

ORIGINAL ARTICLE

Similarities and seasonal variations in bacterial communities from the blood of rodents and from their flea vectors

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Vector-borne microbes are subject to the ecological constraints of two distinct microenvironments: that in the arthropod vector and that in the blood of its vertebrate host. Because the structure of bacterial communities in these two microenvironments may substantially affect the abundance of vector-borne microbes, it is important to understand the relationship between bacterial communities in both microenvironments and the determinants that shape them. We used pyrosequencing analyses to compare the structure of bacterial communities in *Synosternus cleopatrae* fleas and in the blood of their *Gerbillus andersoni* hosts. We also monitored the interindividual and seasonal variability in these bacterial communities by sampling the same individual wild rodents during the spring and again during the summer. We show that the bacterial communities in each sample type (blood, female flea or male flea) had a similar phylotype composition among host individuals, but exhibited seasonal variability that was not directly associated with host characteristics. The structure of bacterial communities in male fleas and in the blood of their rodent hosts was remarkably similar and was dominated by flea-borne *Bartonella* and *Mycoplasma* phylotypes. A lower abundance of flea-borne bacteria and the presence of *Wolbachia* phylotypes distinguished bacterial communities in female fleas from those in male fleas and in rodent blood. These results suggest that the overall abundance of a certain vector-borne microbe is more likely to be determined by the abundance of endosymbiotic bacteria in the vector, abundance of other vector-borne microbes co-occurring in the vector and in the host blood and by seasonal changes, than by host characteristics.

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Introduction

The identification of potential ecological factors that affect the abundance of vector-borne microbes could have a marked impact on our ability to prevent and control vector-borne diseases (Lemon *et al.*, 2008). Importantly, vector-borne microbes are subject to ecological conditions and constraints in two different microenvironments: that in the arthropod vector and that in the blood of its vertebrate host. One important factor that affects these conditions—and therefore the microenvironment that vector-borne microbes are subject to—is the resident bacterial

communities, the members of which can have different relationships with the host and vector organisms, ranging from mutualistic to commensalistic or parasitic (Clay *et al.*, 2006). In addition, interactions between different microbes within each microenvironment may affect both the density of a certain vector-borne microbe and its transmission rate and virulence to human hosts (Pumpuni *et al.*, 1993; Lipsitch *et al.*, 1996; Macaluso *et al.*, 2002; de la Fuente *et al.*, 2003; Gonzalez-Ceron *et al.*, 2003; Lively *et al.*, 2005; Telfer *et al.*, 2010; Cirimotich *et al.*, 2011). Thus, characterizing the structure of bacterial communities (that is, richness, diversity and composition of phylotypes) in both the vector and the host, and the factors that shape them, is important for understanding the determinants of the overall abundance of vector-borne microbes.

Most studies of vector-borne microbiota have focused on microbial communities within the microenvironment of the arthropod vector,

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including studies on lice (Reed and Hafner, 2002), fleas (Jones *et al.*, 2010; Hawlena *et al.*, 2013), mosquitoes (Rani *et al.*, 2009; Osei-Poku *et al.*, 2012), flies (Hornok *et al.*, 2008), ticks (Heise *et al.*, 2010; Andreotti *et al.*, 2011; Hawlena *et al.*, 2013), mites (Netusil *et al.*, 2005; Reeves *et al.*, 2006) and leeches (Worthen *et al.*, 2006). Diverse microbial communities were found in these vectors, and most microbial species were represented sparsely or occurred in only a few individual vectors. Some studies also indicated that the composition of these microbial communities often varies across time and space (Kirstein *et al.*, 1997; Wielinga *et al.*, 2006; van Overbeek *et al.*, 2008; Jones *et al.*, 2010) and that these variations are linked largely to the characteristics of the arthropod (for example, its species, life stage, diet and blood engorgement level; Pidiyar *et al.*, 2004; Moreno *et al.*, 2006; Heise *et al.*, 2010; Jones *et al.*, 2010; Hawlena *et al.*, 2013). Nevertheless, because vector-borne microbes spend a part of their lives in the blood of the vertebrate host, it is important to consider also the internal environment of this host. For instance, microbes in the blood—similar to other microbes in the vertebrate gut, secretion glands, plumage, cloaca and skin—may be influenced by host species, diet, personality, body mass, sex, age and health status (see, for example, Alexy *et al.*, 2003; Voigt *et al.*, 2005; Fierer *et al.*, 2008; Turnbaugh *et al.*, 2009; Martin *et al.*, 2010; Koenig *et al.*, 2011; Muegge *et al.*, 2011; Roeselers *et al.*, 2011; Saag *et al.*, 2011; Yatsunenko *et al.*, 2012; Faith *et al.*, 2013; van Dongen *et al.*, 2013; Gavish *et al.*, 2014; Kedem *et al.*, 2014; Kueneman *et al.*, 2014; Leclaire *et al.*, 2014).

Here, we therefore address two main questions. (1) To what extent is the structure of bacterial communities in arthropod vectors similar to that in the blood of their vertebrate host? (2) What are the most important determinants of the structure of the two communities, and to what extent are they stable between host individuals and over time? To answer these questions, we characterized the structure of bacterial communities in bloodsucking arthropod vectors (fleas) and in the blood of their vertebrate hosts (wild gerbils). Female fleas, male fleas and blood samples were obtained from the same gerbils at two different time points: first during the spring, when some individuals were juveniles and others were adults, and then ~4 months later, at the end of the summer, when all individuals were adults. We hypothesized that the structure of bacterial communities in fleas would differ from those in blood samples because the blood may contain directly transmitted bacteria and phylotypes that are transmitted by non-flea vectors (for example, helminths and ticks; Hawlena *et al.*, 2006 and Hawlena H, unpublished data) in addition to the flea-borne bacteria (that should appear in both communities). We also hypothesized that the bacterial communities in fleas would show greater phylotype richness and diversity than those in the blood samples,

because fleas may harbor both bacterial endosymbionts and bacteria from the very rich soil microbiome (that is, bacteria originally acquired during the larval stage of the fleas and then transmitted transstadially to adult fleas; Torsvik *et al.*, 1990). In addition, although the bacterial communities in the gerbils and fleas are largely influenced by the internal environment of the gerbil and flea, respectively, bacterial communities in the fleas are also influenced by the external environment of the gerbil. Hence, we also expected that the stability and determinants of bacterial community structure in the blood samples would show a higher inter-individual variability, a lower seasonal variability and a stronger dependency on host characteristics than those in the fleas.

