 (order Entomoplasmatales, genus *Entomoplasma*) (Fig. 1A). These microbes made up between 82 and 99% of their respective bacterial communities. Phylogenetic analyses showed that one of these bacteria clustered with *Wolbachia* of other ants, while a second was related to ant-associated microbes described previously from the *Entomoplasmatales* (Funaro *et al.* 2011). For the latter case, *Mesoplasma*-like bacteria from leaf-cutter ants were the closest relatives of this potential symbiont (Fig. 2). In contrast to the three aforementioned rare, yet dominant microbes, *L. humile* workers harboured a number of other numerically rare yet somewhat commonly encountered bacteria from the Pseudomonadales and Burkholderiales (Fig. 1A). However, relatedness between these microbes and bacteria found commonly in blank sequence libraries (Fig. S1, Supporting information) raises doubts about their status as true symbionts.

Patterns of beta diversity for Linepithema humile - associated bacterial communities: comparing communities across continents

To better visualize the variation between bacterial communities of Argentine ant workers from native vs. introduced populations, we plotted the results of a principal coordinates analysis using a pairwise Bray–Curtis dissimilarity matrix focused on 97% OTU composition (Fig. S4, Supporting information). Separation of bacterial communities appeared to be primarily associated with differences in the relative abundance of OTU0002, OTU0003 and OTU0007. Permutational ANOVA (Adonis) analyses based on Bray–Curtis distances revealed significant differences between the bacterial communities of Argentine ants from the introduced Large

supercolony (California, USA) and those from native Argentina populations ($F = 3.734$, $R^2 = 0.083$, $P = 0.023$). Communities from native Argentina populations were also significantly different from those in the introduced Lake Hodges ($F = 2.929$, $R^2 = 0.163$, $P = 0.039$) and Lake Skinner supercolonies ($F = 7.853$, $R^2 = 0.344$, $P = 0.001$).

In spite of the detected statistical differences, we should note that only the Lake Skinner workers harboured communities that were strongly divergent from those of native Argentine ant workers. In contrast, workers from the Lake Hodges and Large supercolonies showed overlap with native workers in their prevalence of the same dominant strains from OTU0002 (Lactobacillales) and, to a lesser extent, OTU0003 (Rhodospirillales) (Figs 1A and 3A).

Community composition of Argentine ant-associated microbiota: sampling across time and trophic level within Rice Canyon (CA, USA)

In more closely examining the Illumina sequence data, we partitioned bacterial communities from 28 Argentine ants collected at different time points in Rice Canyon, CA. While trophic position shifted across the studied time span (Tillberg *et al.* 2007), we saw no evidence for a shift in bacterial communities over time (Fig. 1B; permutational ANOVA statistics on Bray–Curtis distances testing for an effect of time: $F = 0.234$, $R^2 = 0.009$, $P = 0.657$). Similarly, when binning samples into pre- vs. post-trophic shift categories (i.e. 1996–1998 vs. 2002–2003), we saw no significant differences in bacterial communities ($F = 0.232$, $R^2 = 0.009$, $P = 0.653$). Within this population, most workers retained a high prevalence of the *Lactobacillus* symbiont (OTU0002), while a few were enriched for the *Asaia*-like OTU0003 symbiont. As alluded to earlier, each OTU was dominated by a single genotype. The genotypes dominating introduced *L. humile* from Rice Canyon dominated those from the native range. Furthermore, at this genotype/strain level, we found no evidence for shifts in symbiont composition over time (Fig. 3B).

Community composition of Argentine ant-associated microbiota: sampling laboratory-reared workers across dietary regimes

Animal diets can have rapid effects on the membership of their symbiotic microbiota (Chandler *et al.* 2011). Accordingly, amplicon sequencing of bacterial 16S rRNA genes suggested strong impacts of dietary regime on the bacterial communities of 30 Argentine ants (Fig. 1C). Permutational ANOVAs of Bray–Curtis distances confirmed the visual patterns, revealing a significant effect of diet on community composition in each of

