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# Changes in Bacterial Diversity, Composition and Interactions During the Development of the Seabird Tick *Ornithodoros maritimus* (Argasidae)

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## Abstract

Characterising within-host microbial interactions is essential to understand the drivers that shape these interactions and their consequences for host ecology and evolution. Here, we examined the bacterial microbiota hosted by the seabird soft tick *Ornithodoros maritimus* (Argasidae) in order to uncover bacterial interactions within ticks and how these interactions change over tick development. Bacterial communities were characterised through next-generation sequencing of the V3–V4 hypervariable region of the bacterial 16S ribosomal RNA gene. Bacterial co-occurrence and co-exclusion were determined by analysing networks generated from the metagenomic data obtained at each life stage. Overall, the microbiota of *O. maritimus* was dominated by four bacterial genera, namely *Coxiella*, *Rickettsia*, *Brevibacterium* and *Arsenophonus*, representing almost 60% of the reads. Bacterial diversity increased over tick development, and adult male ticks showed higher diversity than did adult female ticks. Bacterial networks showed that co-occurrence was more frequent than co-exclusion and highlighted substantial shifts across tick life stages; interaction networks changed from one stage to the next with a steady increase in the number of interactions through development. Although many bacterial interactions appeared unstable across life stages, some were maintained throughout development and were found in both sexes, such as *Coxiella* and *Arsenophonus*. Our data support the existence of a few stable interactions in *O. maritimus* ticks, on top of which bacterial taxa accumulate from hosts and/or the environment during development. We propose that stable associations delineate core microbial interactions, which are likely to be responsible for key biological functions.

**Keywords** *Ornithodoros maritimus* · Bacterial interactions · Network analyses · 16S rRNA gene

Pablo Tortosa and Karen D. McCoy contributed equally to this work.

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## Introduction

Symbiotic associations between microorganisms and their invertebrate hosts, where partners share essential biological functions and work as a holobiont, are the subject of an increasing number of studies [1, 2]. However, identifying these associations can be difficult given the general richness of the microbiome in most individuals. Quantifying within-host variation in microbial composition over time can help detect these associations, along with the drivers that shape them. Such studies require multifaceted investigations that target different aspects of the host biology and ecology and take into account changing environmental conditions [3].

In arthropods, essential microbial interactions required for completion of the host life cycle have been repeatedly demonstrated, but most investigations focus on one or a limited number of microbial taxa, particularly obligate endosymbionts [4–6]. The advent of next-generation sequencing (NGS) technologies has led to major advances in the characterisation of microbiota [7–10]. These technologies enable the description of microbial communities in terms of both taxonomic diversity and relative abundance. They also allow us to infer microbial interactions using positive and negative correlations between the numbers of reads obtained for each bacterial taxon [11, 12] and can be explored in related host species or within a single host species throughout its development. For example, Hegde et al. [12] have shown that bacterial network associations in the mosquitoes *Aedes albopictus* and *Culex quinquefasciatus* are less complex than in *Aedes aegypti*. Interestingly, this study also demonstrated that some bacterial interactions were common to several mosquito species and stable regardless of the environment, while others appeared more dynamic [12].

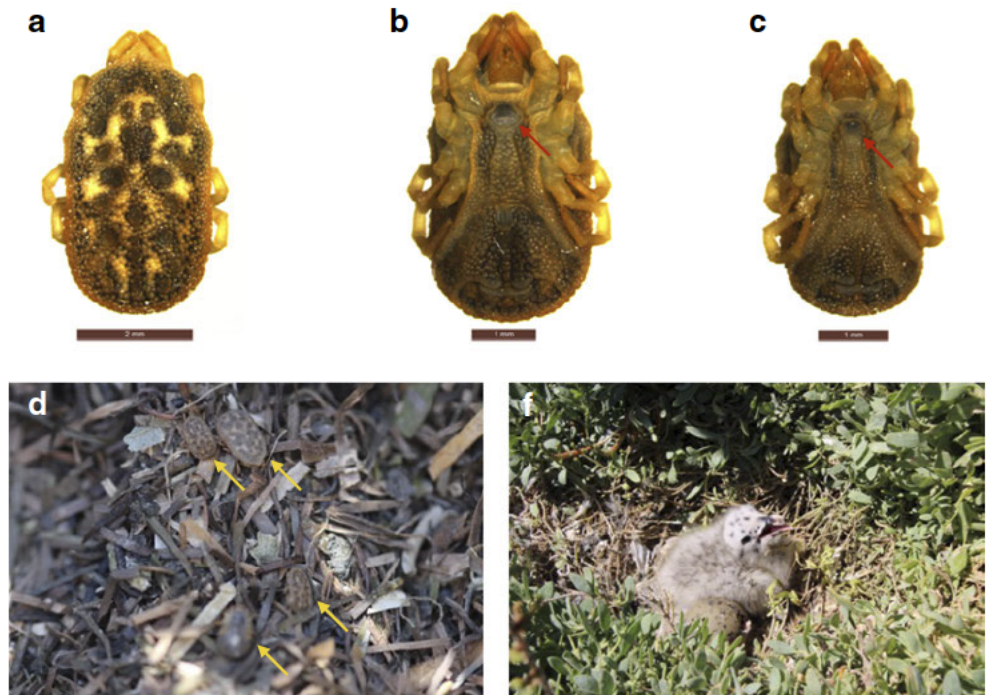
Ticks (order: Ixodida) are haematophagous ectoparasites that exploit a large range of vertebrate hosts (i.e. reptiles, birds and mammals, including humans) and are distributed across the globe [13]. They represent an important group of vectors and transmit numerous diseases of significant importance for animal and human populations (e.g. Lyme disease, relapsing fever, Crimean-Congo haemorrhagic fever virus, etc.) [13, 14]. Given the medical and veterinary importance of ticks, understanding the diversity of their microbiota and the nature of within-holobiont interactions (i.e. among microbiota and between host and microbiota) may provide tools for the control of tick-borne diseases [15, 16]. Although relatively few NGS studies have examined tick microbiomes to date, most of these focus on bacterial communities sheltered by hard ticks (family: Ixodidae) [7, 16, 17], and little is known about these communities in soft ticks (family: Argasidae) [18–20]. These NGS studies have shown that tick microbiota include a remarkable diversity of endosymbiotic, commensal and pathogenic bacteria. Some bacterial taxa are overwhelmingly dominant and considered as essential endosymbionts in several tick species

