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Microbiome changes through ontogeny of a tick pathogen vector

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Abstract

Blacklegged ticks (*Ixodes scapularis*) are one of the most important pathogen vectors in the United States, responsible for transmitting Lyme disease and other tick-borne diseases. The structure of a host's microbial community has the potential to affect the ecology and evolution of the host. We employed high-throughput sequencing of the 16S rRNA gene V3-V4 hypervariable regions in the first study to investigate the tick microbiome across all developmental stages (larvae, nymphs, adults). In addition to field-collected life stages, newly hatched laboratory-reared larvae were studied to determine the baseline microbial community structure and to assess transovarial transmission. We also targeted midguts and salivary glands due to their importance in pathogen maintenance and transmission. Over 100 000 sequences were produced per life stage replicate. *Rickettsia* was the most abundant bacterial genus across all sample types matching mostly the *Ixodes* rickettsial endosymbionts, and its proportion decreased as developmental stage progressed, with the exception of adult females that harboured a mean relative abundance of 97.9%. Laboratory-reared larvae displayed the lowest bacterial diversity, containing almost exclusively *Rickettsia*. Many of the remaining bacteria included genera associated with soil, water and plants, suggesting environmental acquisition while off-host. Female organs exhibited significantly different β-diversity than the whole tick from which they were derived. Our results demonstrate clear differences in both α- and β-diversity among tick developmental stages and between tick organs and the tick as a whole. Furthermore, field-acquired bacteria appear to be very important to the overall internal bacterial community of this tick species, with influence from the host bloodmeal appearing limited.

Keywords: 16S rRNA, bacterial community, blacklegged tick, developmental stages, *Ixodes scapularis*, microbiome

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Introduction

The human microbiome project was launched by the National Institutes of Health (NIH) in 2008 to identify and classify microorganisms associated with healthy and diseased people (The Human Microbiome Project Consortium 2012). This project has served as a model for subsequent work on a variety of vertebrate taxa, for example fish (Sanchez *et al.* 2012), amphibians

(Kueneman *et al.* 2014), reptiles (Keenan *et al.* 2013), birds (Roggenbuck *et al.* 2014) and mammalian carnivores (Menke *et al.* 2014). Among invertebrates, studies of microbiome effects on insect behaviour (e.g. Weiss & Aksoy 2011, are especially noteworthy given their emphasis on aspects of the host's biology other than digestive physiology. Research on the microbiome of ticks is of particular importance, considering the role that many of these species play as pathogen vectors and parasites of humans and other vertebrates.

Studies utilizing PCR amplification of clones, coupled with Sanger sequencing or denaturing gradient gel electrophoresis, have attempted to explore internal microbial communities or identify endosymbionts in tick species (Noda *et al.* 1997; Schabereiter-Gurtner *et al.* 2003; Benson *et al.* 2004; Moreno *et al.* 2006; Clay *et al.* 2008; Van Overbeek *et al.* 2008; Heise *et al.* 2010). These studies have provided valuable information as to the general bacterial diversity and identity of common internal bacteria and potential endosymbionts of some tick species. However, cloning is of limited throughput and requires a high amount of effort and cost to identify rare bacteria and assess relative abundances. High-throughput sequencing (HTS) technologies have allowed for in-depth research on microbial community composition and diversity, and inference of microbial relationships (Hovatter *et al.* 2011; Costello *et al.* 2012).

A preliminary report by the Institute of Medicine Committee on Lyme Disease and Other Tick-Borne Diseases examined the diversity of prokaryotes in three tick species (*Amblyomma americanum*, *Dermacentor variabilis* and *Ixodes scapularis*) using 454 pyrosequencing technology (Clay & Fuqua 2010). Although the report provided useful insight into the microbiomes of adults of these three species, it primarily emphasized the overall gap in knowledge regarding the identity of microorganisms and the roles that microbial communities play in pathogen transmission, infectivity and pathogenicity within tick vectors (Clay & Fuqua 2010). Since then, studies using HTS technologies to classify microbiomes in tick species have been conducted on *Ixodes ricinus* in Italy (Carpi *et al.* 2011) and France (Vayssier-Taussat *et al.* 2013); *A. americanum*, *Amblyomma maculatum*, *Rhipicephalus microplus* and *D. variabilis* in the United States (Yuan 2010; Andreotti *et al.* 2011; Hawlena *et al.* 2013; Menchaca *et al.* 2013; Budachetri *et al.* 2014; Ponnusamy *et al.* 2014; Rynkiewicz *et al.* 2015); *I. ovatus*, *I. persulcatus* and *Haemaphysalis flava* in Japan (Qiu *et al.* 2014); *R. turanicus* in Israel (Lalzar *et al.* 2012); and *I. persulcatus* in China (Zhang *et al.* 2014).

Given the importance of the blacklegged tick (*I. scapularis*) as a pathogen vector in the United States, responsible for >30 000 reported cases of Lyme disease each year (CDC 2015), studies on its internal microbial community have also been increasing in recent years (Hawlena *et al.* 2013; Narasimhan *et al.* 2014; Rynkiewicz *et al.* 2015; Van Treuren *et al.* 2015). Although the role of the blacklegged tick as a vector of the Lyme disease spirochete (*Borrelia burgdorferi*) and the babesiosis protozoan (*Babesia microti*) has been known since the late 1970s (Spielman 1976; Steere *et al.* 1978; Spielman *et al.* 1979), its ability to transmit *Anaplasma phagocytophilum* and *Borrelia miyamotoi* has been known only for the past 15–20 years (Pancholi *et al.* 1995; Scoles *et al.* 2001). The 2001 discovery of *B. miyamotoi* in blacklegged ticks (Scoles *et al.* 2001), only recently determined to be a human pathogen (Platonov *et al.* 2011; Krause *et al.* 2013), underscores how little is currently known about the variety of microbial species occurring in this vector and the potential for more of these species ultimately to be identified as pathogens.

Studies that investigate symbiotic relationships between internal microorganisms and *I. scapularis* have focused on commonly studied human pathogens. For example, infection of blacklegged ticks by *A. phagocytophilum* (the causative agent of granulocytic anaplasmosis in humans) has been shown to induce protein expression that increases cold tolerance and, depending on weather patterns, may aid in tick survival (Neelakanta *et al.* 2010). Furthermore, infection with *B. burgdorferi* has been shown to affect questing behaviour and movement of this tick, possibly affecting survival and/or pathogen transmission (Lefcort & Durden 1996). However, little is known regarding the range of microbes the tick comes in contact with or maintains throughout its entire life cycle. This tick experiences a 2-year life cycle, feeding once per life stage (larva, nymph, and adult) on vertebrate blood. These bloodmeals are necessary for the immature stages (larva, nymph) to moult to the next stage and for adult females to lay eggs (Needham & Teel 1991; Fish 1993). Thus, there are numerous opportunities for this tick to acquire microbes either during any of its three bloodmeals or possibly from the environment, as it spends 98% of its life cycle off-host in a forested setting (Needham & Teel 1991; Fish 1993). Consequently, the potential for additional symbioses involving microbes that benefit *I. scapularis* may be quite high.