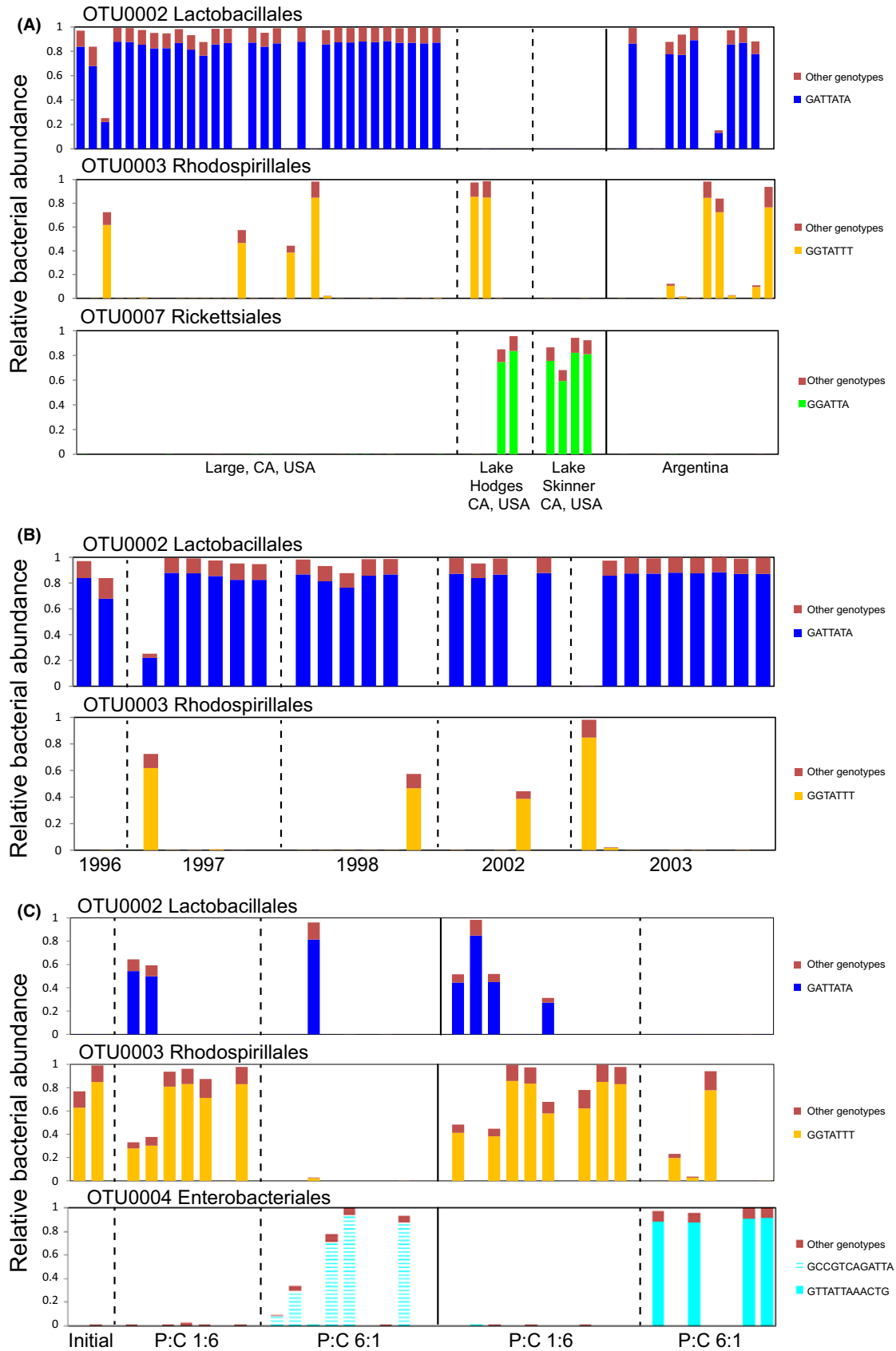


Fig. 3 16S rRNA genotype-level analyses of four indicator OTUs reveal highly related symbiont strains are retained across time, broad geographic scales, and in laboratory-reared colonies. OTU0002, OTU0003, OTU0004 and OTU0007 were the four indicator taxa distinguishing enterotypes in our study. When present, each tended to dominate the bacterial community of its worker host. (A) Native and introduced populations possess the same 16S rRNA genotypes of two indicator OTUs (OTU0002 from the Lactobacillales and OTU0003 from the Rhodospirillales). Three of the four common natural OTUs show just a single dominant genotype across all sampled locations/supercolonies. Note, the dashed line within the Large supercolony portion separates Rice Canyon (1996–2003) samples from workers sampled subsequently (2011) in different locations of this supercolony's range. (B) When we partition out Large supercolony workers from the Rice Canyon population, we see no shift in symbiont strains in conjunction with the multiyear trophic shift in this population. (C) Bacterial communities from laboratory-reared *Linepithema humile* fed diets with low P:C ratios were dominated by the OTU0003 genotype that was fairly common in native and introduced habitats. Those from workers fed high P:C ratios possessed one of two dominant genotypes of a laboratory-enriched taxon, OTU0004 (Enterobacteriales), with the dominant genotype differing between experimental colonies. Note that the names of genotypes correspond to the haplotype at variable 16S rRNA positions for the given out.

two experimental colonies ($F = 5.864$, $R^2 = 0.369$, $P = 0.0003$ for the Los Higueros, CA colony; $F = 8.239$, $R^2 = 0.388$, $P = 0.003$ for the Mast, CA colony).

The *Asaia*-like, Rhodospirillales bacterium (OTU0003) dominated gut communities of workers consuming high quantities of carbohydrates, with sevenfold greater abundance in workers on the 1:6 protein:carbohydrate ratio diet than in those on the 6:1 protein:carbohydrate ratio diet (ANOVA test implemented in QIIME 1.8.0, Bonferroni-corrected P -value = 0.002, Table S9, Supporting information). This OTU and its single predominant strain (Fig. 3) were identical to those found in *L. humile* from natural populations, revealing some resemblance between natural vs. laboratory-reared symbiont communities. In contrast, the dominant microbes from ants on protein-heavy diets were extremely rare in the field. First among these was Enterobacteriales OTU0004, which had 200-fold greater average relative abundance in Argentine ants fed on the protein-rich vs. carbohydrate-rich diet (ANOVA test implemented in QIIME 1.8.0, Bonferroni-corrected P -value = 0.036; Table S9, Supporting information). In addition, two Argentine ants fed on the protein-rich diet harboured dominant bacteria from the order Pseudomonadales that clustered within OTU0001. The strain identities of the proliferating Enterobacteriales bacteria differed between workers from the two experimental colonies (Fig. 3C). Of the two detected 16S rRNA genotypes, only one was present in appreciable quantities in naturally collected ants – in this case within just a single pupa from the Lake Hodges 2014 collection (Fig. S3, Supporting information).

