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Endosymbiont interference and microbial diversity of the Pacific coast tick, *Dermacentor occidentalis*, in San Diego County, California

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The Pacific coast tick, *Dermacentor occidentalis* Marx, is found throughout California and can harbor agents that cause human diseases such as anaplasmosis, ehrlichiosis, tularemia, Rocky Mountain spotted fever and rickettsiosis 364D.Previous studies have demonstrated that nonpathogenic endosymbiotic bacteria can interfere with *Rickettsia* co-infections in other tick species.We hypothesized that within *D. occidentalis* ticks, interference may exist between different nonpathogenic endosymbiotic or nonendosymbiotic bacteria and Spotted Fever group Rickettsia (SFGR). Using PCR amplification and sequencing of the *romp*A gene and intergenic region we identified a cohort of SFGR-infected and non-infected *D. occidentalis* ticks collected from San Diego County. We then amplified a partial segment of the 16S rRNA gene and used next-generation sequencing to elucidate the microbiomes and levels of co-infection in the ticks. The SFGR *R. philipii* str. 364D and *R. rhipicephali* were detected in 2.3% and 8.2% of the ticks, respectively, via *romp*A sequencing. Interestingly, next generation sequencing revealed an inverse relationship between the number of *Francisella*-like endosymbiont (FLE) 16S rRNA sequences and *Rickettsia* 16S rRNA sequences within individual ticks that is consistent with partial interference between FLE and SFGR infecting ticks. After excluding the *Rickettsia* and FLE endosymbionts from the analysis, there was a small but significant difference in microbial community diversity and a pattern of geographic isolation by distance between collection locales. In addition, male ticks had a greater diversity of bacteria than female ticks and ticks that weren't infected with SFGR had similar microbiomes to canine skin microbiomes. Although experimental studies are required for confirmation, our findings are consistent with the hypothesis that FLEs and, to a lesser extent, other bacteria, interfere with the ability of *D. occidentalis* to be infected with certain SFGR. The results also raise interesting possibilities about the effects of putative vertebrate hosts on the tick microbiome.

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Abstract

The Pacific coast tick, *Dermacentor occidentalis* Marx, is found throughout California and can harbor agents that cause human diseases such as anaplasmosis, ehrlichiosis, tularemia, Rocky Mountain spotted fever and rickettsiosis 364D. Previous studies have demonstrated that nonpathogenic endosymbiotic bacteria can interfere with *Rickettsia* co-infections in other tick species. We hypothesized that within *D. occidentalis* ticks, interference may exist between different nonpathogenic endosymbiotic or nonendosymbiotic bacteria and Spotted Fever group Rickettsia (SFGR). Using PCR amplification and sequencing of the *romp*A gene and intergenic region we identified a cohort of SFGR-infected and non-infected *D. occidentalis* ticks collected from San Diego County. We then amplified a partial segment of the 16S rRNA gene and used next-generation sequencing to elucidate the microbiomes and levels of co-infection in the ticks. The SFGR *R. philipii* str. 364D and *R. rhipicephali* were detected in 2.3% and 8.2% of the ticks, respectively, via *romp*A sequencing. Interestingly, next generation sequencing revealed an inverse relationship between the number of *Francisella*-like endosymbiont (FLE) 16S rRNA sequences and *Rickettsia* 16S rRNA sequences within individual ticks that is consistent with partial interference between FLE and SFGR infecting ticks. After excluding the *Rickettsia* and FLE endosymbionts from the analysis, there was a small but significant difference in microbial community diversity and a pattern of geographic isolation by distance between collection locales. In addition, male ticks had a greater diversity of bacteria than female ticks and ticks that weren't infected with SFGR had similar microbiomes to canine skin microbiomes. Although experimental studies are required for confirmation, our findings are consistent with the hypothesis that FLEs and, to a lesser extent, other bacteria, interfere with the ability of *D.*

occidentalis to be infected with certain SFGR. The results also raise interesting possibilities about the effects of putative vertebrate hosts on the tick microbiome.

Introduction

The Pacific Coast tick, *Dermacentor occidentalis* Marx (henceforth *D. occidentalis*) is the most widely distributed tick in California and is found in chaparral and shrubland areas from northern Baja California, through to California and Oregon (Furman & Loomis, 1984). It is a three-host, hard-shell tick that feeds on a variety of vertebrates such as rodents, rabbits, cattle, deer, horses and humans. Surveys of this tick have uncovered human pathogens such as *Francisella tularensis* (tularemia), *Coxiella burnetii* (Q fever), *Anaplasma phagocytophilum* (human granulocytic anaplasmosis), *Ehrlichia chaffeensis* (human monocytic ehrlichiosis), *Rickettsia rickettsii* (Rocky Mountain spotted fever, RMSF) and *Rickettsia philipii* 364D (hereafter *R. philipii*) as well as the non-pathogenic spotted fever group *Rickettsia, R. rhipicephali* (Parker & Brooks, 1929; Cox, 1940; Lane, Emmons, Dondero & Nelson, 1981; Holden, Boothby & Anand, 2003; Wikswo et al., 2008; Shapiro et al., 2010). *Rickettsia philipii*, was originally described as an unclassified *Rickettsia* found by Bell in *D. occidentalis* from California (Philip et al., 1978). It is closely related to *Rickettsia rickettsii* but can be serologically and genetically distinguished (Philip, Lane & Casper, 1981; Karpathy, Dasch & Eremeeva, 2007). Although discovered in 1966, and long suspected of being able to cause disease, it was only recently confirmed to be associated with eschars and lymphadenopathy in people at the site of a tick bite (Lane et al., 1981; Shapiro et al., 2010; Johnston et al., 2013).

Francisella-like endosymbiotic bacteria (FLEs) have also been detected in *Dermacentor occidentalis* as well as other tick species (Burgdorfer, Brinton & Hughes, 1973; Noda, Munderloh & Kurtti, 1997; Scoles, 2004; Kugeler et al., 2005). FLEs share 16S rRNA gene homology with *Francisella* spp., are vertically transmitted, have been observed within tick ovaries and Malpighian tubules, and vary by tick species (Rounds et al., 2012). Although Burgdorfer *et al*. demonstrated pathogenicity of a *Francisella* endosymbiont derived from *Dermacentor andersoni* Stiles ticks (previously categorized as *Wolbachia persica,* Forsman, Sandström, & Sjöstedt, 1994) to guinea pigs and hamsters via injection, most FLEs are not transmitted by tick bites and are considered non-pathogenic (Burgdorfer, Brinton & Hughes, 1973; Niebylski et al., 1997).

Interestingly, the inability of different endosymbiotic *Rickettsia* species to co-infect the same organ in the same tick, called "interference," has been demonstrated, although the exact mechanisms are unknown. Early studies seeking to understand the epidemiology of RMSF in the Bitterroot Valley in Montana demonstrated that the non-pathogenic tick endosymbiont *Rickettsia peacockii* (found on the east side of the valley and originally called the East side agent) colonized the ovaries of *D. andersoni* ticks and excluded pathogenic *Rickettsia rickettsii* (more prevalent on the west side of the valley) from infecting the ovaries and being transmitted to eggs (Burgdorfer, Hayes, & Mavros, 1981). Similarly, studies of *Dermacentor variabilis* (Say) infected with *R. montanensis* or *R. rhipicephali* demonstrated resistance to transovarial transmission of the reciprocal *Rickettsia* in challenge experiments (Macaluso et al., 2002). Negative influences between co-infecting species of *Rickettsia* and other symbionts has been suggested in other vectors such as fleas as well (Azad & Beard, 1998, Jones et al., 2012).