Most studies of tick microbiomes have focused on whole ticks (Yuan 2010; Carpi *et al.* 2011; Hawlena *et al.* 2013; Menchaca *et al.* 2013; Vayssier-Taussat *et al.* 2013; Narasimhan *et al.* 2014; Ponnusamy *et al.* 2014; Zhang *et al.* 2014; Rynkiewicz *et al.* 2015; Van Treuren *et al.* 2015), while only a few have examined specific tissues and organs (Andreotti *et al.* 2011; Budachetri *et al.* 2014; Qiu *et al.* 2014); all have been limited to one or two

developmental stages. This is the first study to assess the bacterial community profile of blacklegged ticks across all developmental stages (larvae, nymphs, and adults). Our comparison of the microbial community in field-collected larvae and freshly hatched, laboratory-reared, unfed larvae allowed us to examine the tick microbiome prior to exposure to the field environment, thus examining the potential for transovarial transmission of bacteria. In addition to investigating the internal microbial communities found within whole ticks, we focused on the microbiomes within two specific tissues in adult female ticks, salivary glands and the midgut, as these are important for maintenance and transmission of many tick-borne pathogens associated with *I. scapularis* (Spielman *et al.* 1987; De Silva & Fikrig 1995; Telford *et al.* 1996; Piesman *et al.* 2001; Liu *et al.* 2011).

A more comprehensive approach to the tick microbiome, exploring all developmental stages and specific organs, will allow for a greater understanding of the bacteria that share the same internal environment with the heavily studied pathogenic species that have dominated previous research efforts.

Materials and methods

Tick collection

Host-seeking blacklegged ticks were collected in the summer and fall of 2014 at Fordham University's Biological Field Station in Armonk, NY, an area where blacklegged ticks are highly prevalent (Diuk-Wasser *et al.* 2006, 2012). Nymphs were collected in June and July, larvae in August and adult females and males in October and November, corresponding to each life stage's peak activity season (Fish 1993). Ticks were collected by dragging a 1-m^2 cloth along the forest floor in randomly selected 20-m transects. After each transect, ticks were removed from the cloth, placed into a glass vial and brought back to the laboratory for taxonomic and life stage identification before being stored at −80 °C until further processing.

To obtain freshly hatched larvae, engorged female blacklegged ticks were collected from hunter-killed white-tailed deer (*Odocoileus virginianus*) in November 2013. Deer were harvested in Bedford, NY (~16 km from Armonk, NY), and were examined for ticks at a butcher shop the same day. Engorged females were placed separately into individual culture tubes and stored in an incubator at 20 °C and a 12:12 [L:D]-h photoperiod until they laid eggs. Eggs were transferred to a sterile vial and kept in the same incubator until they hatched, at which time the new larvae were sacrificed immediately by placing them in −80 °C and stored until processed.

DNA isolation

All laboratory procedures were performed under a laminar flow hood (AirClean Systems) to ensure that microorganisms from the surrounding environment did not contaminate the samples; negative controls were used throughout. Prior to DNA isolation, each tick was surface-sterilized in three washes of 90% ethanol followed by one wash of sterile, nuclease-free, deionized water to remove any external/surface microbes that might be present from plant or soil matter. Whole ticks were grouped into three pools of 10 individuals each, for the following five classifications: freshly hatched laboratory-reared larvae and field-collected larvae (hereafter termed 'lab' and 'field' larvae, respectively, for brevity), field-collected nymphs, field-collected adult females and field-collected adult males. A total of 30 ticks per life stage were tested. Pooling was carried out to maximize microbial yield and provide a representative sample of microbial taxonomic diversity for each life stage.

Larvae were physically disrupted using Lysing Matrix S (MP Biomedicals) on a Benchmark Bead Blaster 24 (Benchmark Scientific) for 3 min at 4 m/s. Nymphal ticks were bisected and adult ticks were quadrisected using a sterile scalpel blade, following recent work by our group (Ammazzalorso *et al.* 2015). An additional 30 adult females were dissected to remove midguts and salivary glands. Organs were then combined into three pools of 10 per organ type. DNA isolation was performed using the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's protocol, with the addition of an overnight incubation at 56 °C in ATL buffer and proteinase K, and a final elution in 100 μL of ddH_2O after a 5 min, room temperature (18–22 °C) incubation step.

16S rRNA PCR amplification

PCR was conducted for the hypervariable regions V3 and V4 of the bacterial 16S rRNA gene using primers Bakt_341F and Bakt_805R, following Illumina's 16S Metagenomic Sequencing Library Preparation protocol (Illumina) and a review by Klindworth *et al.* (2013). This primer set, resulting in a 450- to 550-bp PCR product, was determined to be the best candidate among 512 sets tested in terms of domain and phylum coverage for use in 16S rRNA bacterial sequencing (Klindworth *et al.* 2013). Illumina adaptor sequences were attached as tails to the PCR primers. The PCRs were performed in triplicate with 25 μL Phusion High-Fidelity PCR Master Mix (Thermo Fisher Scientific), 2.5 μL (final concentration of 0.5 mM) for both primers, 15 μL nuclease-free PCR-grade water and 5 μL of DNA

template. Based on Illumina's protocol and the outcomes of preliminary PCR optimization procedures, the thermal cycling conditions consisted of an initial denaturation at 98 °C for 3 min, followed by 30 cycles of 98 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, followed by a final extension for 5 min at 72 °C on a Techne Prime Elite Thermal Cycler (Bibby Scientific). The three PCR product replicates for each sample pool were combined before running 10 μL of each combined pooled product on a 1.5% w/v agarose gel at 120 V for 40 min. The ~460-bp bands were excised from the gel and purified using QIAquick Gel Extraction Kit (Qiagen), with elution in 40 μL of nuclease-free, PCR-grade ddH_2O.

Library preparation. Library preparation of the purified PCR products was performed using Illumina's Nextera XT Index Kit, providing unique dual indices for each sample, thus allowing all samples to be sequenced on the same Illumina MiSeq reagent cartridge and flow cell. Prior to indexing, a supplementary quantitative PCR (qPCR) was performed to determine the optimal number of cycles for the indexing reaction. A subset of the PCR products for all sample pools and the one negative were indexed using 25 μL KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems), 10 μL sdH_2O, 5 μL Nextera XT Index 1 Primer, 5 μL Nextera XT Index 2 Primer and 5 μL purified PCR product. This subset was run on an ABI 7300 Real-Time PCR System (Applied Biosystems, Inc.) with an initial denaturation of 95 °C for 3 min followed by 40 cycles of 95 °C for 10 s and 55 °C for 30 s, and a plate read after each cycle. The optimal cycle number for each sample was determined based on the number of cycles needed for the qPCR amplification curve to begin to peak.