[7, 16, 21, 22]. Among these genera, *Coxiella* and *Francisella* have been shown to play a key role in tick biology by provisioning essential nutrients [18, 23–25]. In addition to *Coxiella* and *Francisella*, many other maternally inherited bacteria, such as *Arsenophonus* and *Cardinium*, are present in ticks. Although their action in the host remains to be documented, these bacteria are usually considered facultative symbionts [21, 22], exhibiting variable infection frequencies in tick populations across diverse temporal and spatial scales [22]. While facultative symbionts are not required for host survival, they are known to encode essential traits in many arthropods: some manipulate reproduction to favour their transmission, while others protect their host against natural enemies or facilitate adaptation to changing environments [5, 26]. As hosts can vary in the number and types of facultative symbionts they harbour, heritable and functionally important phenotypic variation can exist within arthropod populations [27, 28].

Tick bacterial assemblages studied to date have been shown to vary according to different biological factors such as species, life stage, age and sex, as well as by the investigated tissue and environmental conditions [7, 29–41]. However, few studies have thus far investigated bacterial interactions within ticks [33, 42–45], with the exception of those focusing on obligate symbionts (e.g. [14]). An analysis of *Ixodes ricinus* microbiota suggested that virtually no bacterial genus is required for the co-occurrence of another genus [43]. However, both negative and positive interactions (i) between endosymbionts, (ii) between vertebrate pathogens (referred herein as pathogens) and (iii) between endosymbionts and pathogens have been reported in ticks. For instance, Duron et al. [22] detected co-occurrence and mutual exclusion patterns within the symbiotic bacterial communities of 81 tick species. *Francisella* and *Rickettsia* endosymbionts appeared mutually exclusive in *Dermacentor occidentalis* [44], and exclusion patterns were demonstrated for *Francisella* and *Coxiella* endosymbionts on different *Amblyomma* species [46]. Moutailler et al. [47] reported a positive association between the Lyme disease pathogens *Borrelia garinii* and *Borrelia afzelii* in *I. ricinus*. In *D. andersoni*, Gall et al. [48] found a negative correlation between the endosymbiont *Rickettsia bellii* and the pathogen *Anaplasma marginale* and a positive correlation between *Francisella* endosymbionts and the pathogen *Francisella novicida*. Previous studies also reported exclusion between symbiotic and pathogenic *Rickettsia* [49, 50]. Understanding the nature of these interactions is thus complex and, as of yet, incomplete.

The purpose of the present study was to characterise the bacterial microbiota associated with the soft tick *Ornithodoros maritimus* (family: Argasidae) and to examine the nature of bacterial interactions according to life stage (i.e. larva, nymph and adult). *O. maritimus* is a nidicolous soft tick species (Fig. 1) living in or around the nests of their seabird hosts [51], where all life stages co-occur. These ticks feed on birds

**Fig. 1** *Ornithodoros maritimus* ticks from the islet of Carteau. **a** Dorsal and **b** ventral views of a non engorged adult female. **c** Ventral view of an adult male. The red arrows indicate the gonopore of each specimen showing the sex specific shape, females with diamond shaped gonopores and males with oval shaped gonopores. **d** *O. maritimus* in a yellow legged gull (*Larus michahellis*) nest indicated by yellow arrows. **e** A yellow legged gull chick and egg in a nest on Carteau. All photo credits: M. Dupraz



during resting periods, generally at night, taking numerous short bloodmeals on the host as nymphs and adults (several minutes to 1h) and one relatively long bloodmeal (several hours) in the larval stage [52]. Reported from southern Great Britain to North Africa, *O. maritimus* has been recorded parasitising a large range of seabird species (auks, cormorants, gulls, kittiwake, petrels and terns) [53, 54]. Hyperinfestations by *O. maritimus* have been associated with negative effects on some seabird species, but the overall impact of these ticks on host fitness is poorly studied [55–57]. Likewise, although known human and animal pathogens have been reported from *O. maritimus* (e.g. *Borrelia turicatae*, Soldado and West Nile viruses), knowledge on the effect of these bacteria on birds is lacking [53]. Several other bacteria, phylogenetically related to known pathogens, may also have pathogenic potential, but require more study [22, 52, 57–62].