Interference has been postulated to have significant effects in altering the distribution of *Rickettsia* pathogens in the environment, and, consequently, the presence of human disease (Burgdorfer, Hayes & Mavros, 1981).

The use of next generation sequencing has allowed further exploration into endosymbionts and complex bacterial communities that colonize different tick species (Nakao et al., 2013), their organs (Budachetri et al., 2014; Qiu et al., 2014), different life stages (Carpi et al., 2011) and different states of nutrition (Menchaca et al., 2013; Zhang et al., 2014). Attention to the microbiome of ticks was driven, in part, by the fact that ticks can transmit the broadest range of diseases, including new and emerging diseases, of any arthropod and the recognition that tick co-infections can have dramatic consequences on the tick host and human patient (Clay & Fuqua, 2010). Microbiome studies using next generation sequencing techniques have demonstrated that each species of tick harbors its own unique bacterial community often dominated by Proteobacteria and one or two endosymbionts (Clay & Fuqua, 2010; Ponnusamy et al., 2014; Hawlena et al., 2012; van Treuren et al., 2015; Narasimhan & Fikrig, 2015). Given these findings, we hypothesized that *Dermacentor occidentalis* ticks would demonstrate patterns of interference or exclusion within their microbiomes that would be associated with the carriage of pathogenic or non-pathogenic bacteria. We also hypothesized that differences between tick microbiomes might be associated with different geographic locations and that possible reservoirs of tick pathogens could be found by analyzing ticks for the host origin of prior blood meals and comparing the tick microbiomes to the skin microbiomes of potential host species. To answer these questions, we used culture-independent PCR amplification of the 16S rRNA gene and next-generation sequencing (NGS) to determine whether the microbiomes of SFGR-infected ticks

differed from non-SFGR-infected ticks and if this microbial diversity was consistent with a hypothesis of interference. Our results did indeed reveal patterns consistent with partial exclusion between SFGR and FLEs and an association of non-endosymbiotic bacteria with geographic locale. Furthermore, the historical blood meal hosts of the ticks were implicated by the composition of bacterial communities within the ticks and were correlated with SFGR infection. The results of this study suggest that the carriage of certain pathogenic SFGR in ticks could be modulated by other non-rickettsial endosymbionts and propose a non-chemical alternative to control SFGR in the environment to protect public health.

Materials & Methods

Sample Collection. Adult ticks were collected from February to May 2014 from 4 different areas of San Diego County: Escondido Creek, Los Peñasquitos Canyon, Lopez Canyon and Mission Trails Regional Park by dragging a 1 m^2 piece of canvas over grass and chaparral and then capturing the ticks with forceps and placing them in individual sterile microfuge tubes. The ticks were transported live back to the Vector Disease and Diagnostic Laboratory at the San Diego County Operations Center where, by visual examination, their species and sex were determined and cataloged before freezing them at -80 °C.

DNA Extraction, PCR Amplification and Next Generation Sequencing. Ticks were processed individually throughout all procedures. The ticks were thawed and washed sequentially in 3% hydrogen peroxide, 100% isopropanol, and sterile distilled water for 1 minute in each solution. The final distilled water wash was aspirated from the ticks and then the ticks were sectioned

sagittally at midline with a sterile scalpel. Half of the tick was saved at -80 °C; the other half was used for DNA extraction. Briefly, 180 µl of ATL buffer (Qiagen, Valencia, CA) and 20 µl of proteinase K were added to each tick and the ticks lysed overnight at 37 °C in an Eppendorf Thermomixer (Hauppauge, NY) with agitation at 1400 rpm for 15 s every 15 min, before centrifuging the lysate for 3 min at 18,400 x g. The supernatant was transferred into a sterile microfuge tube and DNA extracted using a Qiagen DNeasy Blood and Tissue kit in a Qiacube using the DNeasy Blood and Tissue protocol for Tissue and Rodent Tails (Qiagen, Valencia, CA). Negative extraction controls consisted of sterile water processed via the same washing, chopping and extraction procedure used on the ticks.

The ticks were screened for spotted fever group rickettsia using a Power SYBR Green *real-time* PCR Mastermix kit (Life Technologies, Carlsbad, CA) and primers for the *romp*A gene (Eremeeva et al., 2003). Reactions were carried out in a total volume of 20 µL composed of 10 µL Power SYBR Green Mastermix, 0.125 µL each of primers RR190.547F (20 µM) and RR190.701R (20 µM), 7.75 µL of nuclease-free water, and 2 µL of template DNA (Eremeeva et al., 2003; Wikswo et al., 2008). *Real-time* PCR cycling conditions were: 3 min at 95 °C; 40 cycles of: 20 s at 95 °C, 30 s at 57 °C, 30 s at 65 °C; a holding cycle of 5 min at 72 °C; and a continuous cycle of: 15 s at 95 °C, 1 min at 55 °C, 30 s at 95 °C, 10 s at 55 °C; and a final holding temperature of 4 °C.

DNA from ticks that screened positive for SFGR were subjected to semi-nested PCR amplification of *romp*A using primers Rr190-70, Rr190-701, and Rr190-602 and the intergenic

151 region (IGR) using primary and nested primers RR0155-*rpm*B (Eremeeva et al., 2006; Shapiro et
152 al., 2010; Wikswo et al., 2008). Briefly, 20 µL of 2X Taq Master Mix (Qiagen, Valencia, CA), 2
153 µL of forward primer Rr190-70 (20 mM), 2 µL of reverse primer Rr190-701/Rr190-602 (20
154 mM), 14 µL of nuclease-free H_2O, and 2 µL of DNA was amplified using PCR cycling
155 conditions of 95 °C for 3 min followed by 35 cycles of 95 °C for 20 s, 57 °C for 30 s, and 68 °C
156 for 2 min and then 72 °C for 5 min before holding the products at 4 °C. For the IGR PCR
157 amplification, 20 µL of 2X Taq Master Mix (Qiagen, Valencia, CA), 1 µL of forward primer RR
158 0155 PF (20 mM), 1 µL of reverse primer 0155 PR (20 mM), 16 µL of nuclease-free H_2O, and 2
159 µL of DNA was amplified using PCR cycling conditions of 95 °C for 5 minutes followed by 35
160 cycles of 95 °C for 30 s, 50 °C for 30 s, and 68 °C for 1 min and then 72 °C for 7 min before
161 holding the products at 4 °C.

162

163 Amplification products were visualized in a 1% agarose gel stained with ethidium bromide on a
164 UV illuminator and subsequently purified using the PureLink PCR Purification Kit, following
165 the manufacturer's protocol (Life Technologies, Carlsbad, CA). Products were sequenced using
166 the BigDye Terminator v3.1 Cycle Sequencing Kit and purified using the BigDye XTerminator
167 Purification Kit following the manufacturer's protocols on an AB 3500xL Genetic Analyzer
168 (Applied Biosystems, Grand Island, NY). Due to highly conserved 16S rRNA gene sequences
169 between *Francisellaceae*, DNA extracts of the ticks were also tested specifically for the presence
170 of *Francisella tularensis* using a multi-target *real-time* PCR test employing primers IS*Ftu*2, *iglC*
171 and *tul4* that are specific for *F. tularensis* as described in Kugeler et al., 2005 and Versage et al.,
172 2003. All reactions were performed in a final volume of 20 µl and contained LightCycler
173 FastStart DNA Master HybProbe mix (Roche, Mannheim, Germany) at a 1X final concentration,

500 nM forward and reverse primers, 100 nM probes, and 1.25 U of uracil-DNA glycosylase per reaction. For the iglC and tul4 the final MgCl2 concentration was 4 mM, and for the ISFtu2 assay, the final concentration was 5 mM. *Real-time* PCR cycling conditions were: 50 °C for 2 min; 95 °C for 10 min; 45 cycles of: 95 °C for 10 s, 60 °C for 30 s; and 45 °C for 5 min.