Materials and methods

Study design

We trapped and identified juvenile and adult gerbils (*Gerbillus andersoni*) and obtained their fleas (*Synosternus cleopatrae*) and blood samples on two occasions: (1) during spring (April–May), which is the reproductive season of the gerbils, and (2) during the end of summer (September). At the latter sampling period, all individuals were adults. We quantified changes in the structure of bacterial communities in the flea vectors and in the blood of their rodent hosts in relation to the host characteristics (individual identity, age and sex), sample type (female flea, male flea or rodent blood) and sampling season (spring or summer). Because the same individuals were captured in two sampling seasons, this design enabled differentiating between effects related to vector or host characteristics and effects related to temporal (seasonal) changes that occur in all host individuals. In addition, our sampling of individuals belonging to the same species, occupying the same macrohabitat (semistabilized sandy dunes; Abramsky *et al.*, 1985) and feeding on a similar diet (composed mainly of seeds; Bar *et al.*, 1981) deflates the type II error. Thus, our experimental design increased the likelihood of observing age- and sex-related effects in bacterial communities and avoided sampling challenges common to studies in humans or in laboratory specimens.

Rodent capture, tagging and sampling

We trapped rodents in each of ten 1 ha plots located in the Western Negev, Israel (see Supplementary Methods). We tagged each individual and determined its species, sex, weight, age group (juveniles <18 g; adults >18 g, following Hawlena *et al.*, 2006) and, in female rodents, the reproductive status. We collected all fleas (see Supplementary Methods). In addition, using sterile capillaries, we drew 100–200 µl of blood from the retro-orbital sinus of each rodent and stored the blood in EDTA blood

collection tubes at -20°C until DNA extraction. Because of the sensitivity of the eyes, we could not sterilize the local area before drawing the blood. Therefore, to minimize contamination, we performed the bleeding process as quickly as possible. In addition, we always changed capillaries when we had to stab the retro-orbital sinus of a given individual more than once. Such efforts cannot completely prevent surface contamination, and, as a result, some bacterial phylotypes associated with the skin or eye of the rodent could have been artificially added to the pool of detected phylotypes. However, from the 40 phylotypes that we detected, only five represent genera that are associated with the skin or eye microbiomes of rodents (that is, *Staphylococcus* spp. and *Streptococcus* spp.; Cullen, 2003; Coster *et al.*, 2008; Montiani-Ferreira *et al.*, 2008; Scharschmidt *et al.*, 2009; Meekins *et al.*, 2014). From these five phylotypes, three were each confined to one sample, one was detected in four samples and one was detected in five samples. Hence, such a contamination, if occurred, had a negligible effect on our results. Moreover, as all host individuals were subjected to exactly the same sampling procedure, possible surface contaminants should have only increased the number of bacterial phylotypes that are not associated with the flea vectors and increase the variance among bacterial communities in the blood of different host individuals but not across seasons.

The trapping and handling protocol was approved by the Committee for the Ethical Care and Use of Animals in Experiments of Ben-Gurion University of the Negev (permission no. IL-14-03-2011) and by the Nature and National Parks Protection Authority (permission no. 2011/38146).

Characterization of bacterial communities

Fleas were first surface sterilized by washing them thoroughly three times in 70% ethanol (Hawlena *et al.*, 2013). Although this simple method could not completely prevent surface contamination, it allowed us to remove most contaminants without damaging the quality of the DNA (H Hawlena *et al.*, unpublished data). Indeed, of the 40 phylotypes that we have detected, only two phylotypes—*Azovibrio* sp. and *Saccharothrix* sp.—are possible contaminants; the rest of the phylotypes that we found in fleas represent genera that are known as mutualists of arthropods or that are present in the digestive system of arthropods. As each of these candidate contaminants was found in only a single sample, we believe that their presence should have a negligible influence on our findings.

To extract DNA from fleas, we used the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA, USA) according to the QIAGEN 'purification of total DNA from ticks' supplementary protocol for the detection of *Borrelia* DNA (Hawlena *et al.*, 2013). To extract DNA from rodent blood samples, we used a

Bacteremia DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA). We added 50 μl blood to the Microbead tube and followed the manufacturer's instructions. We added a negative control to each extraction session by adding all reagents to phosphate-buffered saline instead of fleas or blood. We subjected the DNA extracts and nine negative controls to V1–V3 region 16S rRNA PCR, following Hawlena *et al.* (2013), and performed 454 pyrosequencing on a Roche/454 GS-FLX Titanium system (Branford, CT, USA).

We processed the pyrosequencing reads with Mothur (version 1.22.0), as described in Schloss *et al.* (2011). Briefly, we assigned bacterial sequences to specific flea or blood samples only if they perfectly matched the primer barcode sequence. We trimmed the primer and barcode sequences from the remaining sequences, trimmed sequences <200 bp. We performed an additional denoise step to remove low-quality sequences, followed by chimera detection and elimination.

We binned the sequences into phylotypes, where a phylotype was defined as a group of microbes that are commonly characterized by the level of sequence similarity between small subunit (16S) rRNA genes. We grouped two sequences into a single 'species'-level phylotype if they were $\geq 97\%$ identical over their overlap regions (an equivalent for the species level in macroorganisms; Stackebrandt and Goebel, 1994). We classified the phylotypes to the genus level by using the classifier function embedded in the Mothur package, where 60% RDP (Ribosomal Database Project) classifier confidence cutoffs were applied. To confirm the classifications, we also randomly sampled 100 member sequences for each phylotype and compared them with the RDP 10.26 database by using BlastN (Altschul *et al.*, 1997) with an *E*-value cutoff of $1\text{E} - 20$ (Maidak *et al.*, 1996). We included in the analyses only phylotypes that had a significantly higher relative abundance ($\geq 2\%$ of the bacterial community in any one sample) in the flea or blood samples than in the negative controls, following Jones *et al.* (2010). All sequences and the metadata file are available from the SRA (Sequence Read Archive) database (study accession SRP049346).

Data analysis

Details of the data analysis procedures are given in the Supplementary Methods section and are shown here in brief. All analyses were performed at the phylotype level. Specifically, the dependent variables in this study were bacterial community composition (binary Bray–Curtis), richness (number of phylotypes in a sample) and diversity (community richness and evenness), and the independent variables were host characteristics, sample type, sampling season and their second and third interaction terms.

To analyze the effects of the independent variables on community composition, we performed a

nonparametric multivariate multiple regression analysis with a stepwise forward-selection procedure. Community composition was assessed based on the Bray–Curtis dissimilarity index (Magurran, 1988) as follows: first, relative abundances were fourth-root transformed, such that highly abundant phylotypes did not dominate the analyses; then, the ‘phylotype-sample’ abundance matrix was converted to a ‘sample-sample’ distance matrix, by using PRIMER-E (Clarke, 1993).