Samples were indexed using 25 μL KAPA HiFi HotStart Ready Mix (Kapa Biosystems), 10 μL sdH_2O, 5 μL Nextera XT Index 1 Primer, 5 μL Nextera XT Index 2 Primer and 5 μL purified PCR product as suggested by Illumina. The thermal cycling conditions for the indexing PCR consisted of an initial denaturation at 95 °C for 3 min, followed by the optimal number of cycles (8) of 98 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s on a Techne Prime Elite Thermal Cycler. Indexed amplicons were purified with Agencourt AMPure XP beads (Beckman Coulter Genomics) following Illumina's protocol. The indexed and purified amplicons were quantified using a High Sensitivity dsDNA Assay on a QUBIT 2.0 Fluorometer (Life Technologies) with 3 μL of gDNA. The size integrity of the amplicons was validated on a 2100 Bioanalyzer (Agilent Technologies). Equimolar amplicon quantities were pooled, denatured and combined with denatured 20% PhiX at a loading concentration of 5.5 pM. High-throughput amplicon sequencing was conducted on a MiSeq instrument (Illumina) using the v3 Reagent Kit 600-cycle format and 2 × 300 bp paired-end (300PE) reads at the Epigenomics Core Facility of Weill Cornell Medicine (New York, NY, USA).

Data analysis. The sequence processing workflow is depicted in Fig. S1 (Supporting information). Sequences were demultiplexed and converted to fastq files in CASAVA v1.8.2 FASTQ Filter (Illumina) before removing reads that did not pass the Illumina chastity filter using 'fastq_illumina_filter 0.1' (http://cancan.cshl.edu/labmembers/gordon/fastq_illumina_filter). The USEARCH pipeline was used to merge the filtered fastq files with a minimum overlap of 10 nucleotides and length between 250 and 500 bp (Edgar 2010). Merged fastq files were filtered using USEARCH to remove sequences with >0.5 predicted estimated errors per nucleotide (E_{max} = 0.5), as well as removing the first 50 nucleotides to trim indices and primer sequences (Edgar 2010). After dereplication, sequences were sorted according to abundance, operational taxonomic units (OTUs) were clustered de novo using UPARSE (Edgar 2013) with a minimum pairwise identity threshold of 97% (Stackebrandt & Goebel 1994; Huse *et al.* 2010; Kunin *et al.* 2010), and singletons were removed. The UPARSE pipeline includes de novo chimera removal, followed by referenced-based chimera removal using the UCHIME algorithm and the GOLD 16S rRNA gene database (Reddy *et al.* 2014). The final set of sequences was submitted to NCBI under SRA submission SUB1534507.

The OTU table was processed in QIIME (MACQIIME v1.9.0) (Caporaso *et al.* 2010b; Kuczynski *et al.* 2012). OTUs were assigned taxonomy using the RDP CLASSIFIER (Wang *et al.* 2007) against the Greengenes 16S rRNA database v13_8 (McDonald *et al.* 2012; Werner *et al.* 2012), and relative OTU abundances were summarized across taxonomic levels from phylum to genus. Representative sequences were aligned and filtered using PYNAST (Caporaso *et al.* 2010a), and a phylogenetic tree was estimated from the alignment with FASTTREE (Price *et al.* 2009). OTU tables in BIOM format were accessioned to Dryad (doi:10.5061/dryad.mt6m0).

To test for sufficient sequencing coverage, the conditional uncovered probability was calculated for all samples with a confidence interval of 0.95. Multiple rarefactions were performed in QIIME with 100 iterations every 1000 sequences without replacement from a depth of 1000 to 100 000 sequences. Sufficient sequencing depth was determined based on rarefaction curves for observed number of OTUs. In this study, four measures of α-diversity were calculated including the observed number of OTUs, Shannon's diversity index, Simpson's

index and Faith's phylogenetic diversity (PD) measure. The observed number of OTUs is a species richness estimator, while PD takes into account phylogenetic information (Faith 1992). Both the Shannon's index and Simpson's index include abundance information, with Shannon's giving more weight per individual to rare OTUs than common ones, while Simpson's is more sensitive to changes in the dominant taxa (Hill *et al.* 2003). All four measures were calculated based on sequences rarefied at a depth of 100 000 among life stage categories and a depth of 200 000 for whole adult females, female midguts and salivary glands based on the lowest minimum sequence depth for each category. Due to small sample size of replicates, descriptive statistics are presented for comparisons of α-diversity measures. Boxplots were created in R v3.1.2 (R Core Team 2014).

β-Diversity was calculated using jackknifing (use of subsets of available data) at a rarefaction depth of 100 000 sequences for developmental stage categories and a depth of 200 000 for whole adult females and their organs in QIIME. The rarefied data were used to calculate phylogenetically informed weighted and unweighted UniFrac measures (Lozupone & Knight 2005). The *adonis* function, that performs a permutational multivariate analysis of variance (PERMANOVA), was used, with 999 permutations, to determine the per cent variation explained by developmental stages, as well as the significance and effect size for both weighted and unweighted UniFrac distance matrices. Principal coordinate analysis (PCoA) was used to compare differences among groups of samples based on both UniFrac distance matrices.

After analysing the taxonomic summaries for all sample pools, the genus *Rickettsia* was found to be the most abundant. To gauge the identity and potential diversity of *Rickettsia* species and strains in blacklegged ticks, we carried out a phylogeny-informed assessment. First, we isolated 4 315 478 sequences matching the genus *Rickettsia* using the 'filter_fasta.py' script in QIIME and collapsed them into 91 519 unique sequences using FASTX-TOOLKIT (Gordon 2011). We aligned those unique sequences to an alignment of 241 full-length, reference 16S rRNA *Rickettsia* sequences retrieved from GenBank, while accounting for their phylogenetic relationships. This was achieved by aligning the 241 reference sequences in MAFFT (Katoh & Standley 2013) with default parameters for closely related nucleotide sequences. Then, a maximum-likelihood (ML) phylogenetic tree was estimated in RAXML v8.2.8 (Stamatakis 2014) using the general time-reversible nucleotide substitution model and among-site rate heterogeneity modelled by the Γ distribution and four discrete rate categories (Yang 1994). The final alignment employed the reference alignment and tree along with the shorter sequences and was carried out in PAPARA v2.5 (Berger & Stamatakis 2011). The reference ML tree and the large alignment of 91 760 sequences were used to place the shorter sequences onto internal, that is internode, or external, that is terminal, branches on the reference ML tree. This was carried out using the evolutionary placement analysis (EPA) procedure in RAXML. In an effort to reach a good trade-off between speed and accuracy, we restricted the number of candidate branches for placement to 10% of the total 479 branches using a heuristic. The phylogenetic placements were visualized on the iToL webserver (Letunic & Bork 2016) (http://itol.embl.de). The PAPARA alignment and the JPLACE tree file were accessioned to Dryad (doi: 10.5061/dryad.mt6m0).

Results

A total of 21 samples – three pools for each of the five life stage categories (lab-reared larvae, field-collected larvae, field-collected nymphs, field-collected adult females, and field-collected adult males), and three pools for female salivary glands and female midguts – generated 10 896 297 raw reads on one Illumina MiSeq flow cell. A total of 6 908 532 sequences passed filter and were assigned taxonomy. This resulted in 116 941–452 654 (mean = 328 977, SD = 96 960) high-quality, merged sequences per sample (Table S1, Supporting information). The negative control resulted in <0.001% (n = 606) of all sequences and was excluded from further analysis. The conditional uncovered probability of the samples, on average, indicated a high likelihood that over 99.9% of the bacterial taxa in the samples was covered. Rarefaction curves from a depth of 1000 to 100 000 sequences indicated sufficient sequencing coverage, as demonstrated by observed OTU accumulation curves reaching a plateau (Fig. S2, Supporting information).