PCR amplification of the cytochrome b gene was used to query the DNA from the ticks for determining the hosts of their prior blood meals using the primers UNFOR403 and UNREV1025 (Kent & Norris, 2005; Lah et al., 2015). PCR reactions were conducted using 2X Taq PCR Master Mix (Qiagen, Valencia, CA) with primer concentrations at 0.2 µM, 8 µL of template per reaction and a total reaction volume of 40 µL. PCR cycling conditions were: denaturation at 94 °C for 3 min followed by 35 cycles of 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min; then final extension at 72 °C for 7 min before holding the PCR products at 4 °C.

For the bacterial community analysis, a segment of the conserved bacterial 16S rRNA gene was amplified from the individual tick DNA extractions using universal primers 515F and 806R that flank the V4 region (Caporaso et al., 2012). The 806R primers also contained a unique 12-nucleotide Golay ‘‘barcode’’ for each sample that allowed us to pool the PCR products from all the samples into one Illumina MiSeq sequencing run but then to identify sequences derived from each individual tick. PCR reactions were conducted in a total volume of 40 µL using Taq98® Hot Start 2X Master Mix (Lucigen, Middleton WI) with primer concentrations at 0.2 µM. PCR cycling conditions were: denaturation at 98 °C for 2 min followed by 35 cycles of 98 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; then final extension at 72 °C for 10 min before

196 holding the PCR products at 4 °C. The PCR products were visualized under UV light on 1%
197 agarose gels stained with ethidium bromide before being normalized and sequenced on an
198 Illumina MiSeq instrument by The Scripps Research Institute DNA Array Core Facility using
199 their standard protocols (TSRI, San Diego, CA).

200

201 Computational and statistical analyses. The sequence data was analyzed using the QIIME
202 (Quantitative Insights Into Microbial Ecology) version 1.8.0 software program (Caporaso et al.,
203 2010b). Raw sequence data was demultiplexed into samples by barcode and filtered by mean
204 quality score below 25, homopolymers greater than 6, uncorrected barcodes, barcodes not found
205 in the mapping file, chimeric sequences and mismatched primers. Sequences were grouped into
206 operational taxonomic units (OTUs) at the 97% sequence similarity level using UCLUST
207 (Edgar, 2010) and a consensus taxonomic classification was assigned to each representative
208 OTU using the UCLUST classifier with a Greengenes 13_8 reference database (DeSantis et al.,
209 2006) in which at least 90% of the sequences within the OTU matched the consensus taxonomic
210 classification 16S rRNA gene. Sequences were aligned using PyNAST (Caporaso et al., 2010a)
211 against the Greengenes 13_8 reference core set and a phylogenetic tree of the OTUs inferred
212 using FastTree (Price, Dehal &Arkin, 2010). In order to remove spurious OTU's and samples
213 with low numbers of sequences, OTU's that occurred only once in the data and samples with less
214 than 150 OTUs were removed. *Rickettsia, Francisella* and other selected taxonomic sequence
215 identifications were crosschecked against the NCBI nucleotide database using BLASTn.
216 Sequence, OTU table and map files can be downloaded from Figshare:
217 10.6084/m9.figshare.2056275, 10.6084/m9.figshare.2068644, and
218 10.6084/m9.figshare.2056272, respectively.

219

220 The OTU dataset was rarefied to an even sampling depth of 150 and weighted and
221 unweighted UniFrac distance measures between all pairs of microbial communities were
222 calculated and visualized by principal coordinate analyses (PCoA) (Lozupone & Knight, 2005).
223 Rarefaction at 1500 even sampling depth resulted in similar results. Several analyses were
224 performed to determine possible factors related to microbiome differences observed within the
225 ticks and if interference between bacteria was observed. The Pearson product-moment
226 correlation coefficient (PPMC) was calculated using Social Science Statistics calculator to
227 determine if a statistically significant relationship existed between the number of sequences of
228 *Rickettsia* and *Francisella* found in the ticks and that could be consistent with the hypothesis of
229 interference (http://www.socscistatistics.com/tests/Default.aspx). To elucidate which bacteria in
230 the microbiome were associated with the presence of SFGR (as identified by *romp*A and IGR
231 sequences), Random Forest supervised learning was performed in QIIME using 1000 trees and
232 10 times cross validation. Whether non-*Rickettsia,* non-*Francisella* genera within the tick
233 microbiomes that were associated with high *Rickettsia* to *Francisella* ratios (>5), even *Rickettsia*
234 to *Francisella* ratios (0.2-5) and low *Rickettsia* to *Francisella* ratios (<5) were determined via a
235 Kruskal-Wallis H test. In order to assess whether physical separation of the sampling locations
236 was related to phylogenetic differences of the microbiomes at each location, microbiome
237 UniFrac distances were compared to the distances between sampling locations using the isolation
238 by distance (IBD) web service http://ibdws.sdsu.edu/~ibdws/distances.html (Jensen, Bohonak &
239 Kelley, 2005). IBD tests the linear relationship between geographic distance and genetic distance
240 of a population, or, in our case, geographic distance and the microbial community phylogenetic
241 distance. It uses a pairwise Mantel test to assess the relationship between location and UniFrac

phylogenetic distances. To determine which of the abundant genera were responsible for differences in UniFrac measures between locations, OTUs that occurred in less than 10% of the samples were removed and the null hypothesis that abundances of OTUs were the same for all locations was tested using a Kruskal-Wallis H test in QIIME. A Procrustes least squares orthogonal mapping analysis was performed in QIIME to determine if the *beta* diversity of *Rickettsia* and *Francisella* populations was similar to non-*Rickettsia* non-*Francisella* populations with respect to location (Gower, 1975). Procrustes analysis is a statistical scaling method that transforms multidimensional shape data, in this case, *beta* diversity matrices, into maximal superimposition (least squared distances) to determine the concordance between the matrices. Furthermore, Analysis of Similarity (ANOSIM), which compares the ranked Bray-Curtis similarity between and within groups, was used to determine whether microbial population *beta* diversity between locations differed significantly.

SourceTracker was used to compare the tick microbial profiles to microbiome datasets of dog, fish, iguana, human, pigeon, rat, and soil. SourceTracker is a tool that uses Bayesian methods to predict the source(s) of microbial communities in a set of samples (sink) (Knights et al., 2011). To test for sources of the tick microbiomes (sink), microbial source tracking was performed on the merged sink and source OTU file. In order to determine if tick infection with *R. philipii* or *R. rhipicephali* was associated with a particular host source, differences in the mean percentage similarity to these sources were compared between ticks positive for *R. philipii* or *R. rhipicephali* and those that were negative for these *Rickettsia* using a Student's *T*-test. SourceTracker was also used as a quality control measure to identify possible sample contamination. SourceTracker version 1.0 was implemented in QIIME (version 1.9.1) with default settings. As source datasets, we used publicly

265 available sequence data in QIITA (https://qiita.ucsd.edu/) that included 16S rRNA data from a wide range
266 of samples such as canine skin, mouth, and feces (Study ID 1684), human skin, mouth and stool (Study
267 ID 1684), soil (Study ID 1684, 10363), fish, frog, iguana, pigeon, and rat skin (Study ID 1748) and
268 negative water controls (Study ID 10363) as sources. All source and sink samples were sequenced using
269 Illumina and the same 16S rRNA V4 region primers.
270