We sampled different life stages of *Ornithodoros maritimus* from a single breeding colony of the Yellow-legged gull (*Larus michahellis*) found on the islet of Carteau in the Gulf of Fos (Camargue region, Southern France). This sampling scheme enabled us to collect ticks within a relatively closed system, controlling for factors such as host preference (only one host species present), seasonality (sampling over a 6-week period) and geographic scale (one small and homogeneous island). In addition, a previous study suggested no structure in the presence of targeted infectious agents in adult female ticks among nests within this colony [52]. Thus, we hypothesised that any differences in tick bacterial communities and interactions would be related to changes over the course of tick development. Using 16S ribosomal RNA

Illumina sequencing, we first described the bacterial microbiota sheltered by field-collected ticks. We then tested whether the developmental stage (larva, nymph and adult) and/or sex influenced the bacterial microbiota composition. Finally, we constructed bacterial networks and examined co-occurrence in order to understand how bacterial interactions shift through tick development and across sexes.

## Materials and Methods

### Tick Collection

The ticks used in the present work were sampled in the context of a study on the seasonal dynamics of *Ornithodoros maritimus* and its associated pathogens; see Dupraz et al. [52]. In this study, the authors sampled ticks from 30 yellow-legged gull nests in the colony of Carteau during a single breeding season (GPS coordinates: 43° 22' 39" N 4°51' 28" E). Carteau is a small sandy islet within the Gulf of Fos that harbours a monospecific yellow-legged gull-breeding colony. Ticks were collected between March and May 2015 with one collection session per week during six successive weeks. Following collection, the ticks were stored in 70% ethanol and brought back to the lab where they were morphologically identified based on classical criteria [54]. The sex of adult ticks was determined based on gonopore shape (Fig. 1). The ticks then were kept frozen at –20 °C until nucleic acid extraction and molecular analyses. A

subsample of 50 specimens from this tick collection was used in the present study and corresponded to 11 larvae, 12 nymphs and 27 adults (14 females and 13 males).

### DNA Extraction, 16S Ribosomal RNA Gene Sequencing and Bioinformatic Analyses

Each tick was rinsed in two successive baths of sterile water to remove ethanol. Whole ticks were individually crushed in a 1.5-mL tube containing 180  $\mu$ L of ATL buffer and incubated overnight at 56 °C with 20  $\mu$ L of proteinase K. DNA was then extracted using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Les Ulis, France) following the manufacturer's recommendations. One negative control was included in the extraction protocol to identify DNA contaminations produced during the extraction process.

The bacterial microbiota of each tick was investigated through deep sequencing of the V3–V4 hypervariable region of the bacterial 16S ribosomal RNA gene (16S rRNA gene). In summary, the V3–V4 region (V3, forward primer: 5'-TACGGRAGGCAGCAG-3' and V4, reverse primer: 5'-CTACCNNGGTATCTAAT-3') was amplified from 5 ng of DNA from each sample and the negative DNA extraction control following the MetabioTE® protocol for a standard amplicon library preparation (Genoscreen, Lille, France). A positive and a negative PCR control, corresponding to an assemblage of known bacteria and pure water, respectively, were added during library preparation by Genoscreen. The final amplification products (each containing a nucleotide index to differentiate the samples, as well as the adapters necessary for carrying out the sequencing) were purified on beads and then mixed in equal concentrations. Sequencing was realized with a 2  $\times$  250-bp paired-end chemistry using Illumina MiSeq technology (Genoscreen, Lille, France). The Illumina's CASAVA software 1.8 was used to de-multiplex the sequence reads and produce fastq files. Primers were removed, and sequences were trimmed when the Phred quality score (Q score) was inferior to 30. Assembly of the two-paired sequences was realized with the FLASH tool [63], with a minimum overlap of 30 bases and at least 97% identity in the overlapping area. The generated sequences were analysed with the software MOTHUR (version 1.35.1) [64] following the MiSeq SOP protocol [65]. Briefly, we retained sequences presenting (i) a size comprised between 400 and 440 bp, (ii) no ambiguous bases and (iii) a maximum of eight homopolymers. Chimeras were removed from the analysis with UCHIME [66]. Operational taxonomic units (OTUs) were generated based on 97% sequence similarity using the OptiClust clustering method [67]. The SILVA database release 138 [68] was used for sequence alignments and taxonomical assignments of OTUs. The estimated Good's coverage [69] was calculated in MOTHUR in order to check the representation of the bacterial community in each sample; an

index superior to 0.97 indicates a satisfactory per sample sequencing depth. As described in Minard et al. [9] and René-Martellet et al. [37], we removed all OTUs from the analysis for which the relative abundance was not at least ten times superior to that detected in negative extraction and PCR controls. OTUs represented by only one sequence were removed from the analysis. Finally, sequences were proportionally normalised to the sample with the smallest number of reads ( $n = 2814$  reads). For an overview of the bioinformatics workflow, see Online Resource 1 Fig. S1.