Developmental stages

In all, 1652 distinct OTUs were clustered across the five developmental stage classifications. Lab-reared larvae had the lowest α-diversity across measures, followed by adult females (Table S2, Supporting information, Fig. 1). Field-collected larvae, nymphs and males exhibited similar levels of α-diversity, but males contained the richest bacterial communities when abundance was taken into consideration (Table S2, Supporting information, Fig. 1). OTUs were assigned to 332 taxa (Table S3, Supporting information).

Proteobacteria represented the phylum with the highest relative abundances (90–99.7%) across all developmental stage sample pools. The remaining OTUs were

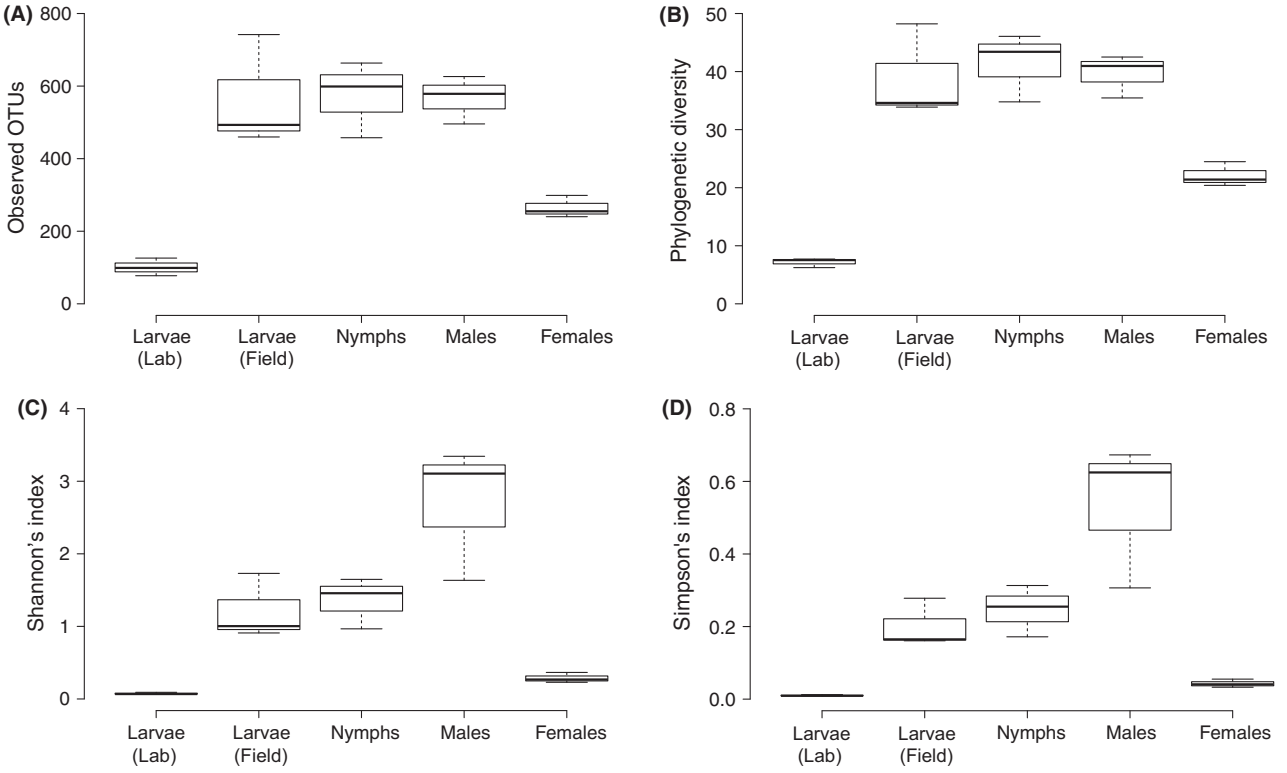

Fig. 1 Measures of α-diversity for each development stage. Each α-diversity measure (A), observed number of OTUs, (B) Faith's phylogenetic diversity, (C) Shannon's index and (D) Simpson's index were calculated at a rarefaction depth of 100 000 sequences.

classified into the phyla Acidobacteria, Actinobacteria, Armatimonadetes, Bacteroidetes, Chlamydiae, Chloroflexi, Cyanobacteria, Firmicutes, Gemmatimonadetes, Planctomycetes, Spirochaetae, Verrucomicrobia, the candidate phyla BRC1, FBP, GN02, OD1, TM6, TM7, WPS-2 and Thermi, or were unassigned at the phylum level.

At the genus level, *Rickettsia* had the highest relative abundance (55–99.5%) across all developmental stage samples. In all, 13 genera (and one family-level taxon) were present with a relative abundance of 1% or greater in at least one sample (Fig. 2). The relative abundance of non-*Rickettsia* taxa increased along developmental stages (i.e. larvae to nymphs to adults) with the exception of adult females, which displayed the highest relative abundance of *Rickettsia* of all field-collected life stages (Fig. 2). Freshly hatched larvae also had a high relative abundance of *Rickettsia* (Fig. 2). Seven taxa were found in all female and all freshly hatched larvae samples (matching to the genera *Rickettsia*, *Phyllobacterium*, *Burkholderia*, *Sphingomonas*, *Pseudomonas* and the families Comamonadaceae and Oxalobacteraceae); however, females harboured more observed OTUs than freshly hatched larvae (Fig. 1). Adult males had the lowest proportion of *Rickettsia* (55–83%) and thus had more genera with a relative abundance of ≥1% of any developmental stage. *Wolbachia* was the genus with the second highest relative abundance in males (mean = 8.4%) and was found in all other life stages, but not in all samples, with a low relative abundance (<0.1%). All field-collected developmental stages contained *Sphingomonas*, *Methylobacterium*, *Hymenobacter*, *Pseudomonas*, *Rhizobium*, *Agrobacterium*, *Luteibacter*, *Mycobacterium* and *Curtobacterium*. Both *Borrelia* and *Anaplasma* were only found in nymphs and adults.

Bacterial communities across the developmental stages were significantly different according to a PERMANOVA (unweighted $F = 4.16$, $R^2 = 0.62$, $P = 0.001$, weighted $F = 9.33$, $R^2 = 0.79$, $P = 0.001$). The unweighted UniFrac (which does not account for abundance data) PCoA explained 41.78% (Axis 1) and 12.03% (Axis 2) of the variation among developmental stages and showed clustering separation for lab-raised larvae and females (Fig. 3A). The weighted UniFrac PCoA explained 82.36% (Axis 1) and 9.55% (Axis 2) of the variation, with two male samples clustering together (Fig. 3B).