Bacterial densities

While not initially a focus of our investigation, another variable that emerged in the course of this study was the quantity of bacteria harboured by individual workers. Questions surrounding this attribute arose as we found more and more specimens yielding weak-to-no

PCR amplification with universal bacterial 16S rRNA primers even though many had already proven sufficient for PCR in assays targeting the ants' *abdA* genes. One of the first notable observations from our qPCR analyses is that Argentine ant workers harbour bacteria at substantially lower abundances than workers of *Cephalotes varians* (Fig. 4), which belongs to a genus renowned for its highly integrated and ancient relationships with gut symbionts (Sanders *et al.* 2014; Lanan *et al.* 2016). In fact, the average standardized 16S rRNA copy number estimate for *C. varians* was between ~1.9 and 66.7 times greater than those estimates from Argentine workers (t -test, $P \ll 0.05$ for all comparisons between *C. varians* and Argentine ant categories shown in Fig. 4; see Tables S10–S11, Supporting information for qPCR data and statistics).

In comparing 16S rRNA results among groups of *L. humile* collections, we found higher proportions of quality DNA samples from Argentina (78.6%; $n = 159$) failed to yield 16S rRNA amplification when compared to quality samples from introduced, CA habitats (48.1%; $n = 237$) (Fisher's exact test, $P = 0.0001$). Across these two regions, we also found slightly higher proportions of samples yielding weak 16S rRNA band intensity scores (<0.77) in native Argentina (91.2%; $n = 159$) compared to the introduced CA region (78.9%; $n = 237$) (Fisher's exact test, $P = 0.0012$; data in Table S1, Supporting information). qPCR measurements further suggested that workers from Argentina harboured fewer bacterial 16S rRNA gene copies (per ng of total DNA) compared to those from Rice Canyon, CA, USA (Fig. 4; t -test, $P = 0.007$ vs. 1996–1998 Rice Canyon samples; $P = 0.034$ vs. 2002–2003 Rice Canyon samples). Although some comparisons raise questions on the impacts of overall DNA quantity (i.e. the denominator in the normalization equations) in driving these results (Table S10, Supporting information), even the newest collections from Argentina (2009), which were of clear sample quality, showed low rates of 16S rRNA amplification, while even the oldest CA samples (e.g. Rice

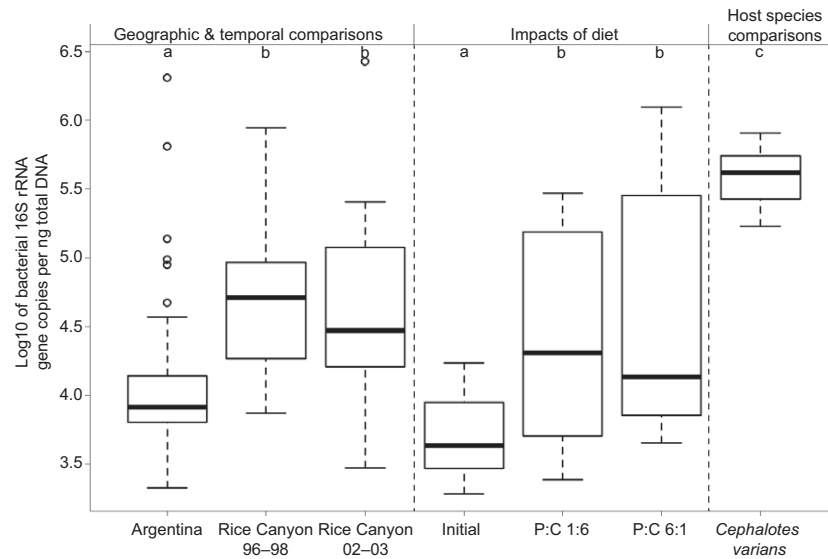


Fig. 4 Bacterial densities in Argentine ants are typically low, but highly variable, differing across geography and with diet. Box plots illustrate 16S rRNA gene copy number normalized by dividing by total DNA (in ng) for each individual worker extraction. Different letters at the top of the figure illustrate groups of ants with significant differences in this measure of bacterial quantity. Ants from Argentina were collected in 2009. Invasive ants from Rice Canyon, CA, are split into two time points (with year abbreviations 96–98 and 02–03) corresponding to times when they were intermediate vs. low on the trophic scale in this habitat. P:C indicates protein:carbohydrate ratios in the administered artificial diets. 'Initial' reveals data from laboratory-reared ants sampled immediately before the dietary manipulation experiment. Normalized bacterial 16S rRNA gene copies per ng total DNA of *Cephalotes varians* (far right) were used for comparison.

Canyon 1996 & 1998) showed comparatively higher 'success' rates of bacterial amplification (Table S8; Appendix S2, Supporting information).

We were interested in determining whether there was any evidence for shifts in bacterial density across the trophic shift in Rice Canyon, experienced between the early 1996–1998 collections and those obtained several years later, in 2002–2003. Workers from before the shift with quality DNA templates showed lower rates of 16S rRNA amplification failure (21.9%; $n = 32$) than those collected subsequently (49.1%; $n = 53$) (Fisher's exact test, $P = 0.02$). They also showed lower, although non-significant, proportions of samples yielding weak 16S rRNA amplification (56.3% of $n = 32$ with band intensities <0.77), compared to the lower trophic level workers from the later time point (73.6% with weak amplification out of $n = 63$) (Fisher's exact test, $P = 0.15$). However, when examining qPCR data we found no significant differences in normalized bacterial densities across these time points (Fig. 4, Table S11, Supporting information; t -test: 0.614), arguing against dramatic changes in bacterial densities in association with the Rice Canyon trophic shift (Tillberg *et al.* 2007).