271 **Results**

272

273 Four hundred seventy four adult *D. occidentalis* ticks were collected. No immature ticks
274 were caught. Although no ticks were positive for *Francisella tularensis*, 39 ticks (8.2%) were
275 positive for *R. rhipicephali* and 12 (2.3%) were positive for *R. philipii* 364D as identified by
276 sequencing of the *romp*A gene and IGR. No significant difference in infection rate between male
277 and female ticks by *R. rhipicephali* and *R. philipii* was observed (Fisher's exact test; P=0.47).
278 From this group, 114 ticks were selected for Illumina sequencing. Amplification and gel
279 electrophoresis of the V4 segment of the 16S rRNA gene produced visible PCR products of the
280 expected 300 bp size from all ticks, while negative PCR and DNA extraction controls yielded no
281 visible bands and were not sequenced. After quality filtering, 102 ticks remained: 44 positive for
282 SFGR (as identified by *romp*A and IGR sequencing) and 59 negative for SFGR (forty-five male
283 and fifty-seven female) from the four locations (Table 1); the total number of sequences was
284 6,799,927 with sample depths ranging from 2013 to 250403 reads (Supplemental table 1).
285 Clustering sequences at the 97% level of similarity and discarding OTUs that occurred only once
286 yielded 105,174 different OTUs and 535 different taxa including one unassigned taxon.

Rickettsia and *Francisella* genera were the most prevalent genera present in the ticks, representing 46.8% and 41.4%% of all genera, respectively. The next most frequently occurring genera were *Sphingomonas* (3%), *Methylobacterium* (1%) and *Hymenobacter* (0.4%) (Fig. 1).

One *Rickettsia* sp. (OTU 83718) accounted for 89% of all *Rickettsia* OTUs and matched 100% to *R. rhipicephali* (GenBank accessions CP013133.1, NR_074473.1, CP003342.1, NR_025921.1, and U11019.1). The next closest matches were to *Rickettsia* sp. Tenjiku01 (GenBank acc. LC089861.1) and several uncultured *Rickettsia* partial 16S rRNA gene sequences (GenBank accs. KF981787.1, KF981786.1) as well as other *Rickettsia* species *Rickettsia aeschlimannii* (GenBank acc. KT318741.1), *R. prowazekii* (GenBank acc. CP004888.1), *R. felis* (GenBank acc. NR_074483.1) and others. The second most abundant OTU (553807) accounted for 0.7% of all Rickettsia OTUs and matched most closely with several different *R. rickettsii* strains including *R. philipii* str. 364D (GenBank NR 074470.1) and other strains of *R. rickettsii* (including GenBank accs. CP006010.1, NR_102941.1, and CP003311.1). All other *Rickettsia* OTUs comprised less than 0.09% of total *Rickettsia* OTUs. OTU 840032 comprised 87.4% of all *Francisella* OTUs and matched 100% with *Francisella*-like endosymbiont (FLE) of *D. occidentalis* (GenBank accs. AY805304.1, and AY375402.1). The next closest matches were *Francisella* endosymbionts of other tick species *D. albipictus*, *D. andersoni* and *D. variabilis* (GenBank accs. GU968868.1, FJ468434.1, and AY805307.1, respectively). The next most abundant OTU (399541) (GenBank acc. KU355875.1, this paper) accounted for 3.1% of all *Francisella* OTUs and matched 97% with gene sequences of endosymbionts previously determined from a spectrum of *Dermacentor* species including *Dermacentor occidentalis (*AY375403.1*), D. albipictus (*GU968868.1), *D. variabilis*

310 *(*AY805307.1), *D. nitens (*AY375401.1) *and D. andersoni (*AY375398.1). All other *Francisella*
311 OTUs accounted for less than 0.4% of the total *Francisella* OTUs.
312
313 Female ticks had significantly less microbial diversity (alpha diversity) than male ticks as
314 measured by Faith's Phylogenetic Diversity which measures diversity based on phylogenetic tree
315 lengths (Faith's PD, two sample t-test; t=3.63, $P<0.01$; Fig. 2). Although there was no significant
316 difference between the mean number of *Rickettsia* and *Francisella* sequences in male versus
317 female ticks (Student's *t* test $P=0.36$, 0.06, respectively), *Rickettsia* and *Francisella*
318 endosymbionts comprised a greater percentage of the microbiome of female ticks than male ticks
319 74.9% and 60.1%, respectively (Student's *t* test $P=0.02$, Fig. 3).
320
321 Escondido Canyon had lower average alpha diversity than Lopez and Peñasquitos
322 canyons, $P=0.05$ (Fig. 4). Beta diversities of unweighted and weighted tick microbiomes had
323 small but statistically significant associations with location as measured by analysis of similarity
324 (ANOSIM) of UniFrac distances and visualized on Principal coordinate analysis (PCoA) (Figs.
325 5.A and 5.B). When only *Rickettsia* and *Francisella* were assessed for association with location,
326 ANOSIM results were not statistically significant (ANOSIM, unweighted UniFrac; R=-0.06,
327 $P=0.92$; ANOSIM, weighted UniFrac R=0.02, $P=0.13$). However, when *Rickettsia* and
328 *Francisella* were removed from the analysis of tick microbiomes, the remaining microbiome
329 association with location was low but statistically significant (ANOSIM, unweighted UniFrac;
330 R=0.20, $P<0.01$; ANOSIM, weighted UniFrac; R=0.28, $P<0.01$). Procrustes analysis also
331 demonstrated that the beta diversity of microbiomes in which *Rickettsia* and *Francisella* were

removed had a different association with location than *Rickettsia* and *Francisella* endosymbionts (error, M^2=0.91, P<0.01). Isolation by distance (IBD) analysis of pairwise microbiome unweighted UniFrac distances compared to distances between each of the sampling locations revealed little geographic IBD (Mantel test, unweighted UniFrac, R=0.09, P < 0.01) whereas IBD of microbiomes after excluding *Rickettsia* and *Francisella* was slightly greater (Mantel test, unweighted UniFrac, R=0.14, P<0.01). IBD of *Rickettsia* and *Francisella* only was not significant and the null hypothesis was not rejected (Mantel test, unweighted UniFrac, R=-0.03, P=0.74). After removing *Rickettsia* and *Francisella* genera from the dataset, significant differences in the abundances of *Nevskia*, *Curtobacterium* and *Sphingomonas*, but not other bacteria, were observed between locations (Kruskal-Wallis H=25.7, 24.2, 22.9; Bonferroni corrected P<0.01, respectively) with Peñasquitos and Lopez Canyons having higher abundances of *Nevskia* than Mission Trails and; Peñasquitos and Lopez Canyons having more *Curtobacterium* and *Sphingomonas* than Escondido Creek and Mission Trails (Table 2).