We determined the overall contribution of individual phylotypes within a community to the observed differences by using SIMPER, an analysis tool within the PRIMER-E software, and visualized patterns in multivariate data with an unconstrained nonmetric multidimensional scaling plot. We analyzed associations between the independent and the other two dependent variables by using the model selection approach (Burnham and Anderson, 2002; see Table 1 and Supplementary Methods for details). We based all models on generalized estimating equations for the repeated analysis of individuals across time, with a gamma distribution for the diversity data and a negative binomial distribution for the richness data. Because the dependent variables may have potentially been affected by the number of sequences, we normalized the number of sequences in each sample for the composition and richness analyses and used Fisher’s α -diversity index (Fisher *et al.*, 1943)—a reliable index of species diversity that is independent of sample size (Hubbell, 2001)—for the diversity analyses.

To characterize the stability of bacterial communities in individual rodents, we used the Jaccard index (Jaccard, 1912; see Supplementary Methods). For each individual rodent, we then compared the ‘intraindividual stability’ and the ‘interindividual stability’. We used a generalized estimating equation model to evaluate the effect of the source of variation (within versus between host individuals), sample type and their interaction (independent variable) on the Jaccard index (dependent variable) and compared each model with an intercept-only model by using the model selection approach.

Results

Blood and flea microbiomes

Details on the gerbil and flea samples are given in the Supplementary Results. Despite capturing 511 individuals, only 8 of the juvenile gerbils were recaptured during the summer, possibly because of a high dispersal rate. The minimal number of fleas per host individual was four. Accordingly, we balanced the numbers of juvenile and adult hosts and of flea and blood samples: for molecular analyses, we used 16 individuals (4 from each sex–age combination) and collected, from each individual and at each sampling period (spring/summer), one blood sample, two female fleas and two male fleas. The DNA from the two samples of the same flea sex were pooled. This sampling procedure yielded in total 32 DNA extracts from blood and 64 from fleas.

Table 1 Model selection results for the effects of host characteristics (individual identity, age and sex), sample type (female fleas, male fleas or rodent blood), sampling season (spring or summer) and the interactions of these variables on (1) bacterial phylotype richness (number of distinct phylotypes per sample) and (2) bacterial phylotype diversity (Fisher’s α -index) of bacterial communities

Effect tested	Phylotype richness		Phylotype diversity	
	$\Delta QICc^a$	w_i^b	$\Delta QICc^a$	w_i^b
No effect (intercept only)	0	36	8 (1)	0 (32)
Individual host identity	28	0	25 (18)	0 (0)
Host age	2	13	2 (11)	13 (0)
Host sex	2	14	2 (25)	14 (0)
Sample type	1	20	0 (37)	31 (0)
Sampling period	2	11	1 (1)	18 (22)
Host identity \times host age	28	0	25 (18)	0 (0)
Host identity \times host sex	28	0	25 (18)	0 (0)
Host identity \times sample type	84	0	72 (48)	0 (0)
Host identity \times period	59	0	54 (41)	0 (0)
Host age \times period	7	1	5 (5)	2 (4)
Host sex \times sample type	6	1	3 (23)	6 (0)
Sample type \times period	6	2	3 (0)	8 (41)
Host identity \times host age \times sampling period	59	0	54 (41)	0 (0)
Host identity \times host sex \times sampling period	59	0	54 (41)	0 (0)
Host age \times host sex \times sampling period	44	0	53 (18)	0 (0)
Host age \times sample type \times sampling period	18	0	15 (12)	0 (0)
Host sex \times sample type \times sampling period	17	0	12 (7)	0 (1)

Numbers in parentheses represent results of analyses performed on bacterial communities excluding the most dominant and influential phylotypes, namely one *Bartonella* and two *Wolbachia* phylotypes. The best models are strongly supported by the data and are marked in bold and underlined.

^a $\Delta QICc$: difference in QICc (corrected Akaike information criterion; Burnham and Anderson, 2002) between current and best model.

^b w_i : Akaike weight, namely, the relative likelihood of the current model, given the data and the set of models. Akaike weights are normalized across the set of candidate models to summate to one, and are interpreted as probabilities.

DNA extracts from 59 of these flea samples and from all blood samples were successfully subjected to pyrosequencing, yielding 138–1139, 213–1173 and 155–1167 sequences/sample in the blood, female flea and male flea samples, respectively. The cleaned sequences had lengths ranging from 400 to 494, with a mean of 449 and a s.d. of 7.2. Altogether, 40 bacterial phylotypes were significantly more

abundant in the field samples than in the negative controls and represented at least 2% of the total number of sequences detected in any sample. These phylotypes represented 22 genera (Table 2), but only 8 of these were represented by sequences that comprised >20% of the total sequences in a given sample. *Bartonella*, *Wolbachia* and *Mycoplasma* were the most prevalent of these genera, that is,

Table 2 Taxonomic affiliations and other relevant information regarding the 40 phylotypes detected in rodent blood samples, flea samples or both