We placed 91 519 sequences on a phylogenetic tree composed of 241 full-length 16S rRNA reference sequences using the EPA ML procedure. The vast majority (i.e. >89 000) of our sequences were placed on the *Ixodes* tick endosymbiont clade, and its component subclades composed of rickettsial endosymbionts

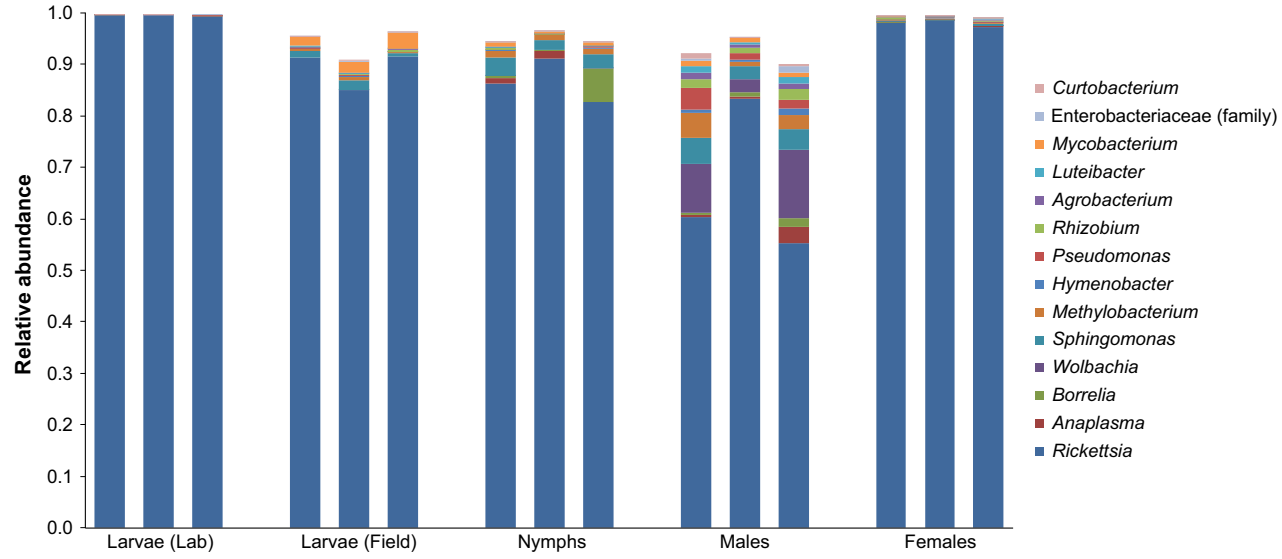

Fig. 2 Relative abundance of genera occurring in whole ticks pooled by developmental stage. Only taxa with a relative abundance ≥1% in at least one sample were considered. Taxonomic classification includes genus when possible. *Rickettsia* represents the most abundant genus for all samples, but decreased in relative abundance from larvae to nymphs to adult males. The notable exception is adult females with a high relative abundance of *Rickettsia*. [Colour figure can be viewed at wileyonlinelibrary.com]

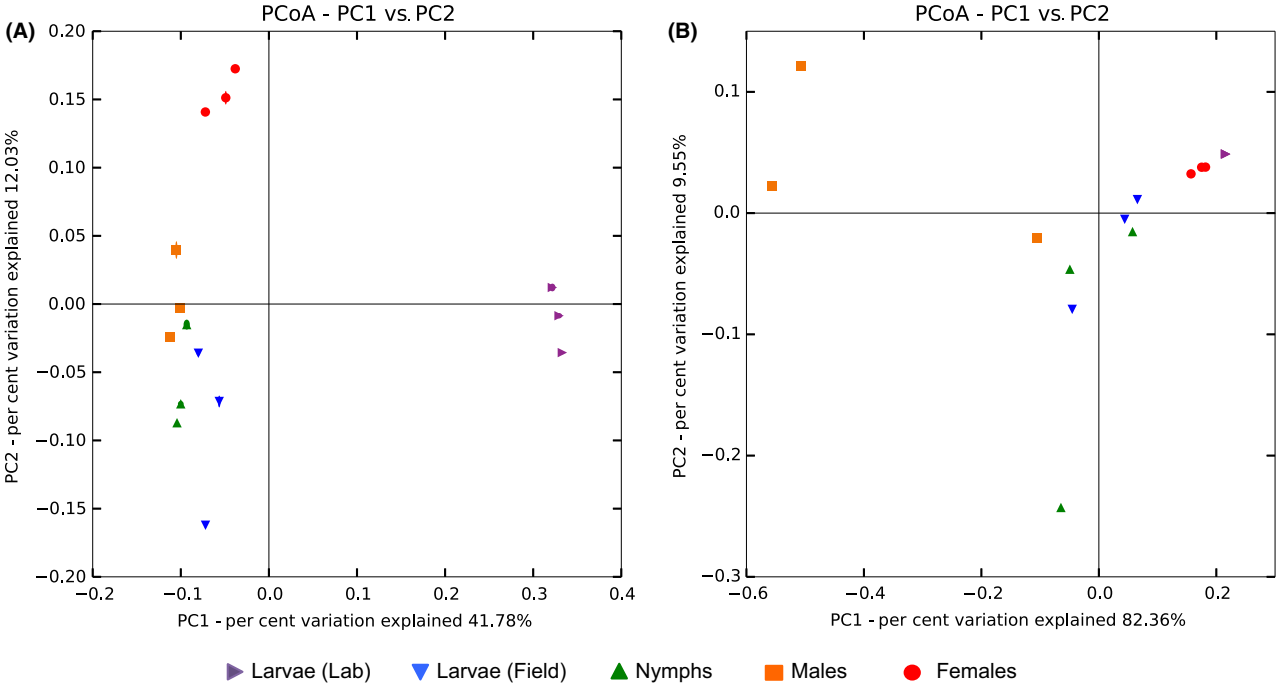

Fig. 3 Principal coordinate analysis of β-diversity measures for all developmental stages. A sequence depth of 100 000 was used. (A) Unweighted Unifrac distances, abundance is ignored; (B) weighted Unifrac distances, abundance is considered. Confidence ellipsoids around samples display degree of variation.

isolated from *Ixodes scapularis* ticks, their relatives in the western U.S.A. *I. pacificus* and the European tick *I. ricinus*. Fewer sequences in the order of a few hundred were placed on other clades related to *Ixodes* ticks, for example *Rickettsia monacensis* (Simser *et al.* 2002), non-*Ixodes* ticks, that is *Rickettsia sibirica* found primarily in

Dermacentor and *Haemaphysalis* ticks (Piesman & Gage 2004), *Rickettsia amblyommii* isolated from the American lone star tick *Amblyomma americanum* (Stromdahl *et al.* 2008), *Rickettsia conorii* and *Rickettsia rhipicephali* isolated from the brown dog tick *Rhipicephalus sanguineus* (Hayes & Burgdorfer 1979; Parola *et al.* 2009). Overall, short sequences could not be unambiguously placed on single branches, but were rather assigned along a clade, which lends a certain degree of confidence. Global placement uncertainty was elevated, as captured by the EPA's entropy measure (mean = 0.7767, SD = 0.0699). The placement of our sequences on the reference tree can be explored interactively on the iToL webserver (http://itol.embl.de/tree/28623420119269146883420 0).