Our quantitative PCR experiments also showed that *L. humile* workers from the beginning of the dietary experiment – fresh from the field and before switching to imbalanced diets – had significantly fewer 16S rRNA

gene copies (per ng total DNA) than those consuming experimental diets, suggesting bacterial proliferation under both treatments (Fig. 4; t -test results: $P = 0.047$ for pre-experiment ants vs. ants on carbohydrate-rich diet; $P = 0.048$ for pre-experiment ants vs. ants on protein-rich diet). Bacterial densities were similar across the two dietary regimes (Fig. 4; t -test $P = 0.581$ for high protein vs. high carbohydrate); hence, parallel trends of symbiont proliferation occurred in spite of clear differences in the types of bacteria favoured by these two diets (Fig. 1C).

While means and medians of bacterial densities were clearly different between various ant categories, we also found trends in the variance of normalized 16S rRNA copy number. Variance was high for introduced Argentine ants in Rice Canyon and in ants fed in the laboratory on experimental diets. In contrast, variance was generally low (with some outliers) for field-caught Argentine ants from their native range and for the small number of surveyed, introduced ants from California when reared under standard laboratory conditions (Fig. 4). *Cephalotes varians* workers were similar to these latter Argentine ant categories with little variation in single worker bacterial density. Further analyses suggest that these patterns of variation in normalized 16S rRNA copy number are driven by 16S rRNA copy number variation and not variation in total DNA quantity

(Table S10, Supporting information). It is interesting to point out that the high variance in normalized 16S rRNA copy number for some Argentine ant groups suggested some *L. humile* workers have higher bacterial densities than any *C. varians* worker. Thus, while most Argentine ants have few bacteria there are several strong exceptions under both natural and artificial conditions.

Functional analysis

To infer the potential functions of common bacteria from Argentine ants, we utilized published genomes from close relatives, addressing whether these microbes encoded intact pathways for nitrogen metabolism. Bacteria from the dominant Lactobacillales strain of Argentine ants had 100% 16S rRNA identity to two *L. florum* strains with sequenced genomes (2F and 8D; GOLD Analysis Project IDs Ga0011978 and Ga0024727, respectively). While more distantly related members of this genus possess capacities to synthesize some amino

acids, the two *L. florum* genomes lack complete pathways for the focal aspects of nitrogen metabolism, including nitrogen recycling, nitrogen fixation, and biosynthesis of all but three amino acids, which happened to each be nonessential (glutamine, asparagine, glycine) (Fig. 5A). These findings fit with prior studies suggesting that *Lactobacillus* from this group are fastidious organisms requiring exogenous amino acids, vitamins and nucleic acid derivatives (Kandler & Weiss 1986; Pridmore *et al.* 2004; Altermann *et al.* 2005). Overall, then, the close relatedness between the focal symbiont (OTU0002) and *L. florum* – with 100% 16S rRNA identity – leads us to suggest that the identified symbiont from these ants is not able to function as a major nitrogen-provisioning symbiont.

In considering the other dominant bacteria in the introduced portion of Argentine ant's range, the Rickettsiales associate (OTU0007) shared an identical 16S rRNA gene sequence with *Rickettsia bellii*, a species that also has two sequenced genomes. Yet, with the exception of glycine and aspartate biosynthesis, neither