Genus name	Sample type	No. of phylotypes	Previous records	References
<i>Aquabacterium</i>	Blood*	1	Found in the blood of other vertebrates	Moriyama <i>et al.</i> (2008)
<i>Azovibrio</i>	Fleas*	1	Found in soil	Reinhold-Hurek and Hurek (2000)
<i>Bartonella</i>	Fleas*	2	Flea-borne bacteria	Chomel <i>et al.</i> (2009); Morick <i>et al.</i> (2013)
	Both	3		
<i>Bifidobacterium</i>	Fleas*	1	Found in the digestive system of arthropods and in rodent blood	Olofsson and Vasquez (2009) Gavish <i>et al.</i> (2014)
<i>Bradyrhizobium</i>	Blood**	1	Found in soil, organic matter and in rodent blood	Bottomley <i>et al.</i> (1994); Gavish <i>et al.</i> (2014)
	Both**	1		
<i>Catenuloplanes</i>	Blood*	1	Found in soil	Yokota <i>et al.</i> (1993)
Cyanobacterium phylum)	Fleas*	1	Found in the digestive system of arthropods and rodent blood	Gavish <i>et al.</i> (2014); Sharma <i>et al.</i> (2014)
<i>Diaphorobacter</i>	Fleas*	1	Found in tick gut	Yuan (2010)
	Both	1		
<i>Halomonas</i>	Blood**	1	Found in the blood of vertebrates, including rodents	Stevens <i>et al.</i> (2009); Gavish <i>et al.</i> (2014)
<i>Lactobacillus</i>	Blood*	1	Found in the blood of vertebrates, including rodents and in mosquitoes	Salminen <i>et al.</i> (2004) Gavish <i>et al.</i> (2014); Sharma <i>et al.</i> (2014)
<i>Massilia</i>	Fleas*	1	Found in organic matter, in invertebrates fed on it and in rodent blood	Moquin <i>et al.</i> (2012); Gavish <i>et al.</i> (2014)
	Blood*	1		
<i>Methylobacterium</i>	Fleas*	1	Found in organic matter, in invertebrates fed on it and in rodent blood	Gavish <i>et al.</i> (2014); Sharma <i>et al.</i> (2014)
	Both	1		
Hemotropic <i>Mycoplasma</i>	Blood*	1	Flea-borne bacteria	Woods <i>et al.</i> (2005, 2006)
	Both	1		
<i>Neisseria</i>	Blood*	1	Found in other vertebrates and in rodent blood	Beachey (1980); Gavish <i>et al.</i> (2014)
<i>Ralstonia</i>	Blood*	2	Found in the blood of vertebrates, including rodents and in mosquitoes. Potentially skin infection abundant in dog skin	Ryan <i>et al.</i> (2011); Gavish <i>et al.</i> (2014); Hoffmann <i>et al.</i> (2014); Sharma <i>et al.</i> (2014)
<i>Rhizobiales</i> (order)	Blood*	1	The genus is unknown; previous records are not applicable	
<i>Rickettsia</i>	Fleas*	1	Endosymbionts of arthropod vectors or vector-borne parasites; found in rodent blood	Azad and Beard (1998)
<i>Saccharothrix</i>	Fleas*	1	Found in soil and in rodent blood	Labeda <i>et al.</i> (1984); Gavish <i>et al.</i> (2014)
Sphingobacteria (class)	Blood**	2	Found in other vertebrates and in rodent blood	Gill <i>et al.</i> (2006); Gavish <i>et al.</i> (2014)
<i>Spirosoma</i>	Fleas*	1	Found in the digestive system of arthropods and rodent blood	König (2006); Gavish <i>et al.</i> (2014)
<i>Staphylococcus</i>	Fleas*	2	Found in fleas and in rodent blood. Potentially contamination (part of the flora in the conjunctival sac and skin of rodents)	Cullen (2003); Coster <i>et al.</i> (2008); Montiani-Ferreira <i>et al.</i> (2008); Scharschmidt <i>et al.</i> (2009); Hawlena <i>et al.</i> (2013); Gavish <i>et al.</i> (2014); Meekins <i>et al.</i> (2014)
	Both**	1		
<i>Streptococcus</i>	Blood*	1	Found in mosquitoes but was also found in rodent blood. Potentially contamination (part of the flora in the conjunctival sac and skin of rodents)	Cullen (2003); Coster <i>et al.</i> (2008); Scharschmidt <i>et al.</i> (2009); Gavish <i>et al.</i> (2014); Meekins <i>et al.</i> (2014); Sharma <i>et al.</i> (2014)
	Both	1		
<i>Wolbachia</i>	Fleas ^a	4	Reproductive parasites of arthropod vectors; found in rodent blood	Werren <i>et al.</i> (2008); Gavish <i>et al.</i> (2014)

Phylotypes marked with one asterisk were detected in only one sample (1% prevalence), whereas those with two asterisks were detected more than once but in less than five samples (between 1% and 5% prevalence), and all the rest were detected in at least five samples.

^aOne phylotype was detected in only one flea sample, another phylotype in three flea samples and two other phylotypes were detected in >29 samples.

were common in more than one DNA sample and outnumbered other genera in the sample by a factor of ≥ 10 (Supplementary Figure S1).

The composition of detected phylotypes is detailed in Table 2, where we distinguish between phylotypes found in only one sample, rare phylotypes and phylotypes that appear in at least 5% of the samples. The latter group, of the most prevalent phylotypes, consisted of only nine phylotypes, of which all but two (namely, *Wolbachia* spp.) were shared by fleas and host blood samples, and represented five genera. Of these genera, *Bartonella* and hemotropic *Mycoplasma*, which are known vector-borne bacteria (Willi et al., 2005; Morick et al., 2010, 2011), were the most common (Figure 1 and Supplementary Figure S1). *Methylobacterium* and *Streptococcus* have been previously detected in arthropods and in vertebrates, and may thus be vector-borne bacteria as well. *Diaphorobacter* was previously found in arthropods but not in vertebrate blood; it is not known as a member of the skin or eye microbiome (Cullen, 2003; Coster et al., 2008; Montiani-Ferreira et al., 2008; Scharschmidt et al., 2009; Grice and Segre, 2011; Hoffmann et al., 2014; Meekins et al., 2014; Tomic-Canic et al., 2014), and therefore its occurrence in the gerbil blood is probably not a result of contamination and deserves further investigation. Seven additional phylotypes were detected in 2–4 samples, representing five genera. Of these seven phylotypes, two shared phylotypes belonged to the genera *Staphylococcus* and *Bradyrhizobium*, which were detected previously in rodents. *Staphylococcus* was also detected in fleas and *Bradyrhizobium* was also

detected in soil and, therefore, could have been acquired by fleas from the soil (for example, via food consumption during the free-living life stages of the fleas). One phylotype was unique to fleas and represents *Wolbachia*, a genus with known mutual associations with arthropods. Finally, four phylotypes were unique to blood, representing three taxonomic groups (*Bradyrhizobium*, *Halomonas* and a member of the Sphingobacteria class) with representative isolates from blood or from other tissues of vertebrate species.

Determinants of the bacterial community structure

The ‘sample type’ and the ‘sample type’ \times ‘sampling season’ interaction were important predictors of the composition of the bacterial community (pseudo- $F > 6$, $P < 0.0005$ for each variable; Figures 1a and 2), and together explained 33% of the variability in phylotype composition among bacterial communities. A SIMPER analysis revealed that two *Wolbachia* phylotypes (which were detected only in female fleas; Figure 1a and Supplementary Figure S1) and one *Bartonella* phylotype (which was more abundant in male fleas and in host blood samples than in female fleas; Figure 3) were primarily responsible for the differences between bacterial communities in fleas and in the blood of their hosts. The higher relative abundance of *Wolbachia* species and the lower relative abundance of *Bartonella* species in female fleas, as compared with that in male fleas, may result from differences in the number of *Bartonella* sequences, in the number of *Wolbachia* sequences or both. However, the mean