### Bacterial Diversity and Community Structure

We assessed alpha diversity at the OTU level and performed non-parametric comparisons between the different tick life stages, i.e. larvae, nymphs and adults (three groups). In addition, we performed comparisons between sexes in adult ticks (two groups). Previous studies indicated that the relatively high abundance of *Rickettsia* in female ticks could explain low bacterial diversity in this sex [39, 40]. To test this hypothesis, we compared the alpha bacterial diversity between male and female ticks with three additional datasets, corresponding to data with the dominant taxa removed: (i) *Rickettsia* sequences removed, (ii) *Coxiella* sequences removed and (iii) both *Rickettsia* and *Coxiella* sequences removed (Online Resource 1 Fig. S1). Alpha diversity was evaluated using Shannon's diversity index, which was converted into an equivalent number of species [70]. Differences in alpha diversity across tick life stages were tested with a Kruskal-Wallis test, followed by post-hoc pairwise comparisons to assess the significance of differences between each tick life stage. The pairwise comparison tests were realised using the function "kruskalmc" from the R package "pgrimess" [71]. For sex, differences in alpha diversity were tested using Wilcoxon tests. To examine differences in community composition among life stages, non-metric multidimensional scaling (NMDS) ordination based on Bray-Curtis dissimilarities was performed. In order to test if microbial communities differ according to the life stage or sex, we then performed permutational multivariate analysis of variance (PerMANOVA) on the Bray-Curtis dissimilarities. All analyses were performed using the R software (version 3.4.3) [72]. We used a linear discriminant analysis (LDA) of effect size (LEfSe) [73] under Galaxy (<http://huttenhower.sph.harvard.edu/lefse/>) to identify bacterial genera whose relative abundance significantly differed according to life stage and sex, and that best discriminated each tick group ( $p$  value  $\leq 0.05$  and LDA score  $> 2.0$ ).

### Bacterial Network Analyses

One bacterial network was generated at the genus level for each tick life stage (larva, nymph and adult) and sex in adults.

For each of the five groups, network construction was performed using bacterial taxa present in at least 50.0% of tick specimens and whose relative abundance was superior to 0.1% of reads in order to discard rare taxa in the generated data. Based on the procedure described by Faust and Raes [74], bacterial networks were generated using CoNet plug-in (version 1.1.1.beta) [74] implemented in Cytoscape (version 3.3.0) [75]. Significant associations were identified using five methods: two correlation methods (Pearson and Spearman), two dissimilarity distance methods (Bray-Curtis and Kullback-Leibler) and mutual information similarity. The *p* values calculated for all methods were merged using Brown's method [76], and false-discovery rates were corrected using Benjamini and Hochberg's procedure [77]. Finally, bacterial associations in the networks were represented as either co-occurrence (positive correlation) or co-exclusion (negative correlation) considered herein as positive or negative interactions, respectively. Investigating the presence of significant interactions and their sign across the different groups allowed us to detect changes in bacterial interactions over tick development and in relation to sex.

## Results

### Bacterial Diversity and Community Structure

For each sample, a Good's coverage index superior to 0.98 was obtained, indicating a satisfactory representation of the bacterial assemblage present in the samples (Table 1 and Online Resource 2 Table S1). In negative PCR and DNA extraction controls, 11 sequences (represented by 5 OTUs) and 17,998 sequences (represented by 37 OTUs) were detected, respectively (Online Resource 2 Table S2). In the negative DNA extraction control, 75.1% and 11.7% of the sequences were classified as *Escherichia Shigella* and *Sphingomonas*, respectively. These genera have been previously reported as contaminants [78, 79]. Thus, based on our cleaning criteria, we removed a total of 38 OTUs (represented by 104,716 reads) from the tick sample (Online Resource 2 Table S3). The remaining 4 OTUs were not removed from the data, as their relative abundance was ten times higher in ticks compared with controls. We also removed 1819 OTUs represented

by a single read (Online Resource 2 Tables S4 and S5). Finally, after the different bioinformatic cleaning steps, a total of 886,359 reads were retained from the sampled ticks, with a mean of 17,727 reads per specimen (range 2814–26,748) (Online Resource 2 Table S5). Based on the normalised data, the phyla Proteobacteria (71.0%), Actinobacteria (13.8%), Firmicutes (9.4%) and Bacteroidetes (5.4%) represented more than 99.0% sequences. Sequences were distributed into 2575 OTUs and 864 genera among which four, namely *Coxiella*, *Rickettsia*, *Brevibacterium* and *Arsenophonus*, accounted for almost 60% of all sequences. *Coxiella* (37.0% of reads), *Rickettsia* (10.8% of reads), *Brevibacterium* (6.6% of reads) and *Arsenophonus* (5.5% of reads) were detected in 100.0%, 96.0%, 98.0% and 90.0% of the specimens, respectively (Fig. 2 and Online Resource 2 Table S6). The relative abundance of each genus varied at the individual level (Fig. 2). Sequences belonging to *Coxiella* were represented mainly by one OTU whose 16S sequence corresponded to the *Coxiella* endosymbiont previously reported in *O. maritimus* [58]. The genera *Kocuria* and *Rickettsiella* were detected in our data with 330 and 28 sequences respectively, based on normalised data. The genera *Wolbachia* was considered as absent with only 1 sequence reported from the normalised data. In addition, none of the following previously described tick-associated bacteria was found in our samples: *Anaplasma*, *Bartonella*, *Borrelia*, *Cardinium*, *Ehrlichia*, *Francisella*, *Lariskella*, *Midichloria* and *Spiroplasma*.