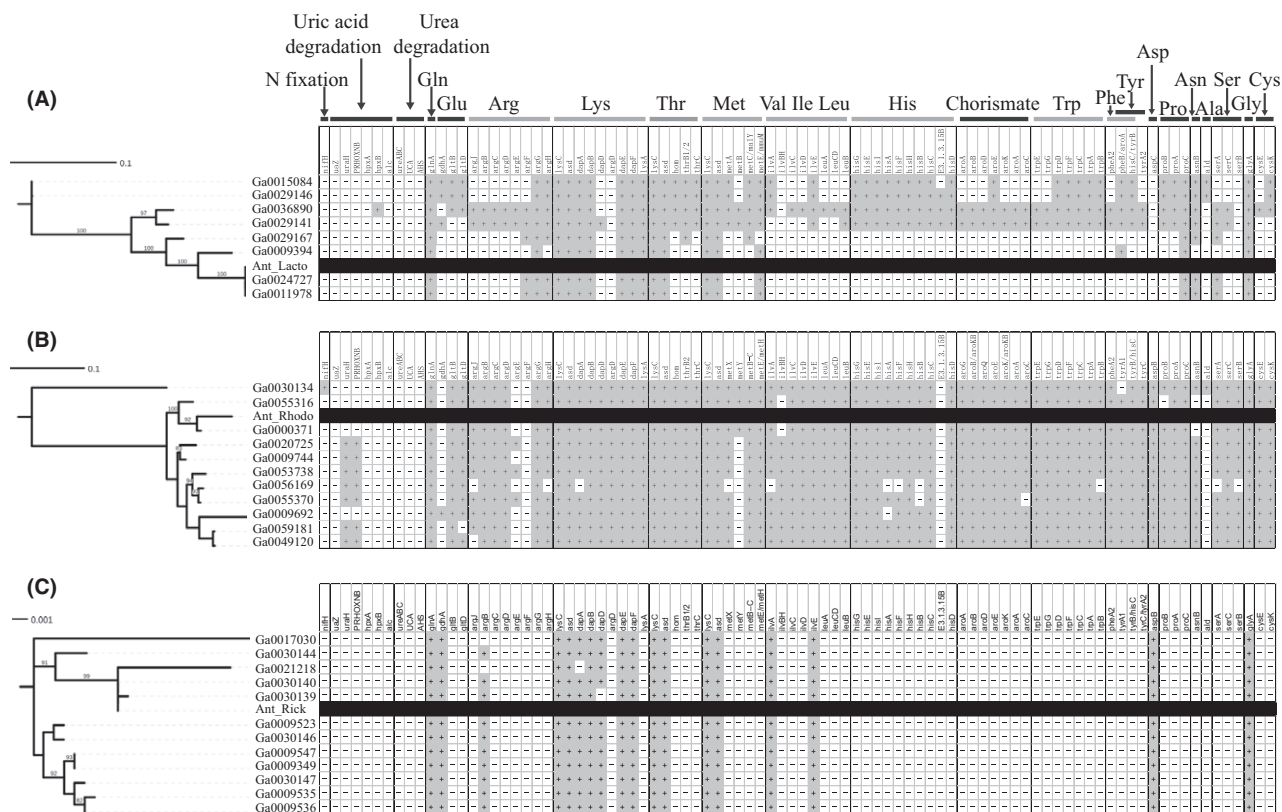


Fig. 5 Presence or absence of genes involved in pathways of nitrogen fixation, nitrogen recycling and amino acid synthesis for relatives of the three dominant symbionts of Argentine ants. Symbionts hail from the orders Lactobacillales (A), Rhodospirillales (B) and Rickettsiales (C). White and grey in each heatmap respectively represent the absence and presence of genes associated with the focal metabolic pathways. Black bars denote the lack of pathway information for the Argentine ant associates. Maximum-likelihood phylogenies on the left were inferred based on an alignment of 16S rRNA sequences. Three ant-associated 16S rRNA sequences were named by Ant-lacto (OTU0002), Ant-Rhodo (OTU0003) and Ant-Rick (OTU0007).

R. bellii strain nor any of their relatives encoded intact pathways for the focal aspects of nitrogen metabolism (Fig. 5C).

Finally, the *Asaia*-like Rhodospirillales strain (OTU0003) from natural and laboratory-reared Argentine ants had no identical 16S rRNA sequences among bacteria with sequenced genomes. However, phylogenetic evidence suggests that this bacterium may be capable of useful nitrogen-provisioning; genomes from close relatives encode pathways for the biosynthesis of most amino acids, although not the pathways required for nitrogen recycling. While nitrogen fixation has been cited as a possible function for ant-associated bacteria in this group (Samaddar *et al.* 2011), only one distantly related Rhodospirillales bacterial genome possessed the *nifH* gene encoding the dinitrogenase reductase enzyme used in this process (Fig. 5B).

Discussion

Argentine ants have been introduced widely and encounter a variety of resources throughout their diverse range. Relative to native populations, these ants consume greater quantities of nitrogen-poor foods in their introduced habitats (Tillberg *et al.* 2007). The importance of symbionts in such exploitation and the impacts of invasion on symbiont diversity are topics that have largely gone unexplored (but see Reuter *et al.* 2005; Tsutsui *et al.* 2003a), helping to motivate the present study on this widespread invasive pest. Another major goal of our investigation was to better understand the macroevolutionary correlations between diet and symbiosis across the ants (Russell *et al.* 2009; Anderson *et al.* 2012). At question is whether symbionts have been routinely required to initiate trophic shifts, or whether ants may transition towards greater reliance on nitrogen-poor diets by their own devices. The recency of changing diet in Argentine ants makes the system ideal for addressing this query, while the widespread precedent for nitrogen-provisioning by bacterial symbionts in animals with marginal diets (Sabree *et al.* 2009; Douglas 2011; Brune & Dietrich 2015) helps to further justify our focus.

Deviations from the herbivores' symbiotic syndrome

Several groups of functionally herbivorous ants (e.g. *Camponotus*, *Cephalotes*, *Tetraponera* or *Dolichoderus*) exhibit features of a symbiotic syndrome, housing large masses of symbionts in their guts or their gut-associated cells, using elaborate mechanisms for symbiont transfer (i.e. oral–anal trophallaxis or transovarial transmission), and associating with long-standing symbiont specialists that group into host-specific lineages (Sauer *et al.* 2000;

Cook & Davidson 2006; Stoll *et al.* 2007; Russell *et al.* 2009, 2017; Anderson *et al.* 2012). In at least one case, these ants derive nitrogen in the form of essential amino acids from their gut-associated symbionts (Feldhaar *et al.* 2007). Such benefits are hypothesized to have been pivotal in the successful exploitation of nitrogen-poor liquid diets, like floral nectar and honeydew, by diverse groups of herbivorous ants (Davidson *et al.* 2003).

Our findings on the bacterial associates of *Linepithema humile* present a sharp contrast to the symbiotic syndrome of herbivorous ants. First, Argentine ants harboured low-density bacterial communities, with low qPCR-based estimates of symbiont titre and a surprising prevalence of failed bacterial 16S rRNA amplification across castes and stages of development. Second, the bacteria identified as recurrent associates of Argentine ants were all very closely related to bacteria from other hosts or habitats, arguing against long-term trends of specialization. These microbes also showed little overlap with the taxa found commonly in several herbivorous ant groups (e.g. *Blochmannia*, *Bartonella*-like Rhizobiales, Burkholderiales). Third, comparative genomics suggested that two of the three dominant symbionts are unlikely to provision essential nitrogen. While the third symbiont (an *Asaia*-like acetic acid bacterium; OTU0003) could play such a role, its frequency and abundance argue against a large impact of any such provisioning on both native and invasive *L. humile* populations.