Whole female adult ticks, salivary glands and midguts

A total of 392 OTUs was found across whole females, female salivary glands and female midguts. At a rarefaction depth of 200 000 sequences, whole females exhibited the highest α-diversity for observed number of OTUs and phylogenetic diversity, with female midguts exhibiting the lowest measures (Fig. 4). However, female salivary glands exhibited the highest α-diversity out of all three categories for both Shannon's and Simpson's indices (Fig. 4).

Rickettsia was the most abundant genus found in whole females and their organs. Other than that, no bacterial genera were found with a relative abundance ≥3%, although 47 taxonomic classifications – genus being the lowest – were present. In salivary glands, the predominant genera after *Rickettsia* were *Anaplasma*, *Borrelia* and *Wolbachia*, with average relative abundances of 12.5%, 7.2% and 1.9%, respectively. *Anaplasma* and *Borrelia* were also the most prominent genera found in midgut tissue, after *Rickettsia*, but at low average relative abundance (0.3% and 0.8%, respectively; Fig. S3, Supporting information).

Whole females and female organs differed significantly for both unweighted ($F = 5.044$, $R^2 = 0.63$, $P = 0.01$) and weighted UniFrac measures ($F = 2.04$, $R^2 = 0.41$, $P = 0.001$) according to a PERMANOVA. Distinct clustering was observed for each organ and the whole tick when abundance data were not considered (unweighted UniFrac PCoA) explaining 39.53% (Axis 1) and 22.35% (Axis 2) of the variation (Fig. 5A). Clustering of whole females and midguts away from salivary gland samples was observed when abundance

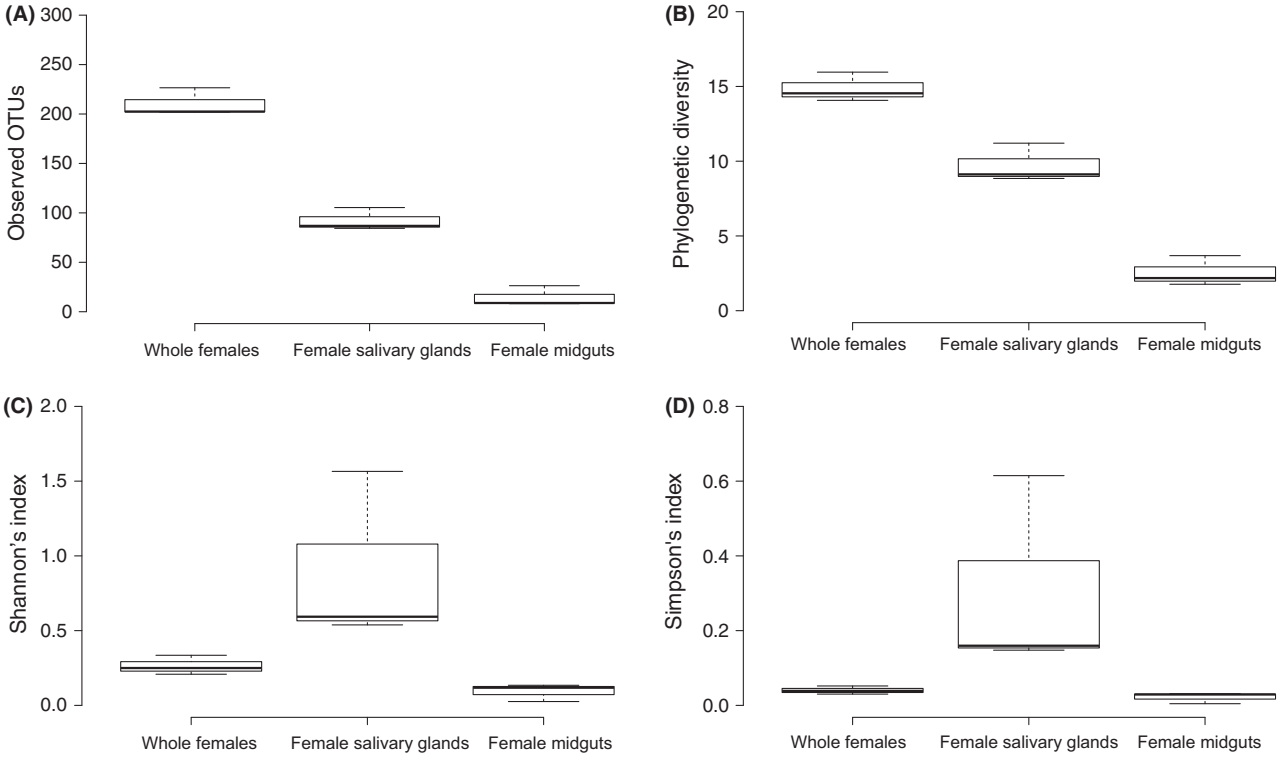

Fig. 4 Measures of α-diversity for whole female ticks, midguts and salivary glands. Each α-diversity measure (A) observed number of OTUs, (B) Faith's phylogenetic diversity, (C) Shannon's index and (D) Simpson's index were calculated at a rarefaction depth of 200 000 sequences.