Interestingly, *Rickettsia* and *Francisella* were negatively correlated in the ticks (Pearson's product moment correlation; R=-0.44, P<0.01; Fig. 6). In order to assess whether the tick microbiomes were predictive of infection with spotted fever group *Rickettsia* (as determined by *real-time* PCR of the *romp*A gene and IGR sequences), a Random Forests supervised learning analysis using 1000 trees and 10x cross validation was performed on the OTU dataset minus *Rickettsiaceae* and *Rickettsia* OTUs. The ratio of baseline error to the estimated generalization error was 8.8, which indicates that the classifier was greater than eight times more predictive

than random chance. A ratio greater than 2 is accepted as a good classifier result (http://qiime.org/tutorials/running_supervised_learning.html, (Knights, Costello & Knight, 2011). The most predictive OTU was the FLE OTU 840032 and it accounted for 13% of the model. OTUs 866436 and 639277 each accounted for 3% of the model and the closest database matches to it were the Firmicutes *Geobacillus* and *Aeribacillus (Geobacillus*), respectively (Miñana-Galbis, Pinzón, Lorén, Manresa, & Oliart-Ros, 2010). Non-*Rickettsia,* non-*Francisella* bacteria associated with *Francisella* to *Rickettsia* >2 (range 2.4-119.0) were *Planococcaceae* and *Geobacillus* (Kruskal-Wallis test; H=23.8, 14.2, Bonferroni $P<0.001$ and $P=0.011$, respectively).

Amplification of vertebrate cytochrome b gene was attempted to determine the origin of the ticks' host blood meals, however, no cytochrome b was amplified from the ticks. This may have been due to ticks being captured before feeding as they were questing for a blood meal. However, SourceTracker analysis revealed that 31% of ticks had microbiomes that were 1.1-27.4% similar to dog skin microbiomes (Supplemental table 2). Ticks negative for *R. philipii* or *R. rhipicephali* were more likely to have microbiomes similar to dog skin than ticks that were infected with *R. philipii* or *R. rhipicephali* (Student's *T*-test; $t=2.90$, $P<0.01$). *Sphingomonadaceae*, *Oxalobacteraceae*, and *Comamonadaceae* were the most abundant families of bacteria shared between tick and dog skin microbiomes. *Geobacillus* (OTU 4414596) and *Planococcaceae* (OTU 219154) were also present in both microbiomes. The tick microbiomes were less than 1% similar to microbiomes of the skins of fish, iguana, pigeon, rat, and human, as well as human oral, plant and soil microbiomes (Supplemental table 2).

Discussion

This is the first study of the microbiome of *D. occidentalis* ticks using next generation sequencing techniques to examine geographical associations and pathogen interference within the tick microbiome. *D. occidentalis* is one of the most common tick species found in San Diego and is a vector of human pathogens including *Francisella tularensis* and *Rickettsia philipii*. Although *Francisella tularensis* has been detected previously in ticks in San Diego (Kugeler et al., 2005), none of the ticks harbored this bacterium or genera of other recognized zoonotic tick-borne pathogens such as *Borrelia*, *Anaplasma*, *Ehrlichia*, *Babesia* or *Bartonella*; however, a low percentage of the ticks were infected with spotted fever group *Rickettsia*: 2.5% with *R. philipii* and 8.2% with *R. rhipicephali*. This is a slightly lower prevalence of *R. philipii* than surveys of ticks performed in Orange, Riverside, Los Angeles, Santa Barbara and Ventura counties north of San Diego, that reported an overall 7.5% prevalence of *R. philipii* (Wikswo et al., 2008) but is within the range of *R. philipii* prevalence reported from northern California of 0.4-5.1% (Lane et al., 1981; Philip, Lane & Casper, 1981). Similar to other tick species, the microbiome of *D. occidentalis* was dominated by Proteobacteria, primarily *Rickettsia* or *Francisella,* with much lesser amounts of *Sphingomonas*, *Methylobacterium* and *Hymenobacter* (*Bacteroidetes*). These last three genera are all decomposer microbes found in the soil and except for *Hymenobacter*, have been detected in other tick microbiome studies. Even though the ticks were washed multiple times before DNA extraction, the possibility that some of these represent surface bacteria cannot be completely excluded, however, SourceTracker analysis did not reveal soil microbiome or other contamination, of the samples.

398

399 Although 58 of the ticks were negative for SFGR by real-time PCR of the *romp*A gene
400 and IGR, all of the ticks contained OTUs whose partial 16S rRNA gene segments aligned with
401 SFGR in GenBank. The cause of this discrepancy may be due to the increased sensitivity of the
402 Illumina sequencing platform compared to real-time PCR of *romp*A and IGR sequences and/or
403 the presence of other *Rickettsia* spp. with highly conserved 16S rRNA genes but that lack *romp*A
404 and IGR sequences complementary to the PCR primers used. Analysis of other genes would be
405 required to resolve them at the species level (Eremeeva, Yu & Raoult, 1994; Regnery, Spruil &
406 Plikaytis, 1991). Additional data support that more than two different *Rickettsia* species were
407 present within the tick population tested. *R. rhipicephali* was detected by real-time PCR of the
408 *romp*A gene and/or IGR in ticks that had OTU 837189 counts greater than 5900/tick, except for
409 two ticks, T14-0667 and T14-0769 that had high OTU 837189 counts of 73,527 and 53,714,
410 respectively, but were negative for *R. rhipicephali*. Similarly, *R. philipii* was detected in ticks
411 with OTU 553807 counts ranging from 11 to 2158, except for one sample, T14-0667, that had
412 884 counts of OTU 553807 yet was negative for *R. philipii* by real-time PCR of *romp*A gene and
413 IGR. These findings are consistent with the presence of species of *Rickettsia* different from *R.*
414 *rhipicephali* and *R. philipii* that could not be discriminated by the partial 16S rRNA gene or
415 *romp*A and IGR sequences.

416

417 The two most abundant *Francisella* OTUs, 840032 and 399541, accounted for over 90%
418 of all *Francisella* OTUs and were 100% identical to *Francisella*-like endosymbionts (FLE) of *D.*
419 *occidentalis* (GenBank accs. AY805304 and AY375402 for OTU 840032, and KU355875 for

OTU 399541). Taken as a whole, these results are consistent with tick co-infection with a mixture of *Rickettsias* and FLEs.

The number of unique OTUs detected in *D. variabilis* was 6.4 times higher than found in a study of *Ixodes* ticks, although, sequence depth was approximately two times greater in our study and, as noted, the vast majority of the OTUs occurred at very low frequencies but in at least two different ticks (van Treuren et al., 2015). The reason for this is unclear, but it is not a phenomenon only found in our tick system. However, similar to *Ixodes scapularis* and *Amblyomma americanum* ticks, female *D. occidentalis* ticks harbored a less diverse array of bacteria than males (Fig. 2) (Ponnusamy et al., 2014; van Treuren et al., 2015). Endosymbionts belonging to *Rickettsia*, *Coxiella*, *Francisella* and *Arsenophous* genera have been found in different tick species and are thought to interfere with and partially exclude other bacteria and pathogenic forms of closely related organisms from transovarial transmission leading to lower alpha diversity in female ticks than males (Burgdorfer & Brinton, 1975; Macaluso et al., 2002; Niebylski, Peacock & Schwan, 1999; Noda, Munderloh & Kurtti, 1997; Reinhardt, Aeschlimann & Hecker, 1972; Telford III, 2009). In *D. occidentalis*, a higher percentage of *Rickettsia* and *Francisella* in the microbiomes of female ticks than male ticks may have similarly decreased species richness in female ticks compared to males (Fig 2).