Beyond these results, bacteria in native and invasive populations showed strong overlap, and we found minimal turnover in the microbiota across an observed trophic shift in Rice Canyon. So in addition to a lack of support for symbiont-driven nitrogen-provisioning, our findings argue against wholesale changes in symbiosis with invasion and changing *L. humile* ecology.

Stability of symbioses between Argentine ants and their microbes

Close relatedness between the dominant microbes of Argentine ants and those in other habitats clearly argue against long-term specialization. Yet several aspects of our analyses suggest strong stability in the associations between *L. humile* and their bacterial menageries. First, identical 16S rRNA genotypes of the *Asaia*-like (OTU0003) and *Lactobacillus florum*-like (OTU0002) symbionts were found in Argentine ants from both native and introduced regions. Bacteria with these same genotypes also dominated laboratory-reared ants feeding on carbohydrate-rich diets. While absent from Argentina and laboratory-reared ants, *Rickettsia bellii*-like symbionts (OTU0007) sharing identical 16S rRNA genotypes were found across two introduced supercolonies in

California. These recurring microbiota are distinct from those of the red imported fire ant (Ishak *et al.* 2011), in spite of their parallel shifts towards nectar and honeydew consumption within their introduced ranges (Wilder *et al.* 2011), suggesting that Argentine ants employ a recurrent and possibly specialized cast of bacterial symbionts.

While studies on symbiont localization are needed, at least two of the identified microbes – the *L. florum*- and *Asaia*-like bacteria – are likely to be gut inhabitants. Bacteria from these groups are renowned for their abilities to tolerate the sugar-rich, acidic gut environments that likely arise with sugar consumption and subsequent bacterial fermentation. Yet the lack of more generalized associations with a variety of Acetobacteraceae and Lactobacillaceae, as seen in other insects with sugar-rich diets (e.g. *Drosophila*) (Wong *et al.* 2011), suggests there is more to the observed specificity than just low gut pH and high gut osmolarity. In other words, while the gut habitat of *L. humile* may be highly selective, selectivity alone seems unlikely to explain the recurrence of identical symbiont strains across such broad geographic scales.

So do the widespread distributions of symbiont genotypes and the laboratory persistence of these ‘same’ bacteria suggest strong partner fidelity or effective partner choice mechanisms to be at work? It is tempting to answer in the affirmative, invoking social transmission for the persistence of candidate gut symbionts and transovarial transfer for the *Rickettsia* symbiont, which hails from a group without known affinity for the gut (Perlman *et al.* 2006). At this point, however, we cannot rule out the possibility that these ants regularly acquire related bacterial strains from the environment and that these bacteria, while symbiotic, are not solely confined to the guts of Argentine ants. Indeed, the identity of 16S rRNA between the most dominant Argentine ant gut symbiont (OTU0002) and free-living *Lactobacillus florum* makes it clear that feeding on floral nectar could favour acquisition of bacteria that are well suited for lifestyles in the gut environment. Hence, along with the clear need to demonstrate symbiont lifestyle and localization, the degrees of specificity and modes of symbiont spread/acquisition are important areas of future study for *L. humile*. Genotyping at more rapidly evolving loci will help in these efforts as will further studies of laboratory-reared colonies, with careful tracking of symbiont strains among the ants that have hatched and eclosed in this controlled environment.

What controls symbiont density?

Bacterial cells outnumber the cells of their animal hosts in some vertebrate systems (Sommer & Backhed 2013),

and many insects harbour large masses of bacteria in their guts and internal tissues (Dillon & Dillon 2004; Stevanovic *et al.* 2015). Herbivorous ants can be included among these ranks, with several possessing large quantities of bacteria in their gut cavities or gut-associated cells (Cook & Davidson 2006; Stoll *et al.* 2010). We found that Argentine ants present a strong contrast to these patterns, with most workers harbouring few bacteria. Low-density bacterial communities may in fact be somewhat widespread across the ants, as universal 16S rRNA PCR assays for bacteria are often negative in workers with suitable quality for host nuclear- or mtDNA-targeting PCR (Rubin *et al.* 2014; Russell *et al.* 2017). The drivers of variable bacterial density in these social insects remain unknown. But a potential parallel can be seen for the cuticular symbionts of fungus growing ants. Genera from this group vary in the abundance of actinomycete bacteria housed on worker cuticles. In species where these putative agents of garden pest control are absent or found at low density, the ants seem to invest in alternate strategies for the maintenance of healthy fungus gardens, including heightened grooming and the use of antimicrobial substances secreted from the metapleural gland (Fernandez-Marin *et al.* 2009, 2013, 2015). Might large masses of gut bacteria, then, represent one strategy in a series of trade-offs that ants navigate to meet the challenges imposed by nature?

Beyond the ants, it is worth noting that other insects show variability in microbial biomass and that some, including honeybee larvae (Martinson *et al.* 2012) and adult *Drosophila*, harbour low quantities of gut-associated bacteria. In the latter case, for example, it has been estimated that 4- to 7-day-old adult *D. melanogaster* harbour only ~1000 bacterial cells per gut (Broderick *et al.* 2014). Age and the recency of food consumption both impact bacterial density in this system (Broderick *et al.* 2014), raising questions as to whether such factors could drive the large degree of variability in bacterial density seen for *L. humile*. Under controlled laboratory rearing, we did observe an effect of diet on bacterial densities, with proliferation upon shifting laboratory-reared workers to imbalanced diets. It stands to reason that greater access to sugar-rich foods could explain our observations of higher bacterial densities in at least one invasive population (Rice Canyon, CA) compared to those in workers from native Argentina.