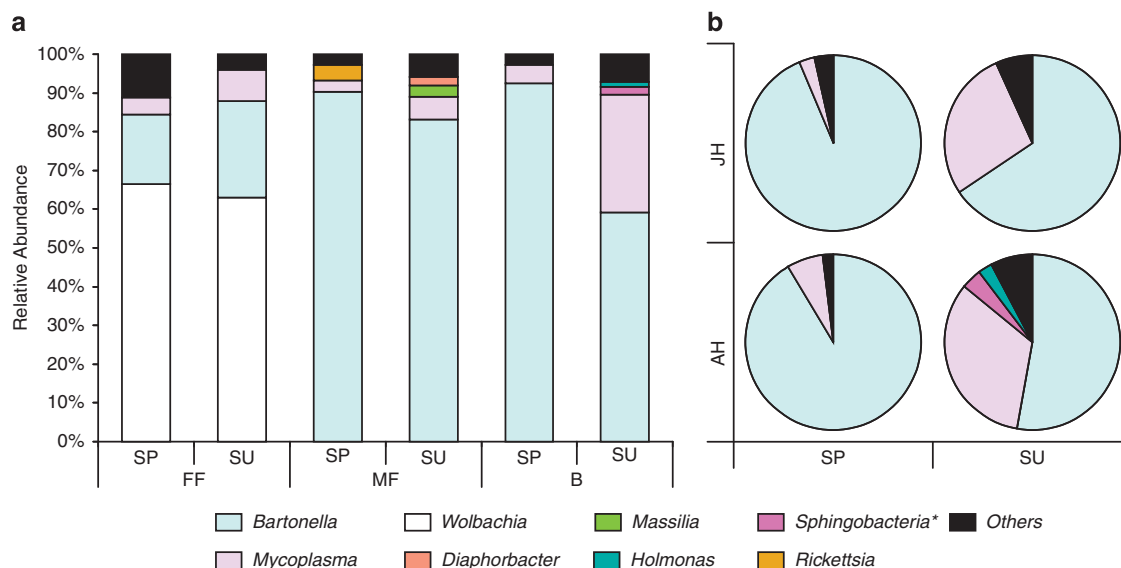


Figure 1 Mean relative abundance of bacterial genera (that is, sequences classified as a certain genus, as percentage of the total number of sequences) detected in the different sample types (female fleas (FF), male fleas (MF) or rodent blood (B)) collected from 16 female and male *G. andersoni* hosts. The hosts were trapped either as juveniles (JH) or as adults (AH) during the spring (SP), and resampled as adults at the end of summer (SU). The most abundant genera (that is, those comprising at least 20% of the total sequences in at least one DNA sample) are specified, whereas all remaining sequences are represented as ‘others’. Collective data are shown (a) from all host individuals and (b) for only blood samples. *Only class is provided because the phylotype was not classified to lower taxonomic levels.

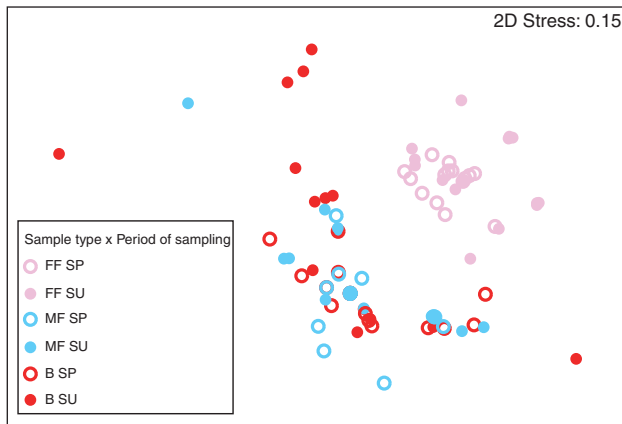


Figure 2 Nonmetric multidimensional scaling (MDS) ordination of bacterial communities in female fleas (FF; pink), male fleas (MF; blue) and host blood (B; red). Samples were collected from *G. andersoni* rodents during the spring (SP; empty circles) or summer (SU; filled circles). MDS is based on Bray–Curtis similarities in fourth root transformed abundance data of the different bacterial phylotypes. Each point represents a bacterial community from each sample, and the proximity between points represents the extent of similarity in bacterial community compositions. One outlier, namely, a bacterial community in a male flea, was omitted.

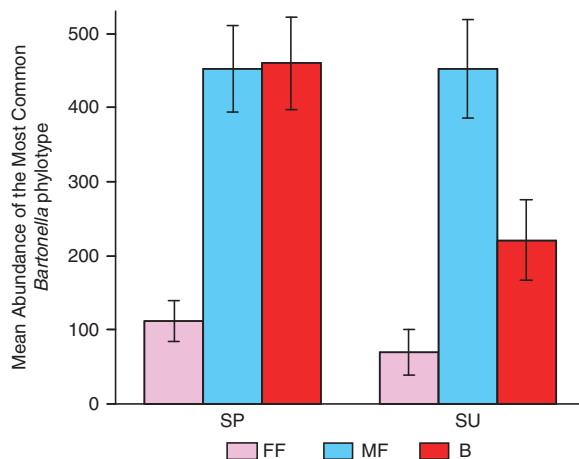


Figure 3 Mean abundance of *Bartonella* sp., which were the most abundant and influential bacterial phylotype found, in female fleas (FF; pink), male fleas (MF; blue) and rodent blood (B; red). Samples were collected from *G. andersoni* hosts, trapped during the spring (SP) and summer (SU). Data from all hosts are combined.

absolute number of *Bartonella* sequences found in *Wolbachia*-free (that is, male) fleas was significantly higher than in *Wolbachia*-infected (that is, female) fleas (574 ± 49 and 247 ± 45 , respectively; Wald $\chi^2 = 16$, $P < 0.0001$).

The significance of the ‘sample type’ \times ‘sampling season’ interaction reflects a seasonal variability in the composition of bacterial communities in female fleas (pseudo- $F = 4$, $P < 0.01$; Figure 2) and in the blood of their hosts (pseudo- $F = 3$, $P < 0.05$; Figures 1b and 2), but not in the male fleas (pseudo- $F = 0.4$, $P = 0.9$; Figure 2). A SIMPER analysis revealed that

one *Wolbachia* phylotype and one *Bartonella* phylotype, which were both more abundant in the spring than in the summer, were primarily responsible for this variability in female fleas. In the host blood, one *Mycoplasma* phylotype and one *Bartonella* phylotype explained 39% of the seasonal variability, with the abundance of *Mycoplasma* increasing and that of *Bartonella* decreasing from spring to summer (Figure 1b). However, the ratio between the average and the s.d. of dissimilarity of these two phylotypes was smaller than one, indicating that their contribution to the seasonal variability between bacterial communities in the blood is not highly consistent. After removing the two *Wolbachia* and one *Bartonella* phylotypes that were mainly responsible for the observed patterns in the composition of the bacterial communities, none of the independent variables significantly affected the composition of the bacterial phylotypes. The identity of the individual host, its gender, age or the second or third interaction terms between these variables did not have a significant effect on the composition of the bacterial phylotypes, either when all bacterial phylotypes were included or when the most common phylotypes were removed (Figures 1 and 3, Supplementary Figure S1).