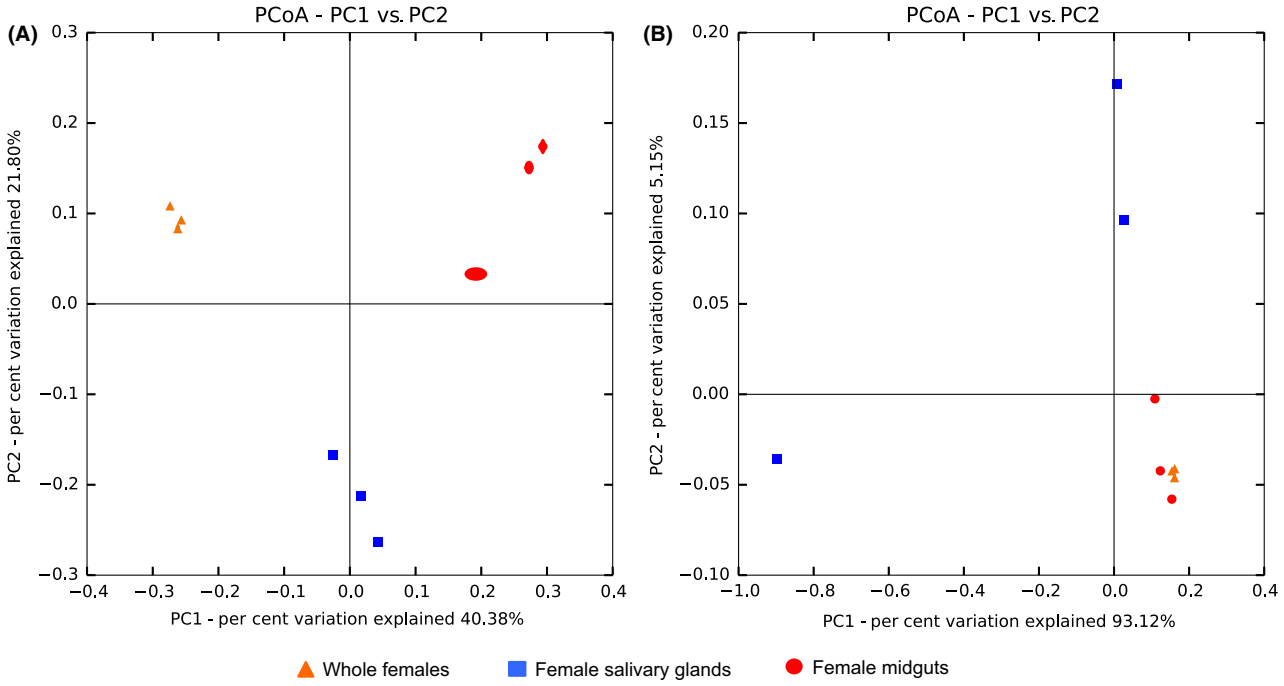

Fig. 5 Principal coordinate analysis of β-diversity measures in females. Measures were rarefied at a depth of 200 000 sequences. (A) Unweighted Unifrac distances, abundance is ignored. (B) Weighted Unifrac distances, abundance is considered. Confidence ellipsoids around samples display degree of variation. [Colour figure can be viewed at wileyonlinelibrary.com]

information was considered in a weighted UniFrac PCoA, which explained 93.1% (Axis 1) and 5.14% (Axis 2) of the variation (Fig. 5B).

Discussion

This study is the first to examine the bacterial microbiome across all developmental stages of the blacklegged tick, the most important disease vector in the United States. This is also the first study of tick microbiomes, to our knowledge, to target both V3 and V4 hypervariable regions of the 16S rRNA gene with longer 300 × 300 bp paired-end reads, thus enhancing our ability to establish OTUs and their taxonomic identity. Many of the previous studies on tick microbiome have used Roche's 454 pyrosequencing technology (Yuan 2010; Hawlena *et al.* 2013; Budachetri *et al.* 2014; Narasimhan *et al.* 2014; Ponnusamy *et al.* 2014; Qiu *et al.* 2014; Rynkiewicz *et al.* 2015) that has a lower read throughput yield (most studies produced 2500–8000 sequences/sample), very likely limiting the ability to sequence rare bacterial taxa and reducing the rarefaction level for diversity analyses.

Our results support previous findings of Proteobacteria being the most abundant phylum in tick species (Yuan 2010; Hawlena *et al.* 2013; Narasimhan *et al.* 2014; Rynkiewicz *et al.* 2015; Van Treuren *et al.* 2015), with *Rickettsia* occurring in the highest proportion in blacklegged ticks (Moreno *et al.* 2006; Yuan 2010; Hawlena *et al.* 2013; Rynkiewicz *et al.* 2015; Van Treuren *et al.* 2015). *Rickettsia* are obligate, intracellular bacteria associated with eukaryotes, including arthropods (Perlman *et al.* 2006). Although many *Rickettsia* species are nonpathogenic (Ahantarig *et al.* 2013), a number of them are known tick-borne pathogens, including *Rickettsia rickettsii*, *R. conorii*, *R. sibirica*, *R. japonica*, *R. australis* and *R. akari* (Azad & Beard 1998). The *Rickettsia* endosymbiont of *I. scapularis*, presumed to be nonpathogenic, has been identified in this tick in previous studies (Noda *et al.* 1997; Benson *et al.* 2004; Moreno *et al.* 2006; Gillespie *et al.* 2012; Kurtti *et al.* 2015) and is believed to be the only *Rickettsia* species found in this tick (Gillespie *et al.* 2012; Kurtti *et al.* 2015). Recently, the name *Rickettsia buchneri* has been proposed for this species (Kurtti *et al.* 2015). The vast majority of our sequences were assigned to the rickettsial clade isolated from *Ixodes* ticks using a phylogenetic tree-guided procedure. Although individual short sequences could not be assigned to single tree branches with full confidence, they were mostly assigned to neighbouring branches within a clade of sister endosymbionts isolated from the same tick species. We interpret this as an indication of sufficient evolutionary informativeness for the identification of our sequences at the clade level *grosso modo*, combined with the observation of greater divergence patterns of this locus in *Rickettsia*.

We found similar α-diversity for field-collected larvae and nymphs, compared to freshly hatched larvae, for all α-diversity measures (Fig. 1). This implies that exposure to the natural environment has a strong effect on the microbiome, as the lab-raised larvae with no exposure to the tick's natural habitat contained a much less diverse bacterial community. More interestingly, nymphs that have taken a single bloodmeal were found to harbour bacterial communities with diversities similar to those of field-collected larvae, a life stage that has not yet fed on a host. This suggests that host bloodmeal has limited influence on the overall diversity of the bacterial community within these ticks, an interesting finding, as host bloodmeals are the only known source of pathogens transmitted to ticks. A recent study by Rynkiewicz *et al.* (2015) found a significant difference between the bacterial communities of *I. scapularis* and *D. variabilis* and their shared host bloodmeal source. Further investigation of the influence of host bloodmeal source on tick bacterial communities is warranted, given the role that reservoir hosts play in maintaining and transmitting tick-borne pathogens to feeding ticks.

The increase in the observed number of OTUs from lab larvae to field larvae, nymphs and adult males, as well as the lower number of observed OTUs in adult females, is concomitant with the relative abundance of *Rickettsia* species throughout the life cycle. As *Rickettsia* represented over 99% of the taxa in lab larvae and steadily decreased to an average of 66% in adult males throughout development, it seems likely that other bacteria replace *Rickettsia*. However, despite similar environmental exposure, including the time off-host and the number and types of host bloodmeals, host-seeking adult male and female blacklegged ticks exhibited markedly divergent bacterial communities from one another. Female ticks harboured more homogeneous bacterial communities than males (Fig. 2). A less diverse microbiome in females has also been recently documented in both the blacklegged tick (Van Treuren *et al.* 2015) and the lone star tick, *Amblyomma americanum* (Ponnusamy *et al.* 2014). An earlier study identified *Rickettsia* in only half of their *I. scapularis* nymphs examined ($n = 10$) compared to all of the larval samples ($n = 10$) and concluded that the decrease in *Rickettsia* between the larval and nymphal stages was due to the possibility that male ticks lose *Rickettsia* species through each subsequent moult (Noda *et al.* 1997). This was supported by the lack of *Rickettsia* in the testes of adult males and its presence in adult female ovaries (Noda *et al.* 1997). However, although we found relatively less *Rickettsia* in males compared to females, our study indicates that *Rickettsia* sp. are found in whole *I. scapularis* adults of both sexes, a finding also recently reported (Van Treuren *et al.* 2015). If all external abiotic and biotic interactions are the same between sexes of the adult stage, it is plausible that internal factors such as hormonal mechanisms leading to sexual maturation and dimorphism in the adult stage may be driving these sex-specific differences in bacterial community diversity.