The beta diversity of the endosymbionts and non-endosymbionts differed with respect to location. Although non-endosymbionts demonstrated a small association with location, geographic association was not observed by the *Rickettsia* and *Francisella* endosymbionts. In

addition, Procrustes analysis results demonstrated that *Rickettsia* and FLE beta diversities had different relationships to geographical locations than the other microbiome components, illustrating that different factors shape *Rickettsia* and FLE components of the *D. occidentalis* microbiome than other non-endosymbiont microbiome members. One factor that appeared to contribute to the geographical differences in the non-endosymbiont microbiome was isolation by distance. Geographical differences in bacterial community composition in the same hematophagous insect species has been seen in fleas and ticks, however, the causes are not completely known (Jones et al., 2010, van Treuren et al., 2015). Differential geographic localization of *Nevskia*, *Curtobacterium* and *Sphingomonas*, genera that are associated with environmental sources such as the air-water interface (*Nevskia*) and soils (*Curtobacterium* and *Sphingomonas*), may be the result of differences in soil microbial ecology at each location (van Treuren et al., 2015). Alternatively, non-endosymbiont microbiome differences could be the result of stochastic or different populations of ticks at each location. In contrast, the dependency of *Rickettsia* and *Francisella* endosymbionts on their *D. occidentalis* host may have restricted the degree of variation that population separation could impart upon these endosymbionts (Budachetri et al., 2014).

One of the primary hypotheses of this study was to determine if negative associations between bacteria, suggestive of interference, occurred within ticks especially with respect to pathogens. Indeed, a strong inverse relationship was observed between *Rickettsia* and FLE infection (Pearson's product moment correlation $R=-0.44$, $p<0.01$, Fig. 6) and a Random Forests supervised learning model successfully predicted the absence of SFGR within the ticks (baseline error:observed error=8.8; an error ratio≥2 is significant). Not surprisingly, FLE OTU 840032

contributed most to the model. FLE and different uncategorized *Rickettsia* co-infection in ticks has been previously observed but not enumerated (Niebylski et al., 1997; Scoles, 2004) and partial interference between co-infection by different *Rickettsia* species has been demonstrated (Burgdorfer, Hayes & Mavros, 1981; Macaluso et al., 2002). Although the quantitative 16S rRNA gene sequence data of FLE and *Rickettsia* co-infection in this study do not directly measure interference between the organisms, they are consistent with interference between FLE and *Rickettsias* and require further experimental studies for confirmation.

The mechanisms by which *Rickettsia* and *Francisella* interfere with each other in co-infections are not known. Although the localization of *R. rhipicephali* and *R. philipii* within ticks has not been determined, FLEs have been found in female tick reproductive tissues and hemolymph (Goethert & Telford, 2005; Scoles, 2004). In addition, non-*Francisella* bacteria were also associated with low *Rickettsia* to *Francisella* ratios. *Planococcaceae* and *Geobacillus* were associated with greater abundance of *Francisella* relative to *Rickettsia* within the ticks (Kruskal-Wallis H=23.8, 14.2; $P<0.001$, 0.011, respectively). Although blood meals of the ticks could not be detected by amplification of vertebrate cytochrome b gene from the ticks, 31% of the tick microbiomes had microbiome components similar to canine skin which may suggest the source of a prior blood meal if they incorporated some of the skin flora into their own microbiome as has been shown with host blood microbiomes following feeding (Zhang et al., 2014). Use of SourceTracker for comparison of tick and skin microbiomes is a novel approach and, interestingly, demonstrated that ticks with canine skin microbiome components were less likely to be infected with *R. philipii* or *R. rhipicephali* which is consistent with *R. rhipicephali* and *R. philipii* being endosymbionts without a canine host. Both *Geobacillus* and

Planococcaccaceae were present in canine microbiomes as well. *Geobacillus* also demonstrated a negative association with *Rickettsia* infection in the Random Forests model. *Geobacillus* are thermophilic gram-positive bacteria and have been explored for use in biofuel synthesis due to their ability to catabolize hemicellulose and starch (Hussein, Lisowska & Leak, 2015). The *Planococcaceae* family belongs to the *Firmicute* phylum and consists of 14 soil dwelling genera, some of which have been proposed to have possible applications in bioremediation (Shivaji, Srnivas & Reddy, 2013). Their interactions with endosymbionts, much less *Rickettsia* or *Francisella,* have not been described, thus, how they might influence *Rickettsia* or *Francisella* co-infection is unknown. Unfortunately, a microbiome dataset of another common tick blood meal host, i.e. deer, was not available for comparison.

Conclusions

The results of this study suggest that FLE and *Rickettsia* endosymbionts partially exclude each other in co-infections of the same *D. occidentalis* tick. Although interference between *Rickettsia* co-infections has been known for many years, this is the first study that points to exclusion between different endosymbiont genera in ticks. The mechanisms for this phenomenon are not known and warrant future research. Since chemical control of ticks with organophosphates, pyrethrins, pyrethroids, or even antibiotics in the environment is neither feasible nor desirable due to deleterious effects on other insects and wildlife, a nonpathogenic, biological control of a tick's ability to transmit pathogens, would desirable. This technique has already been employed for mosquito control by releasing *Wolbachia*-infected mosquitoes to

reduce mosquito abundance and vectoral capacity (Iturbe-Ormaetxe, Walker & O' Neill, 2011). Whether FLEs can be shown to inhibit *Rickettsia* co-infection in the laboratory and could be propagated through a tick population as a means to render ticks unable to vector pathogenic *Rickettsia* or other pathogens is an intriguing prospect that warrants further exploration.

Table 1. Tick collection locations, number of ticks infected with Spotted Fever group *Rickettsia*, and number of male and female *D. occidentalis* ticks collected at each location.

Location	GPS Coordinates	*R. rhipicephali*	*R. philipii*	Negative	M/F*
Escondido Creek	33.060700, -117.179500	7	1	9	8/9
Lopez Canyon	32.906776, -117.202964	14	9	22	23/22
Mission Trails	32.834444, -117.045833	4	1	19	7/17
Peñasquitos Canyon	32.938638, -117.130351	7	1	8	7/9

*No statistically significant association between SFGR infection and male versus female, Fisher's exact test; $P>0.5$. M=male, F=female.

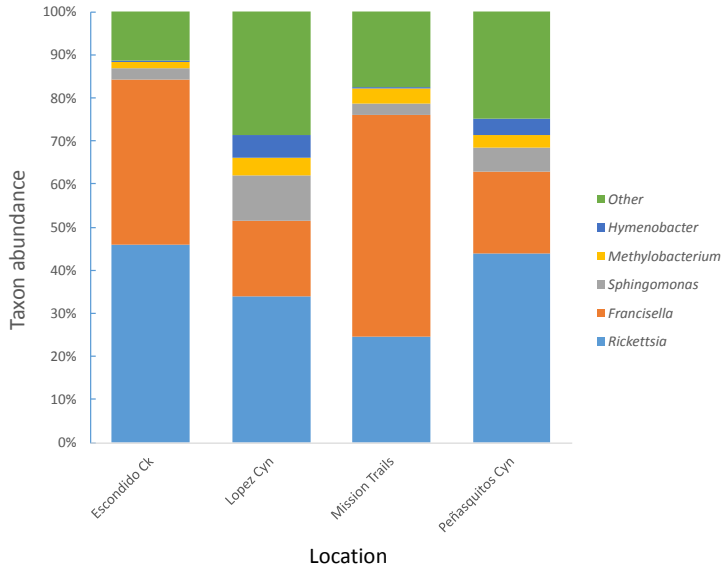
Table 2. OTUs and genera associated with different locations.