The ‘sample type’ was the best predictor of bacterial community diversity (Table 1), and this was higher in female fleas than in male fleas or host blood (Figure 4a). However, after removing the two *Wolbachia* phylotypes and the one *Bartonella* phylotype that were mainly responsible for the observed patterns in the community composition, the ‘sample type’ alone was a poor predictor of community diversity (Table 1). Rather, when ignoring the two common phylotypes, the ‘sample type’ \times ‘sampling season’ interaction was the best predictor of community diversity, decreasing in female fleas and increasing in host blood between spring and summer (Table 1 and Figure 4b). In explaining the observed variation in the richness of the bacterial phylotypes, the weight of the intercept-only model was similar ($\Delta\text{QIC}_C < 2$) to that of the model that included ‘sample type’, and was higher than that of all other models ($\Delta\text{QIC}_C > 2$ for the comparison between the intercept-only model and each of the other models). This result suggests that all the tested variables are weakly associated with phylotype richness (Table 1).

Stability of bacterial communities in individual hosts

The weights of the intercept-only models were higher ($\Delta\text{QIC}_C > 2$) than those of the models that included the ‘source of variation’ or the ‘sample type’ factors, possibly indicating a high variance in the Jaccard index calculated for different combinations of paired communities. Notably, however, the stability of bacterial communities was higher between host individuals than within host individuals (Figure 5).

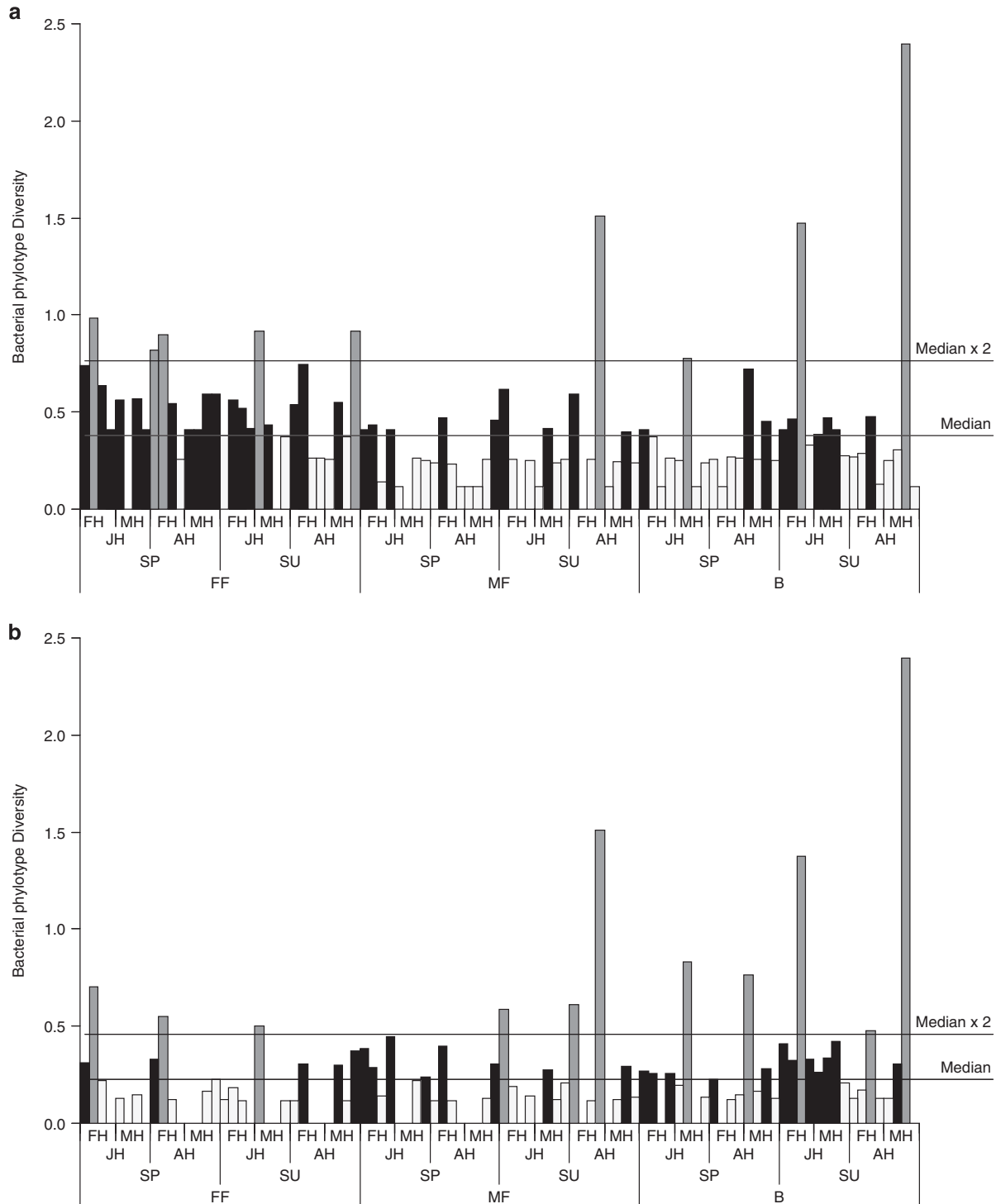


Figure 4 Bacterial phylotype diversity (mean Fisher's α -index) measured for bacterial communities obtained from female fleas, male fleas and host blood samples (FF, MF and B, respectively). Samples were obtained from female (FH) and male (MH) *G. andersoni* rodent hosts, trapped either as juveniles (JH) or as adults (AH) during the spring (SP), and resampled as adults during the summer (SU). Values below and above the median are indicated by a white and black color, respectively, and values that are more than twice the median are indicated by a gray color. (a) Data of all bacterial communities; (b) data of the bacterial communities excluding the most dominant and influential phylotypes: one *Bartonella* and two *Wolbachia* phylotypes.

Discussion

Microbial symbionts play many important roles in the evolution, development, physiology and

behavior of animals (McFall-Ngai *et al.*, 2013). However, as most studies of host-associated symbionts have focused on microbial communities in the mammalian gut (Peterson *et al.*, 2009;

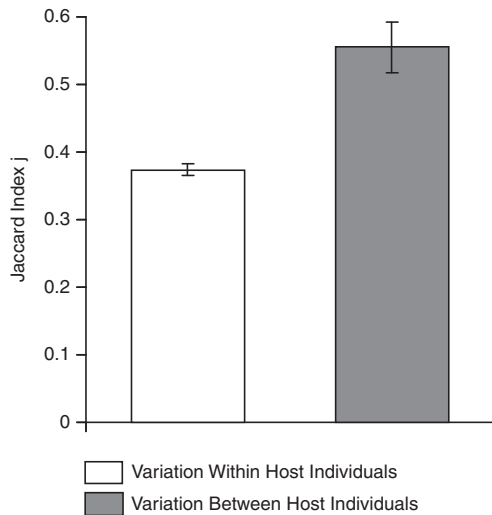


Figure 5 Comparison of microbiota stability (means \pm s.e. of the Jaccard index) within and between host individuals.