Although a recent study by Narasimhan *et al.* (2014) explored the microbiome of two different cohorts of larvae hatched and reared in the lab (reared in sterile conditions or so-called 'normal' containers), our study is the first to compare lab-hatched to field-collected larvae. The decrease in bacterial α-diversity observed in adult females was also observed in freshly hatched, lab-raised larvae. These larvae, having no exposure to the field, presumably contain only bacteria that were transovarially transmitted. The high relative abundance of *Rickettsia* observed in females (mean = 97.8%) was observed at an even higher relative abundance in the lab-raised larvae (99.5%), potentially due to certain bacteria found in females that are not transferred to their offspring. Transovarial transmission of *Rickettsia* has been detected in a number of tick species (Macaluso *et al.* 2001, 2002; Horta *et al.* 2006; Perlman *et al.* 2006), including *I. scapularis* (Narasimhan *et al.* 2014), which our results support. It is possible that environmental bacteria present on the tick's cuticle persisted into the DNA isolation procedure, despite sterilization with ethanol. However, this is unlikely, due to the large differences in *Rickettsia* present in lab-raised larvae compared to field-collected larvae. Furthermore, if bacteria did persist on the tick cuticle following ethanol washes, this would have been observed across all tick developmental stages, including adult females, which was manifestly not the case.

In addition to *Rickettsia*, 12 bacterial genera and one family-level taxon were found in at least 1% of field-collected developmental stages (Fig. 2). *Borrelia* and *Anaplasma* species, for example, were found in nymphs and adults. Within blacklegged ticks, known human tick-borne pathogens *B. burgdorferi* and *A. phagocytophilum* are not transovarially transmitted (Hodzic *et al.* 1998; Rollend *et al.* 2013), nor are they believed to be acquired in any manner other than via bloodmeals. *Borrelia miyomotoi* can be vertically transmitted (Barbour *et al.* 2009; Rollend *et al.* 2013); however, the prevalence of this pathogen tends to be low in natural populations (Barbour *et al.* 2009; Tokarz *et al.* 2010), which may be why *Borrelia* species were not detected in our field-caught larvae. In male *I. scapularis*, the average relative abundance of *Wolbachia* was 8.4%, but remained very low (<0.1%) in all earlier developmental stages and adult females. *Wolbachia* is an invertebrate-associated genus of Rickettsiaceae endosymbionts that has radiated into various supergroups (Noda *et al.* 1997). Many supergroup

members have been shown to cause reproductive manipulation in various arthropods (Perlman *et al.* 2006), while others engage in mutualistic relationships with their hosts, such as *w*Cle of supergroup F, an obligate nutritional mutualist in the hematophagous bed bug *Cimex lectularius* (Hosokawa *et al.* 2010; Nikoh *et al.* 2014). The influence that *Wolbachia* has on tick species, if any, is poorly understood. Other bacterial taxa (>1% relative abundance) found in at least one sample were taxa that have species that are found in water and soil habitats (Conn 1948; Janssen 2006). It is likely that field-collected ticks acquired these bacteria in their host-seeking environment, where they spend the majority of their life cycle, potentially via spiracle, oral or genital openings (adults only), as opposed to during a bloodmeal. Variations in tick microbiome across different field locations or geographic regions may exist owing to differences in environmental bacteria. Furthermore, the distinct clustering of adult females and freshly hatched larvae in the unweighted UniFrac PCoA is likely due to the lower number of OTUs present in these life stages (Fig. 3A), while the more divergent cluster of males in the weighted UniFrac PCoA is most likely due to the higher relative abundance of non-*Rickettsia* genera compared to the other life stages (Fig. 3B).

Both α- and β-diversity measures differed between whole females, female salivary glands and female midguts. Both organs displayed lower α-diversity than the entire female for measures of observed number of OTUs and phylogenetic diversity (Fig. 4), while female salivary glands displayed a higher α-diversity than midguts or even the entire tick for Shannon's and Simpson's index (Fig. 4). This is due to these latter measures taking evenness into account and the increased relative abundance of *Borrelia* and *Anaplasma,* in the salivary gland samples. Furthermore, the unique clustering displayed by all three sample types (Fig. 5A) suggests that the differences in taxa for each of these samples represent unique signatures. When abundance was taken into account with the weighted UniFrac distances (Fig. 5B), the midguts and whole female ticks tended to cluster together, away from the more divergent salivary gland samples, presumably due to the >98% relative abundance of *Rickettsia* species in these two sample types. The higher average relative abundance of *Anaplasma* (12.5%) and *Borrelia* (7.2%) found in the salivary glands compared to the midguts (<1% average relative abundance for both bacterial genera) may be due to differences in tissue tropism of known pathogens within these two genera. *B. burgdorferi* spirochetes are localized in the midgut (Piesman *et al.* 2001) and then migrate to the salivary glands during tick blood feeding (Spielman *et al.* 1987; De Silva & Fikrig 1995), while *A. phagocytophilum* resides in the salivary glands (Telford *et al.* 1996) and then replicates during tick blood feeding (Liu *et al.* 2011).

Overall, this study successfully identified bacterial genera and assessed bacterial diversity across all developmental stages of the blacklegged tick. We used pools of ticks to examine large numbers of ticks for each developmental stage, while maintaining a high sequencing depth for each group. Although pooling may obscure variations between individual ticks, it remains a solid strategy against PCR biases.

Our results indicate that much of the bacterial diversity observed in these ticks is not acquired via a bloodmeal, but transmission from female to offspring (*Rickettsia*), as well as environmental acquisition, plays important roles in shaping the microbial community within *I. scapularis.* Further studies exploring the bacterial composition in nymphal and adult stages are warranted to understand when the decrease in bacterial diversity observed in adult females occurs. Given the hormonal changes that accompany female maturation and subsequent egg production (Rees 2004), it is conceivable that such changes alter the internal environment enough to significantly restrict the growth and/or survival of a large segment of the microbial community.

Given the overwhelming abundance of *Rickettsia* across life stages and organs, we attempted to dissect its subgeneric diversity using phylogenetic criteria and found the vast majority of the sequences to cluster with *bona fide Ixodes* tick rickettsial endosymbionts. It remains unknown whether this is indicative of a genomewide pattern of conservation due to a bottleneck or a massive selective sweep accompanying symbiosis, or a mere localized snapshot of sequence conservation at this locus. *I. scapularis* is known so far to harbour one endosymbiotic rickettsial species (Gillespie *et al.* 2012; Kurtti *et al.* 2015), but finer taxonomic resolution using more informative genetic markers at the subgeneric and strain levels may provide additional insight into population-level variation and adaptation to intracellular life in coexistence with pathogens. Our study focused on an area with one of the highest pathogen prevalence levels in blacklegged ticks. Future studies examining the functional interplay between tick microbiomes and pathogens over a larger geographic region are warranted.

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Data accessibility

DNA sequences: NCBI SRA: SUB1534507

BIOM OTU tables, *Rickettsia* PAPARA alignment, *Rickettsia* JPLACE tree file: Dryad doi: 10.5061/dryad.mt6m0

Supporting information

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

Fig. S1 Bioinformatics workflow.

Fig. S2 Rarefaction curves based on the observed number of OTUs.

Fig. S3 Average relative abundance of genera occurring in whole female ticks and associated organs.

Table S1 Sequence read throughput per sample and life stage.

Table S2 Measures of α-diversity for each developmental stage pool.

Table S3 OTU table with taxonomy assignment.