Alpha diversity increased significantly with life stage ( $n = 50$ , Kruskal-Wallis test,  $X^2 = 11.579$ ,  $df = 2$ ,  $p$  value  $< 0.005$ ) with larvae, nymphs and adults presenting average Shannon number-equivalent diversity values of  $7.33 \pm 5.44$ ,  $16.58 \pm 24.81$  and  $23.54 \pm 22.19$ , respectively (Table 1). Multiple comparison tests indicated that alpha diversity was significantly different between larvae and adults (post-hoc Kruskal-Wallis,  $p$  value  $< 0.05$ ), nymphs and adults (post-hoc Kruskal-Wallis,  $p$  value  $< 0.05$ ), but not between larvae and nymphs (post-hoc Kruskal-Wallis,  $p$  value  $> 0.05$ ). Within adults, alpha diversity differed according to sex ( $n = 27$ , Wilcoxon test,  $W = 12$ ,  $p$  value  $< 0.001$ ), with males displaying higher bacterial diversity (average Shannon diversity index =  $37.68 \pm 24.72$ ) than females (average Shannon diversity index =  $10.40 \pm 4.01$ ) (Table 1). Relative sample sizes did not seem to be an issue for these analyses; power analyses, using the "kwpower"

**Table 1** Summary of Good's coverage index, OTUs and Shannon number equivalent diversity obtained for the microbiota of *O. maritimus*

Type	Good's coverage index	Total OTUs detected	Average number OTUs/sample	Shannon number equivalent diversity
Larvae ( $n = 11$ )	$0.998 \pm 0.001$	345	$57.27 \pm 30.26$	$7.33 \pm 5.44$
Nymphs ( $n = 12$ )	$0.995 \pm 0.005$	1382	$234.25 \pm 185.94$	$16.58 \pm 24.81$
Adults ( $n = 27$ )	$0.994 \pm 0.003$	2185	$359.41 \pm 161.90$	$23.54 \pm 22.19$
Males ( $n = 13$ )	$0.991 \pm 0.003$	1929	$473.92 \pm 128.80$	$37.68 \pm 24.72$
Females ( $n = 14$ )	$0.996 \pm 0.002$	1236	$253.07 \pm 107.00$	$10.40 \pm 4.91$

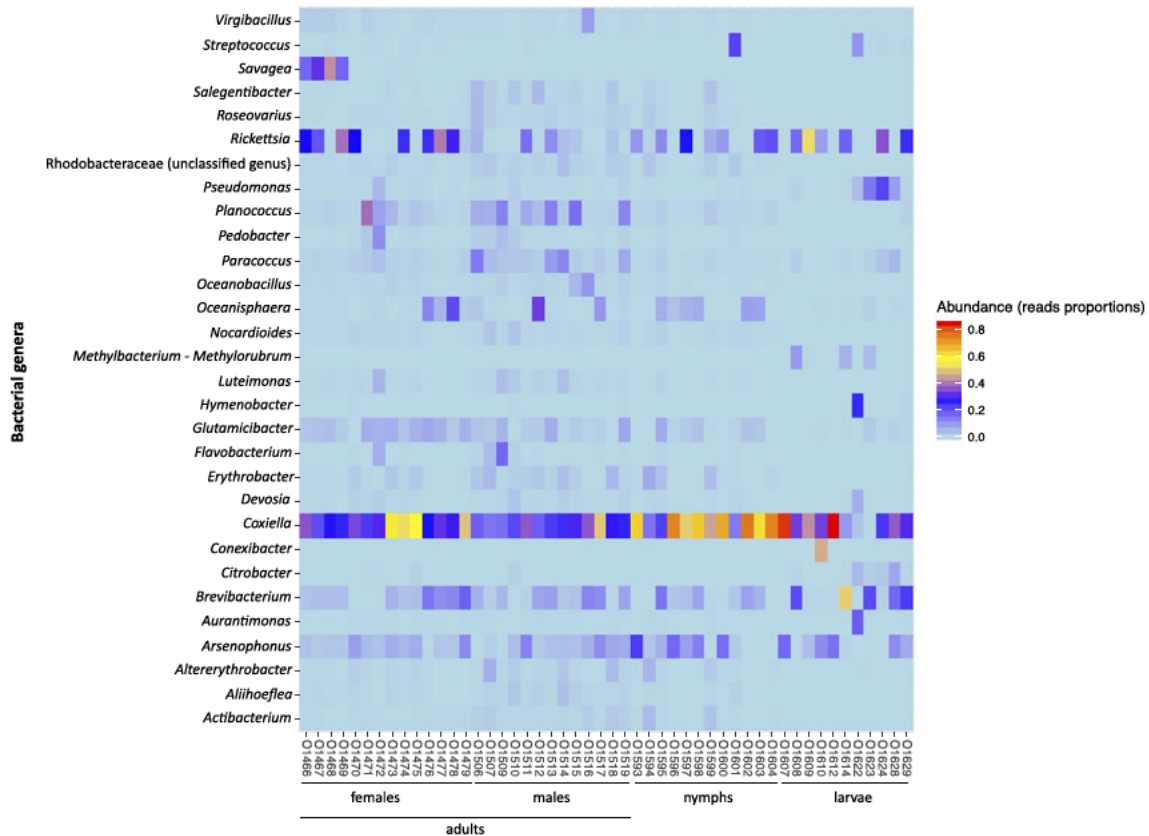


Fig. 2 Heatmap of the top 30 genera detected in *O. maritimus* according to tick life stage and sex