Beyond *L. humile*, changing diet can induce rapid changes in the gut microbiota across a range of animals, with alterations ranging from drastic (Broderick *et al.* 2004; David *et al.* 2014) to subtle (Hu *et al.* 2014). These shifts could partially stem from varying types of bacteria being brought into the gut environment with varying foods (i.e. allochthonous inputs). Yet they may also

reflect changes in the abundances of autochthonous bacteria that persist within the gut. A possible demonstration of this latter phenomenon has been seen for the bullet ant, *Paraponera clavata*. In this case, provisioning of sucrose to colonies for just 2 weeks led to increased detection of ant-specific *Bartonella*-like symbionts among the workers of these experimental colonies (Larson *et al.* 2014). Bacteria from this group are widely known to colonize ant guts (Stoll *et al.* 2007; Sapountzis *et al.* 2015) and some have been hypothesized to provision nitrogen to their ant hosts (Russell *et al.* 2009). Apparent spikes in the abundance of these bacteria, thus, present a parallel to the proliferation of candidate nitrogen-provisioners (i.e. the *Asaia*-like symbionts) in *L. humile* reared on sucrose-rich diets (Figs 1C and 4). While it will be interesting to assess whether these microbes actually provision nitrogen to their hosts, it will be similarly important to assess whether nutritional mutualisms, if present, can be upregulated during times of potential benefit, or whether bacterial proliferation may more simply reflect a response to the presence of a limiting nutrient (i.e. sugar).

Future directions

Findings that (i) bacteria are not abundant and (ii) the most common bacteria likely contribute little to host nitrogen budgets suggest that Argentine ants have navigated their recent dietary changes on their own. Stemming from this likelihood are questions relating to the level of nitrogen limitation experienced by workers in introduced *L. humile* populations. Traditional interpretation of stable isotope data would suggest that, relative to native populations, much of the nitrogen in adult workers' tissues comes from their nitrogen-poor liquid foods, and not from the animal protein they consume (Tillberg *et al.* 2007). Isotope profiling of larvae will be important to understand whether animal protein – rich in all amino acids – might make a larger contribution towards this nutrient-demanding stage of development. Of further pertinence to the question of nitrogen limitation is the observation that at least one other *Linepithema* species exhibits abnormally low %N given their size and trophic position (Davidson 2005). Might the presumably lowered N-requirement be a key to these ants so successfully exploiting their new diets?

Another area of needed study relates to the impacts of recurring symbionts on *L. humile* and, hence, their importance in the global invasions of Argentine ants. While the absence of key metabolic pathways makes it unlikely that *Rickettsia* and *Lactobacillus* symbionts help the ants to navigate the low nitrogen content of honeydew or plant nectar (Auclair 1963; Baker *et al.* 1978; Fischer *et al.* 2002), we cannot rule out alternative

functions of some nutritional importance (e.g. vitamin biosynthesis). Beyond nutrition, it is important to note that gut bacteria of *Drosophila*, with relatedness to those in *L. humile*, shape crucial host physiological functions such as insulin signalling and fat storage (Newell & Douglas 2014). The low biomass of these fly-associated communities makes it clear that we cannot rule out important impacts for the bacteria of Argentine ants. It is also possible that colony-level nutritional benefits could be realized without the ubiquity or abundance of beneficial symbionts in all constituent workers, due to the potential for nutrient flow via trophallaxis.

In addition to nutrient provisioning, heightened defence against parasites and pathogens is a common benefit conferred by microbial symbionts (Oliver *et al.* 2014), including those inhabiting the gut (Dillon & Dillon 2004; Koch & Schmid-Hempel 2011). The arguably gut-associated symbionts of *L. humile* (i.e. *Lactobacillus* and the *Asaia*-like bacterium) could conceivably defend their hosts through direct (i.e. antimicrobial) mechanisms or through indirect means, including colonization resistance or the creation of inhospitable gut physiology (i.e. low pH). Transovarially transmitted *Rickettsia* with close relatedness to the strain found here can have various impacts on their host arthropods (Hagimori *et al.* 2006; Perlman *et al.* 2006; Brumin *et al.* 2011; Himler *et al.* 2011); and their protection of sap-feeding insects against fungal pathogens (Łukasik *et al.* 2013; Hendry *et al.* 2014) suggests an intriguing possibility for the *Rickettsia* of Argentine ants.

Unlike the other symbionts found in our study, the *Rickettsia* symbiont has thus far been found only in invasive *L. humile* populations. Yet prior studies of symbionts in invasive insects have often focused on expectations for symbiont loss upon invasion, given the presumed effects of colonization bottlenecks. This expectation is at least partially exemplified among the ants, with loss of *Wolbachia* from both invasive *L. humile* (Tsutsui *et al.* 2003a; Reuter *et al.* 2005) and red imported fire ants (Shoemaker *et al.* 2000). But in spite of this, *Spiroplasma* symbionts are common in invasive fire ant populations (Ishak *et al.* 2011), and *L. humile* have apparently imported their *Lactobacillus* and *Asaia*-like symbionts from native Argentina. When combined with *Rickettsia*, there is a clear variety of microbiota housed by invasive *L. humile*. Retained or newly acquired symbionts may have important impacts on invasive arthropod pests (Brown *et al.* 2014), raising important questions about the impacts of these bacteria on their host ants' pest status. Broader geographic surveys are clearly warranted to understand the dynamics of symbionts over time and space for *L. humile*. Also warranted will be efforts to study microbiota in

L. humile workers with low symbiont density. Indeed, the majority of ants utilized in our study had insufficient bacterial titres for sequence-based investigation (Fig. S5C, Supporting information), raising the possibility that we have missed some diversity.