McFall-Ngai *et al.*, 2013), basic ecological principles shaping these communities remain unknown for the vast majority of species. The characterization of microbial communities associated with arthropod vector-borne bacteria is of particular interest, because these bacteria are members of two distinct communities that may be influenced, simultaneously, by the characteristics of the vector, the characteristics of its host and the temporal variations to which both the vector and the host may be exposed (Hawlena *et al.*, 2013). We report that bacterial communities in fleas and in the blood of their rodent hosts are dominated by flea-borne bacteria and are thus remarkably similar in terms of community structure and stability. Furthermore, although bacterial communities in both the fleas and the rodent hosts were similar—regardless of being isolated from different rodent individuals—they varied seasonally. In fact, possibly because of the presence of *Wolbachia* only in female fleas, the bacterial communities in the male fleas and in the blood of their rodent hosts were more similar to each other than those in female and male fleas. Taken together, our results suggest that the overall abundance of a certain flea-borne microbe is more likely to be determined by the abundance of endosymbiotic bacteria in the flea, abundance of other flea-borne microbes co-occurring in the flea and in the rodent blood or seasonal changes rather than by rodent characteristics.

Bacterial communities in fleas are remarkably similar to those in the blood of their hosts

Some bacteria in the blood of rodents can potentially be irrelevant to fleas, for example, because they are transmitted via helminths, ticks or other arthropods (Hawlena *et al.*, 2006 and Hawlena, Veciana, and Ribas, unpublished data) or via direct transmission between hosts. Hence, we hypothesized that the

bacterial communities in the flea vectors would be substantially different from those in their vertebrate hosts. In addition, because fleas acquire bacteria from the soil (which is characterized by diverse bacterial communities) and because the blood of the rodent hosts is a relatively sterile environment, we hypothesized that bacterial communities in the fleas would show greater species richness and diversity than those in the blood of their hosts.

Our hypotheses were only partly confirmed: although the bacterial communities in the fleas were different from those in the blood of their hosts, the two communities shared seven of the most common phylotypes and both exhibited low and remarkably similar phylotype richness and diversity. These similarities suggest that flea-borne bacteria form the core microbiota in both communities. Regarding *Bartonella*, experimental transmission confirms that the main *Bartonella* bacterium in our study is, indeed, flea-borne (Morick *et al.*, 2010, 2011, 2013; Gutiérrez *et al.*, 2014). In addition, the two other common *Bartonella* phylotypes were detected in both blood and flea samples (Table 2). Finally, we recently reported a pyrosequencing analysis, conducted on the same field samples used here, that targeted the citrate synthase gene to investigate *Bartonella* phylotype diversity (Gutiérrez *et al.*, 2014). The higher resolution and more specific target gene explored in that study provided us with a greater coverage, including 331 *Bartonella* phylotypes. Nevertheless, all 14 phylotypes that represented 95% of all reads were observed in both fleas and blood samples (Gutiérrez *et al.*, 2014). With respect to the detected *Mycoplasma* phylotype, we already demonstrated rodent-to-flea transmission and we are currently conducting a complement experiment to confirm flea-to rodent transmission (C Cohen *et al.*, unpublished data). Similar experiments should be conducted to confirm that the three other shared phylotypes are also flea-borne. Evidence for inter-specific interactions among vector-borne microbes have been reported in fleas and ticks and in various vertebrate hosts (Cox, 2001; Clay *et al.*, 2006; Steiner *et al.*, 2008; Jones *et al.*, 2010; Telfer *et al.*, 2010; Gutiérrez *et al.*, 2014; Kedem *et al.*, 2014). It is thus likely that the overall abundance of a specific vector-borne bacterium (for example, *Bartonella* sp. in our study) is influenced by the abundance of other phylotypes of vector-borne bacteria co-occurring in the vectors and in the blood of their hosts (for example, *Mycoplasma* sp. in our study).

The Gutiérrez *et al.* (2014) study was the first and only study, to the best of our knowledge, to have compared bacterial communities in vertebrate blood and in arthropod vectors. That study revealed some differences in *Bartonella* phylotype composition between flea vectors and blood samples from their rodent hosts. However, the intersexual distinction (that is between female and male fleas) in bacterial communities was similar or even more pronounced than the distinction between bacterial communities

in fleas and blood in both macropatterns (that is, different genera, in our study) and micropatterns (that is, different species and genotypes in the study by Gutiérrez *et al.*, 2014) of the community structure. This fact suggests that the bacterial communities in the flea vectors and in the blood of their vertebrate hosts are shaped by similar factors. One possibility is that the blood meal remnants in the gut of fleas are sufficiently close to the blood in live gerbils to present similar conditions (for example, similar substrate, thermal conditions or resources). Accordingly, the body of the rodent or flea may not 'filter out' the dominant phylotypes that are introduced by the flea or the rodent, respectively. However, even if bacteria in fleas and in rodents are exposed to different conditions, they may still be exposed to similar selective forces, reducing the possible number of coexisting bacteria via, for example, interspecific competition between bacteria (see, for example, Macaluso *et al.*, 2002; Hawlena *et al.*, 2012; Zouache *et al.*, 2012) or the immunity of the rodent hosts (see, for example, Cox, 2001) and flea vectors (Vallet-Gely *et al.*, 2008) to their bacterial inhabitants. By extrapolation, low bacterial diversity likely characterizes any microbial community in arthropod vectors and in the blood of their vertebrate hosts. This observation may simplify the efforts to control emerged vector-borne pathogens by manipulating the density of their bacterial inhabitants (see, for example, Cirimotich *et al.*, 2011). This is because, in poor bacterial communities, we have only a few candidate species that could affect pathogen densities, and this should ease the screening and experimental procedures.

The composition of bacterial communities in fleas and in the blood of their rodent hosts has only a weak association with host characteristics and exhibits variations from spring to summer

One of the main goals in the study of microbiomes is to investigate the causes for the temporal and spatial variability in the composition of microbial communities. Although some degree of interhost variability was detected (Supplementary Figure S1), we found indications that the variability in bacterial community composition was lower between host individuals than within host individuals, and that it was similar between juvenile and adult hosts and between female and male hosts. From spring to summer, the relative abundance of *Wolbachia* and *Bartonella* phylotypes in female fleas decreased, whereas that of *Mycoplasma* sp. in the blood of the hosts increased, in a manner that was independent of the host identity, their sex or their maturation timeline (Table 1, Figures 1b and 3 and Supplementary Figure S1). Seasonal interhost variability in physiology, immunity and behavioral responses to symbionts is often most pronounced when comparing juveniles with adults and males with females (Hudson and Dobson, 1997; Wilson

et al., 2002; Mooring *et al.*, 2004; Hawlena *et al.*, 2005; Altizer *et al.*, 2006; Grassly and Fraser, 2006; Krasnov *et al.*, 2006). However, the fact that bacterial communities in all gerbil groups have passed through similar seasonal changes suggest that these changes may not be linked to changes in the internal environment of the host. Other studies reported that microbial communities show high variability between host individuals, age groups and sexes and/or a relatively high temporal stability (Moran *et al.*, 2005; Costello *et al.*, 2009; Godoy-Vitorino *et al.*, 2010; Oh *et al.*, 2012; Yatsunenkov *et al.*, 2012; Faith *et al.*, 2013; van Dongen *et al.*, 2013; Kueneman *et al.*, 2014; Leclaire *et al.*, 2014). The lack of interindividual variations in the composition of bacterial communities in our study may result from the transmission of fleas between hosts via direct interactions, replacement of burrows or free dispersal (Krasnov, 2008). These processes may equilibrate the microbial communities across host individuals, given that vector-borne phylotypes are the core of these communities.