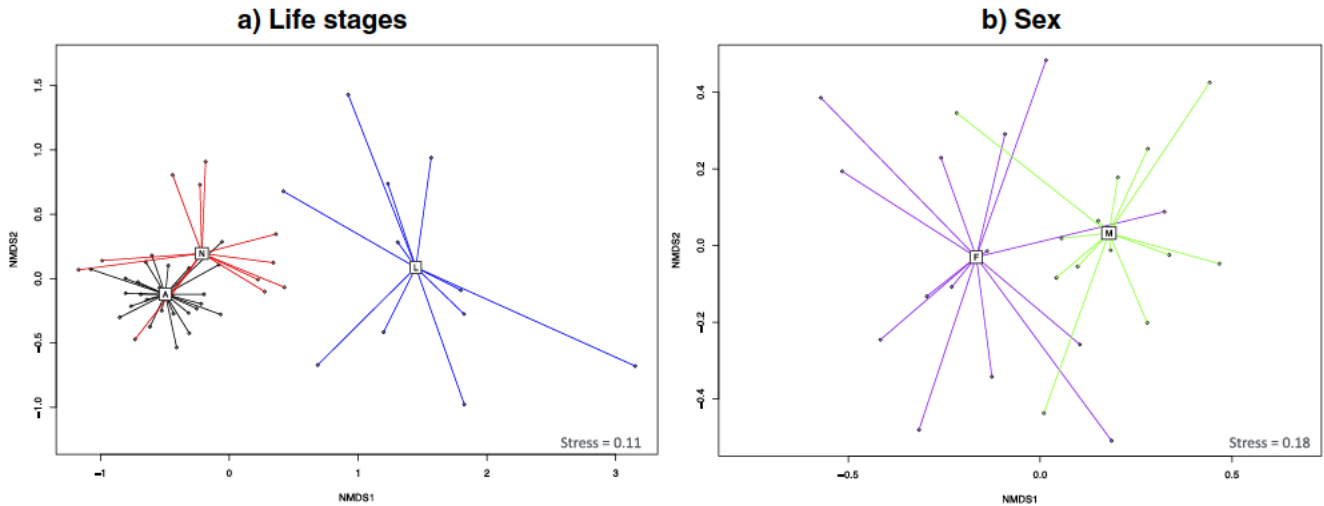
function of the “MultNonParam” R package [80], revealed an estimated power above 0.95 when observed diversity values were used as estimates for effect sizes, but also when effect sizes were lowered by one order of magnitude. *In silico* removal of *Rickettsia* and/or *Coxiella* sequences did not change the results as alpha diversity remained significantly higher in males compared with females for all datasets (without *Rickettsia* sequences:  $n = 27$ , Wilcoxon test,  $W = 14$ ,  $p$  value  $< 0.001$ ; without *Coxiella* sequences:  $n = 27$ , Wilcoxon test,  $W = 21$ ,  $p$  value  $< 0.001$ ; without *Rickettsia* and *Coxiella* sequences:  $n = 27$ , Wilcoxon test,  $W = 29$ ,  $p$  value  $< 0.01$ ).

Bacterial communities were significantly structured across tick life stages (PerMANOVA,  $F$ -stat = 4.03,  $df = 2$ ,  $p$  value = 0.001), between larvae and nymphs (PerMANOVA,  $F$ -stat = 2.65,  $df = 1$ ,  $p$  value = 0.015), between larvae and adults (PerMANOVA,  $F$ -stat = 4.74,  $df = 1$ ,  $p$  value = 0.001) and between nymphs and adults (PerMANOVA,  $F$ -stat = 4.23,  $df = 1$ ,  $p$  value = 0.001) (Fig. 3a, Online Resource 1 Fig. S2). Within adults, the bacterial community was also structured according to sex (PerMANOVA,  $F$ -stat = 4.59,  $df = 1$ ,  $p$  value = 0.001) (Fig. 3b). Some tick specimens seemed to harbour the microbial community of the other sex (Fig. 3b); this pattern may result from mistakes in sexing adult specimens, which can occur for recently moulted ticks.

The LEfSe results indicated that the genus *Devosia* was more abundant in larvae while *Tropicimonas* and *Lacticigenium* genera were more abundant in nymphs (Fig. 4a). Compared with larvae and nymphs, 36 genera were found to be more abundant in adult ticks, such as *Planococcus* and *Pedobacter*. In adults, 135 genera were significantly more abundant in males compared with females, including *Paracoccus*, *Planococcus* and *Erythrobacter*, whereas only nine genera were significantly more abundant in females compared with males, including *Coxiella*, *Savagea* and *Glutamicibacter* (Fig. 4b).

### Bacterial Network Analyses

The networks generated for the three life stages were largely dominated by positive interactions (Table 2, Online Resource 1 Figs. S3 and S4, Online Resource 3 Table S7). Indeed, co-occurrence represented 76%, 97% and 94% of the interactions detected in larval, nymphal and adult datasets, respectively. Similarly, within adults, co-occurrence represented 88% and 96% of the interactions reported respectively in females and males (Table 2). Network complexity (i.e. the number of nodes and edges) increased with life stage, with adults displaying the most complex network (60 nodes and 412 edges) relative to larvae (20 nodes and 66 edges) and nymphs