OTU	Genus	H*	*P***	Escondido Creek⁺	Mission Trails⁺	Peñasquitos Cyn⁺	Lopez Cyn⁺
73481	*Nevskia*	25.7	0.0002	1.59	0.04	2.31	1.09
643513	*Curtobacterium*	24.2	0.0004	0.18	0.25	3.06	1.91
489455	*Sphingomonas*	22.9	0.0007	0.12	0.13	3.69	2.09

* Kruskal-Wallis H value

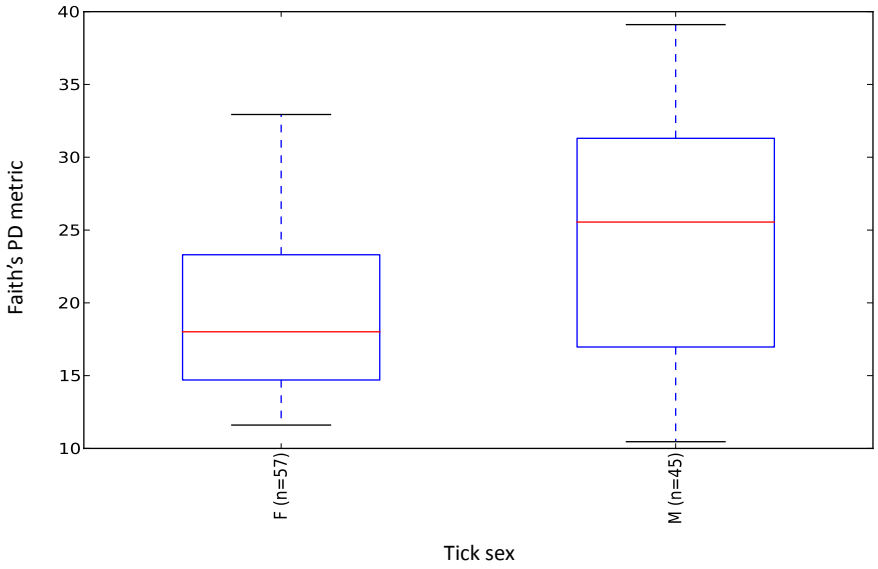
**Bonferroni correction (Bonferroni correction is used to reduce the chances of obtaining false-positive results (type I errors) when multiple pair wise tests are performed on a single set of data because the probability of identifying at least one significant result due to chance increases as more hypotheses are tested.)

⁺ Average number of OTU occurrences per sample



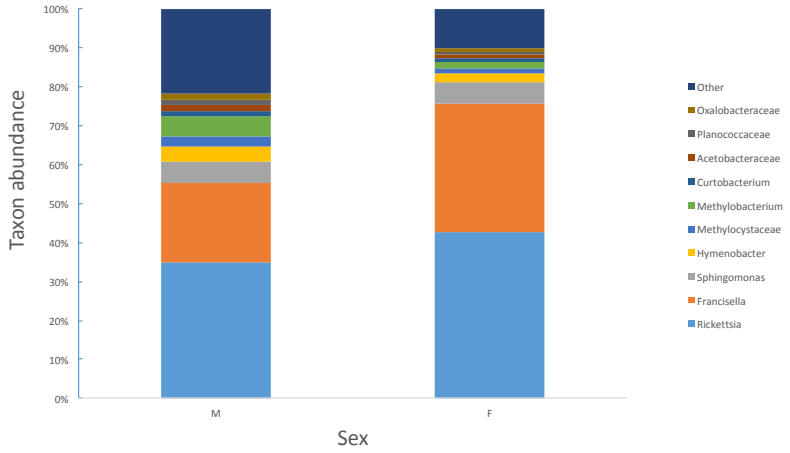
532
533
534 Figure 1. Most abundant bacterial genera detected in *D. occidentalis* from four different
535 locations in San Diego County.
536

537



538

539 Figure 2. Boxplot of microbiome alpha diversity in *D. occidentalis* ticks measured by Faith's
540 phylogenetic diversity (PD) whole tree as implemented in QIIME of male and female *D.*
541 *occidentalis*. Faith's PD, two sample *t*-test, male versus female; t=3.63, P<0.01.



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544 Figure 3. Percent abundance of taxa that comprise at least 1% or greater of the total
545 microbiome in male and female ticks.
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547
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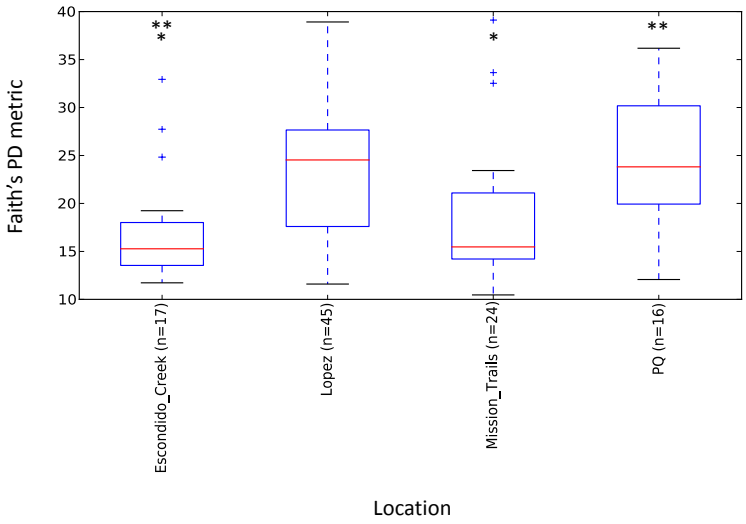

549
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551 Figure 4. Boxplot of microbiome alpha diversity in *D. occidentalis* ticks measured by Faith's
552 phylogenetic diversity (PD) whole tree as implemented in QIIME of four different hiking areas in
553 San Diego County. Stars indicate statistically significant differences between samples; Faith's

PD, two sample *t*-test, Escondido Creek versus Lopez Canyon; t=-3.28, P=0.02; Escondido Creek versus PQ, t=-3.31; P=0.04; other comparisons were not statistically significant. PQ = Peñasquitos Canyon.