The spring-to-summer variations in bacterial communities in the female fleas may be explained, in part, by the nature of ectoparasitic vectors that are affected by the external environment of their host (Krasnov, 2008). Fleas spend time in the burrows of their rodent hosts during both their free-living (when they consume organic matter) and parasitic (adult) life stages. Thus, the bacteria associated with fleas can be affected by extrinsic factors that vary across seasons. For example, it is possible that the composition of microbial communities changes in response to an increase in flea burden over summer months (Lehmann, 1989) or that, similar to other flea species (Krasnov *et al.*, 2002), *S. cleopatrae* fleas in the field undergo physiological changes that affect the relative abundance of *Wolbachia* species in females. The spring-to-summer changes that we found in bacterial communities in the host blood (Figure 1b) may have resulted from accumulation of some bacterial phylotypes—such as the *Mycoplasma* sp. found in both flea and blood samples—that, once acquired, persist in the host. Indeed, our lab experiments show that *Mycoplasma* sp. can persist in the blood of an individual *G. andersoni* rodent for >3 years (C Cohen *et al.*, unpublished data). If resources for these bacterial phylotypes are limited in the blood of the rodent host, then the accumulation of these phylotypes may come at the expense of other phylotypes. To determine whether the observed spring-to-summer variations in flea-borne bacteria reflect a more general temporal pattern, one should sample microbes in additional timeframes and in multiple years and vector species. Reports of climatic influences on microbial abundance and composition in flies, mosquitoes and ticks support a general role of seasonality (Kirstein *et al.*, 1997; Roper *et al.*, 1998; Sutherst, 2004; Wielinga *et al.*, 2006; Ostfeld, 2009; Lázár *et al.*, 2012). If seasonal variability in microbial community structure

indeed plays an important role in determining the overall abundance of vector-borne bacteria, then future investigations of the specific causes of seasonal variability are encouraged.

Wolbachia phylotypes play an important role in shaping the bacterial community in female fleas

The differences in bacterial composition between the sample types and the significantly higher bacterial diversity in female than in male fleas or in the host blood were attributed to the *Wolbachia* phylotypes. *Wolbachia* species are common endosymbiotic bacteria in arthropods, exerting several phenotypic effects on their hosts and generally behaving as reproductive parasites that distort the sex ratio of arthropods toward females. *Wolbachia* bacteria have been previously detected in fleas, but the nature of this association is not yet known (Gorham *et al.*, 2003). In our *S. cleopatrae* flea samples, *Wolbachia* phylotypes were found exclusively in females and comprised a significant portion of the bacterial community in all samples collected from female fleas. This finding suggests that *Wolbachia* are transmitted vertically between fleas and that feminization, male killing or both mechanisms are involved in the *Wolbachia*–flea relationships (Werren *et al.*, 2008). However, despite the high prevalence of *Wolbachia* in the female flea population, the flea sex ratio was not distorted toward females (see Supplementary Results). Regardless of the mechanism through which *Wolbachia* affects flea populations, our results suggest that the presence of *Wolbachia* affects the load of *Bartonella* species—which are the most common flea-borne bacteria in our study system. In our study system, the effects of the flea sex and of *Wolbachia* presence or absence are confounded, as these bacteria were restricted to female fleas. Thus, to confirm a direct negative association between *Wolbachia* and *Bartonella*, one should manipulate the abundance of *Wolbachia* in female fleas and quantify (for example, by using a quantitative PCR analysis) the resulting number of *Bartonella* cells.

Wolbachia bacteria thus appear to play an important role in shaping natural communities that are associated with flea-borne bacteria. Other vector endosymbionts are known to shape microbial communities in mosquitoes, fleas, aphids and ticks (Philip *et al.*, 1976; Noda *et al.*, 1997; Aksoy, 2000; Macaluso *et al.*, 2002; Gonzalez-Ceron *et al.*, 2003; Scarborough *et al.*, 2005; Cirimotich *et al.*, 2011), indicating, more generally, that the abundance of bacterial endosymbionts are good predictors of the abundance of vector-borne bacteria. From an applied perspective, the sex-related differences in bacterial community structure indicate that male but not female fleas are probably the main source of flea-borne pathogens. The same conclusion may hold true for any arthropod vector that harbors a reproductive parasite, suggesting that efforts to control these vectors should focus on the males.

Summary and conclusion

We used deep sequencing to provide a comprehensive catalog of bacteria that naturally inhabit rodent blood and fleas. We report that, in both the rodent blood and the fleas, bacterial communities within an individual are characterized by a very low diversity and are dominated by flea-borne phylotypes. In addition, the structure of bacterial communities is remarkably similar in fleas and in the blood of their rodent hosts, with the exception of *Wolbachia* bacteria that are present exclusively in female fleas. Finally, we show that, whereas the structure of communities in both the rodent blood and the fleas is largely independent of host characteristics, both these communities are prone to seasonal variations. These findings are consistent with data on other vector-borne microbes (Philip *et al.*, 1976; Pumpuni *et al.*, 1993; Kirstein *et al.*, 1997; Noda *et al.*, 1997; Roper *et al.*, 1998; Aksoy, 2000; Cox, 2001; Macaluso *et al.*, 2002; Gonzalez-Ceron *et al.*, 2003; Sutherst, 2004; Scarborough *et al.*, 2005; Clay *et al.*, 2006; Steiner *et al.*, 2006; Wielinga *et al.*, 2006; Ostfeld, 2009; Jones *et al.*, 2010; Telfer *et al.*, 2010; Cirimotich *et al.*, 2011; Lalzar *et al.*, 2012). Collectively, they highlight the following candidate factors as affecting the overall abundance of a specific vector-borne microbe: (1) the abundance of microbial endosymbionts of vectors, (2) the abundance of other vector-borne microbes that co-occur in the vector and in the blood of its host and (3) seasonal variation in the host environment. Our findings thus indicate that efforts to prevent the spread of such vector-borne pathogens should focus on monitoring and controlling the three factors specified above.

Conflict of Interest

The authors declare no conflict of interest.

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