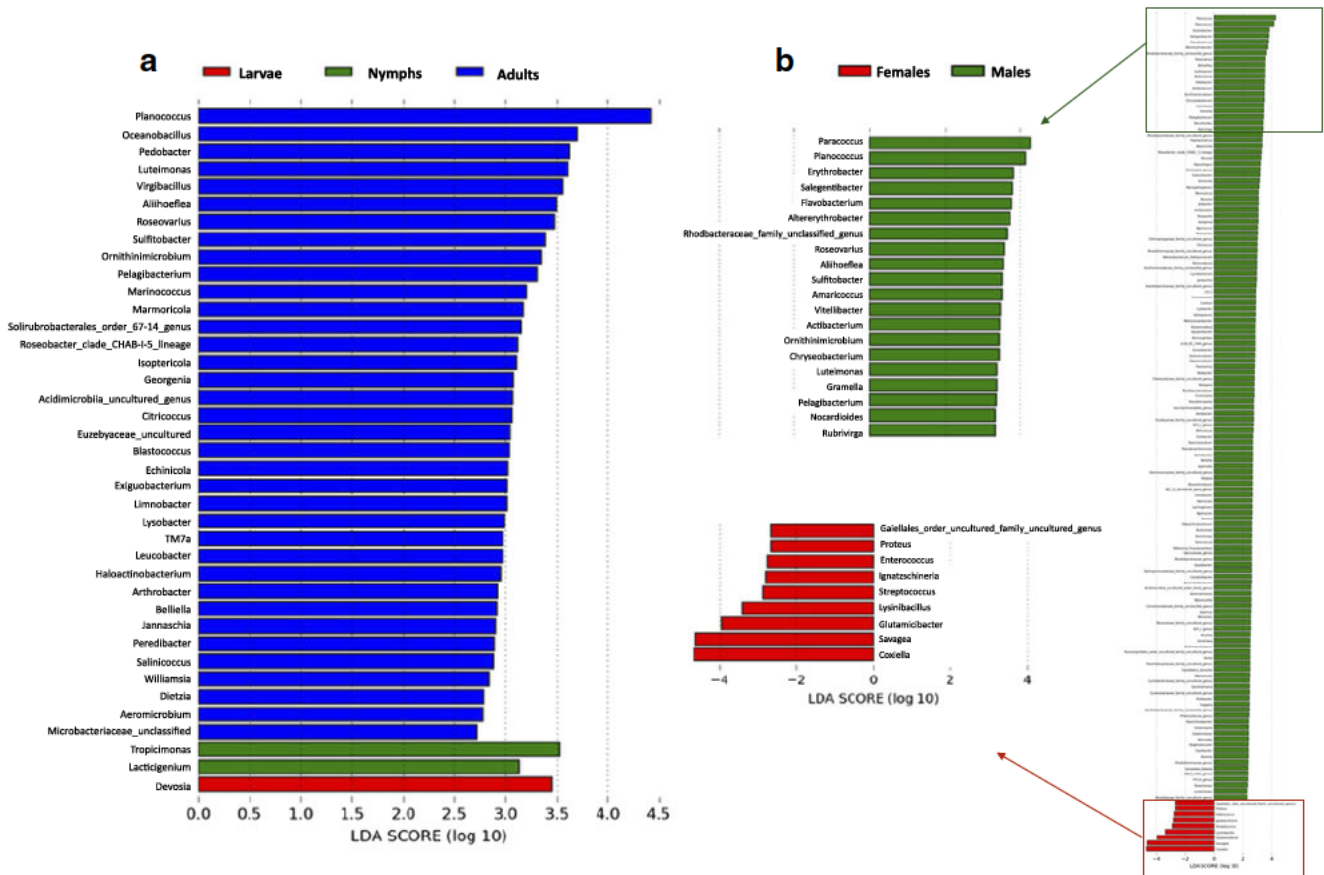


**Fig. 3** Bacterial community composition according to life stage (a) and sex (b) of *O. maritimus* ticks. Non metric multidimensional scaling (NMDS) plots were produced from Bray Curtis distances of OTU matrices. Each point in the NMDS plots corresponds to the microbiota of one

tick specimen. L larvae, N nymphs, A adults, F females and M males. The stress values of each plot are inferior to 0.2 indicating good representation

(55 nodes and 570 edges). Within adults, the network appeared less complex in females (54 nodes and 164 edges) than in males (62 nodes and 177 edges). The reported complexity

of the adult network could be due to the higher number of analysed specimens ( $n = 27$ ) compared with larvae ( $n = 11$ ) and nymphs ( $n = 12$ ). However, larval and nymphal networks



**Fig. 4** The bacterial genera whose abundance differed according to tick life stage (a) and sex (b) of *O. maritimus* ticks. These results are based on LEfSe's analyses ( $p$  value = 0.05 and LDA score > 2.0). The red rectangle

shows the 9 genera which are significantly more abundant in females than in males. The green rectangle highlights the top 20 genera which are significantly more abundant in males than in females



**Table 2** Details on bacterial networks produced from *O. maritimus* according to life stage and sex

Life stage	Number of nodes	Number of edges	Number of co occurrences	Number of co exclusions
Larva ( $n = 11$ )	20	66	50	16
Nymph ( $n = 12$ )	55	570	555	15
Adult ( $n = 27$ )	60	412	388	24
Male ( $n = 13$ )	62	177	170	7
Female ( $n = 14$ )	54	164	144	20

were based on comparable sample sizes and support the observation that network complexity increases with life stage.