One final attribute for future study stems from our discovery that the microbiota of the sampled Argentine ants were dominated by a small number of bacteria. For instance, the sequence libraries from most field-caught workers were dominated by a single bacterial species with over 90% representation among the quality sequence reads (Fig. 1). These species were comprised mainly of a single dominant strain (Fig. 3), further underscoring the simplicity of the microbiota. While not formally quantified in our study, these trends of low diversity resemble those seen for some other insects. For instance, in a broad study across the insects Jones and colleagues found an average of 7.5 bacterial species (97% OTUs) per insect from amplicon sequence libraries rarefied to 500 reads (Jones *et al.* 2013). In a meta-analysis using cloned Sanger sequence libraries with varying depth, Colman and colleagues suggested high species (97% OTU) diversity for microbes of wood-feeding insects, with an average of 102.8 species per sample (Colman *et al.* 2012). Bacterial communities from bees and wasps exhibited the lowest diversity, with 11.8 species per sample. Through our efforts, we found 12.1 OTUs per adult field-caught Argentine ant worker (data derived from Table S4, Supporting information). When considering the large sequence libraries (i.e. avg. = 12 340 quality reads per individual among these ants), combined with the stronger tendencies for dominance by single bacterial species and strains, it would appear that bacterial communities of Argentine ants are near the low end of the diversity spectrum among insects. Much like the forces behind low bacterial densities and strong symbiont strain recurrence, the drivers of such low diversity are unclear and an interesting target for future study.

Conclusions

Our work shows that nitrogen-provisioning bacterial symbionts may not always aid hosts in early stage shifts towards low-quality, nitrogen-poor diets. Given (i) the presence of highly abundant, specialized symbionts across several ant groups with long-standing reliance on nitrogen-poor foods and (ii) nitrogen-provisioning roles by such symbionts in at least one of these ant groups, we argue that highly integrated nutritional symbioses may unfold sometime after shifts towards low-quality diet reliance. Symbionts would still serve as major players in lifestyle success and host diversification of such ants. But under this model, they would be

seen not as gatekeepers enabling trophic shifts, but as latecomers that enhance niche exploitation.

Our findings resemble recent discoveries from Trinidadian guppies, whose shifts towards more detritus-rich diets have occurred without notable changes in gut microbiota (Ley *et al.* 2008; Sullam *et al.* 2015). They further echo work on the panda gut microbiome, which shows greater similarity to gut microbiomes of carnivorous relatives than to those from other herbivorous mammals (Ley *et al.* 2008; Xue *et al.* 2015). Such inertia in host-associated microbiota suggests that specialized associations may be hard to shake and that macroevolutionary correlations between symbiont community composition and diet may be a product of microbial shifts unfolding after animals' own innovations provide the first steps in exploiting marginal diets.

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Y.H. and J.A.R. designed the study, with assistance from D.A.H. and A.V.S. Y.H. performed the experiments, with assistance from L.H. in DNA extraction and P.L. and J.G.S. with quantitative PCRs as well K.A.M. and A.D.K. with dietary manipulation experiments. D.A.H., A.V.S. and E.G.L. collected all ant specimens used in this research. Y.H. and J.A.R. wrote the manuscript and made the figures and Y.H. performed most of the data analyses, with contributions from P.L., J.G.S., and J.A.R.

Data accessibility

Amplicon sequencing libraries (generated with Illumina technology) were deposited in the GenBank Short Read Archive under accession nos. SRR4409538, SRR4409543–SRR4409547 and SRR4420973–SRR4421086. In addition, assembled 16S rRNA sequences generated were Sanger sequencing were also deposited to NCBI under accession KX984917–KX984921.

Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 Supplementary methods, figure and table legends.

Appendix S2 Supplementary document on sample quality and Contamination

Fig. S1 Relative abundance of bacterial communities of blank samples and Argentine ant samples in relation to 16S rRNA band intensity scores.

Fig. S2 Correlation between standardized 16S rRNA gene copies and 16S rRNA band intensities.

Fig. S3 Bacterial communities across Argentine ant castes and developmental stages at the OTU (A) and unique sequence (B) level.

Fig. S4 Principal coordinates analysis plot comparing bacterial communities of Argentine ants collected from different colonies in CA, USA vs. native Argentina.

Fig. S5 Identification of bacterial community enterotypes in *Linepithema humile* as illustrated by PCoA.

Table S1 Collection information for the ant colonies and both 16S rRNA and *abdA* gene screen results for all ant samples utilized in this study.

Table S2 Recipe for dietary manipulation experiment diets.

Table S3 Information on PCR conditions.

Table S4 Unique sequence table from Argentine ant gut community samples and blank samples.

Table S5 OTU table from Argentine ant gut community samples and blank samples.

Table S6 Support for four enterotypes of Argentine ant microbiomes.

Table S7 Statistical results for selection of indicator OTUs.

Table S8 Are rates of 16S rRNA amplification governed by sample degradation?

Table S9 ANOVA results for bacterial OTUs with varying abundance in Argentine ants fed on diets with different protein:carbohydrate ratios.

Table S10 qPCR-estimated 16S rRNA gene copies and DNA quantities across Argentine ant and *Cephalotes varians* samples.

Table S11 *P*-values from statistical analyses comparing standardized bacterial densities (log 16S rRNA copies/total DNA) across geographic locales, the Rice Canyon trophic shift, dietary treatments and host species.