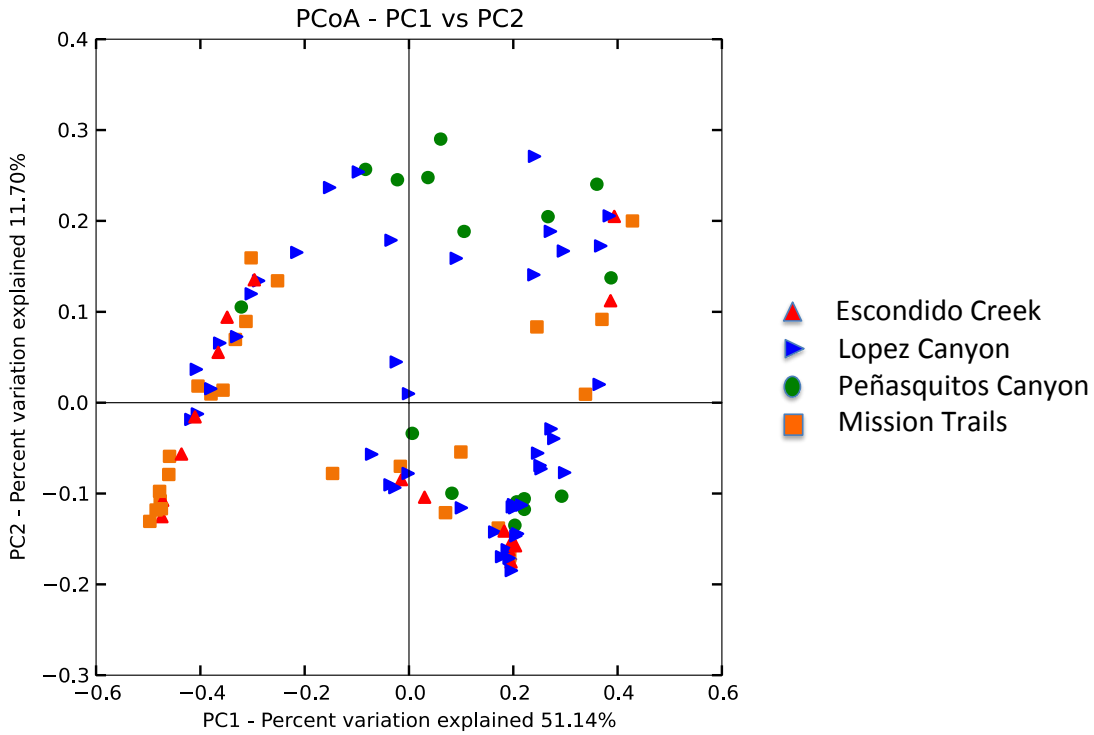
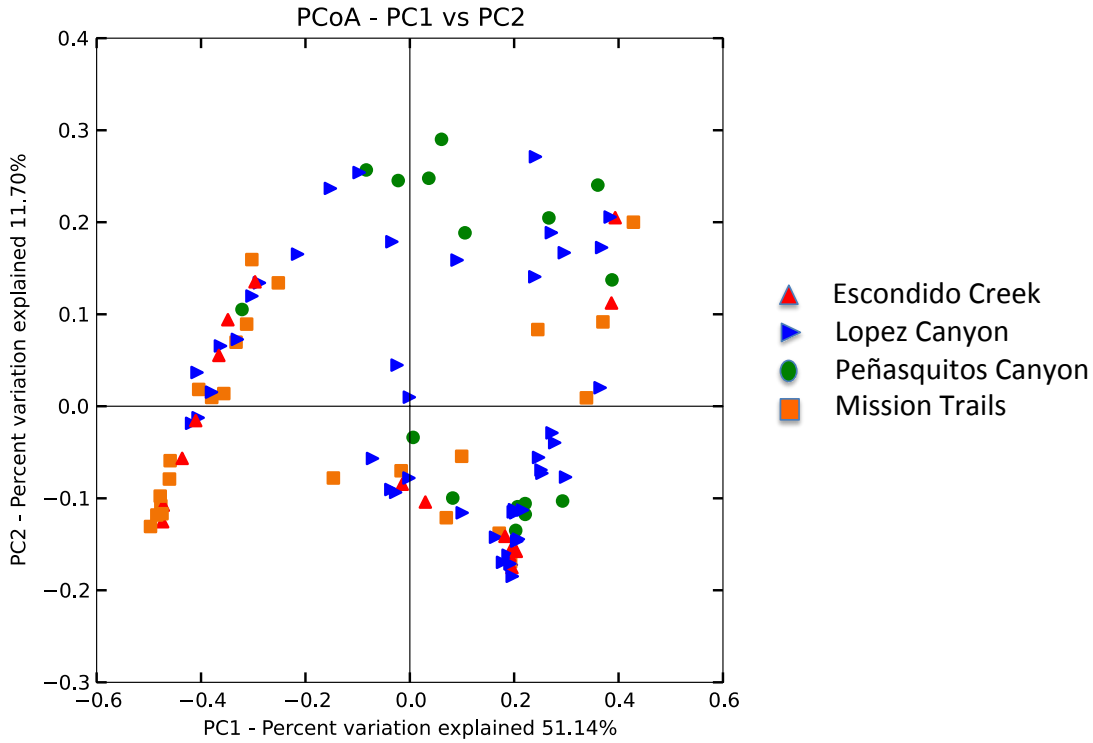


Figure 5.A. Unweighted beta diversity of *D. occidentalis* microbiomes at four different locations in San Diego County. ANOSIM, unweighted UniFrac; R=0.14, $P<0.01$.



565
566
567 Figure 5.B. Weighted beta diversity of *D. occidentalis* microbiomes at four different locations in
568 San Diego County. ANOSIM, weighted UniFrac; R=0.12, *P*=0.01.

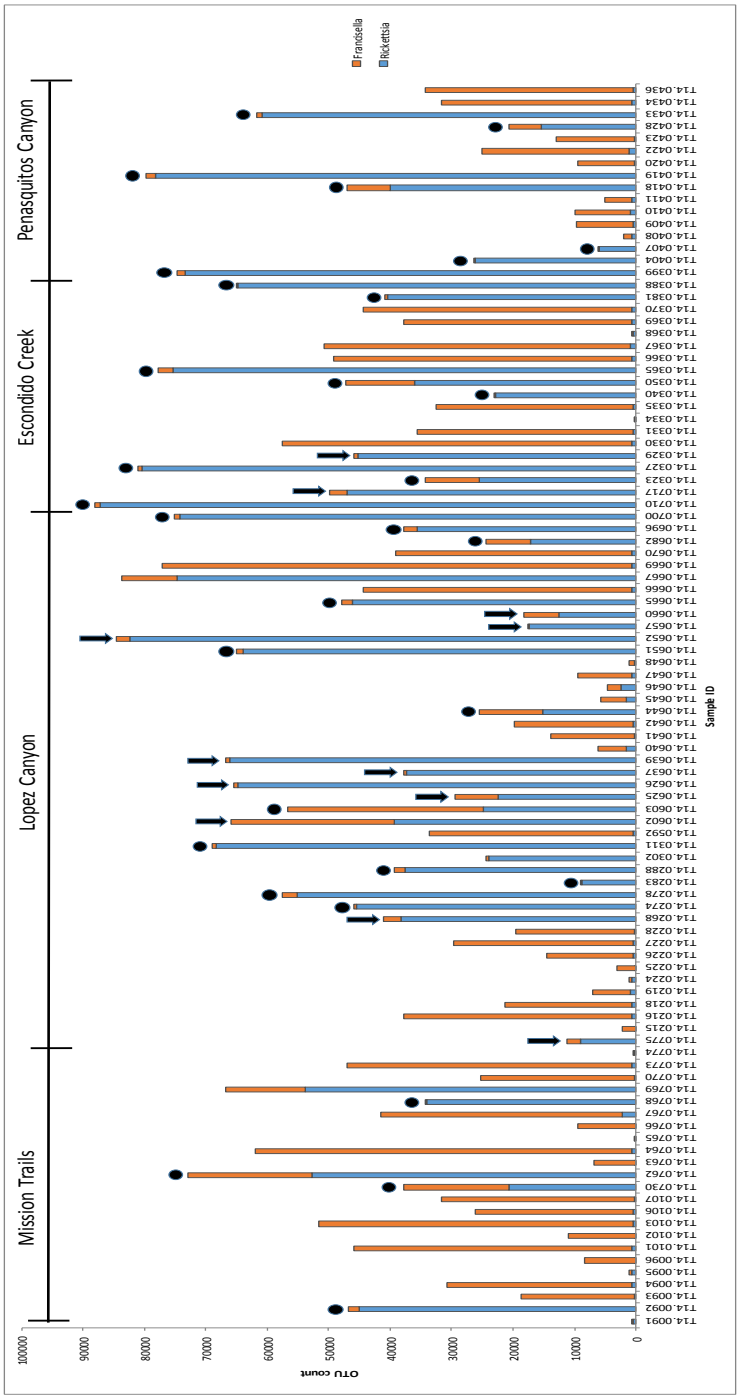


Figure 6. *Rickettsia* and *Francisella* OTU abundance in *D. occidentalis* ticks in San Diego County. Circles indicate ticks infected with *R. rhipicephali* and arrows indicate ticks infected with *R. philipii* 364D. Pearson product moment correlation; R=-0.44, $P<0.01$

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