At a finer level, the comparison of the different networks showed that bacterial interactions considerably changed between life stages and sexes (Online Resource 1 Figs. S3 and S4, Online Resource 3 Table S7). For instance, *Rickettsia* did not systematically display the same interactions in the networks obtained from larvae, adults and females (Online Resource 1 Figs. S3 and S4); no interaction with the genus *Rickettsia* was found in networks generated using nymphal and male datasets, whereas both positive and negative interactions were present in larval and female networks (Online Resource 1 Figs. S3 and S4, Online Resource 3 Table S7). In some cases, the interaction pattern (co-occurrence or co-exclusion) changed according to the development stage. For example, co-exclusion was detected between *Planococcus* and *Paracoccus* in the larval network, whereas co-occurrence was reported in nymphal and adult networks (Online Resource 4 Table S8). Despite these differences, some bacterial taxa displayed the same type of interaction in most networks, suggesting that these interactions are stable across life stages and sexes. For instance, co-occurrence between *Brevibacterium* and *Staphylococcus* was detected in all five networks (Online Resource 4 Tables S8 and S9). Interestingly, co-occurrence between the endosymbionts *Coxiella* and *Arsenophonus* was detected in larvae and in adults, but not in nymphs (Online Resource 4 Table S8). No other interactions involving *Coxiella* and *Arsenophonus* were detected in adult ticks, with similar findings for the individual female and male datasets. In contrast, bacterial interactions involving *Coxiella* and *Arsenophonus* were detected in larvae and nymphs (Online Resource 1 Figs. S3 and S4).

Interestingly, the four most abundant bacterial genera (*Coxiella*, *Rickettsia*, *Brevibacterium* and *Arsenophonus*) did not display a higher number of interactions within the different networks than less abundant genera (Online Resource 5 Table S10). Indeed, the genus with the highest number of interactions varied according to tick life stage and sex. For instance, in larvae, *Corynebacterium* displayed the highest number of interactions (11 interactions), while 36 interactions were reported for the genus *Aliihoflea* in nymphs. In adults, the genera *Altererythrobacter*, *Novosphingium* and

*Sulfitobacter* displayed the highest number of interactions (27 interactions for each taxon). In females, *Brevundimonas* displayed the highest number of interactions (20 interactions), while in males, the highest number of interactions was reported for *Euzebyella* (16 interactions, Online Resource 5 Table S10). These genera appear as relatively central nodes in the corresponding networks, which may indicate important functional roles in microbial interactions.

## Discussion

To date, few NGS studies have investigated the bacterial microbiota associated with soft tick species [18–20], and, to our knowledge, the present work constitutes the first NGS study of bacterial microbiota in *Ornithodoros maritimus*. The results reveal some similarities to the microbiota of hard tick species. For instance, we show that the microbial community of *O. maritimus* is dominated by the genera *Coxiella*, *Rickettsia*, *Brevibacterium* and *Arsenophonus*. The detection of *Coxiella* and *Rickettsia* is consistent with previous targeted studies on *O. maritimus* feeding on the same host species, *Larus michahellis* [22, 52, 58, 59], and is common to the majority of *Ornithodoros* spp. specimens tested to date [22]. *Coxiella* has been recently described as an obligate symbiont providing vitamin B to ticks [23–25]. Some *Rickettsia* species are also known to be maternally inherited symbionts, whereas others are vertebrate pathogens acquired horizontally [81, 82]. Using a microfluidic real-time PCR amplification method, Dupraz et al. [52] detected the presence of *Rickettsia* endosymbionts and the pathogen *Rickettsia helvetica* respectively in 81.6% (164/201) and 6.0% (3/201) of ticks from the same study site. Thus, most *Rickettsia* detected in the present samples likely correspond to the *Rickettsia* endosymbiont. However, given the low discriminatory power of the 16S rRNA gene fragment used, additional molecular information is required to validate this hypothesis. Our data also revealed the presence of the genus *Arsenophonus* in relatively high abundance. *Arsenophonus* is one of the most diverse and abundant maternally inherited bacteria reported in arthropods [83–86] but has been rarely reported in ticks [22, 87, 88]. The tick phenotype associated with *Arsenophonus* infection is

unknown, but nutritional and protective functions have been linked to the presence of this bacterial genus in haematophagous insects and psyllids [89, 90]. In addition, *Arsenophonus* sp. was suggested to reduce insecticide resistance in pest insects [91], while *Arsenophonus nasoniae* is a sex-ratio modifier in wasps [88, 92]. Although previously reported in ticks [39, 93–95], the genus *Brevibacterium* is poorly known. *Brevibacterium* species occurring in haematophagous arthropods (kissing bugs, sand flies and horn flies) have been simply described as non-pathogenic bacteria with low abundance [96–98]. However, the recurrent presence of *Brevibacterium* in diverse unrelated haematophagous arthropods suggests that these bacteria may play a role in blood digestion by their arthropod hosts, and thus possibly in ticks. Experimental work will now be required to test this hypothesis.

Reads corresponding to *Rickettsiella* were also detected in our samples, but in limited numbers and not in all tick specimens. This situation appears consistent with the exclusion pattern previously reported between this genus and *Coxiella*-like endosymbionts in *Ornithodoros* spp. [22]. The absence of *Wolbachia* genera is also coherent with the study of Duron et al. [22] who did not detect these bacteria in *O. maritimus* or other *Ornithodoros* species. As mentioned above, the 16S rRNA gene fragment used in the present study did not allow us to go down to the species level and thus demonstrate the presence of bacterial pathogens (e.g. *Kocuria*, *Rickettsia* and *Rickettsiella*). However, we did not detect sequences for the following bacterial genera, which frequently include pathogenic species: *Anaplasma*, *Bartonella*, *Borrelia*, *Ehrlichia* and *Francisella*. The absence of these genera is consistent with their low prevalence or absence in adult ticks from the same site [52]. Interestingly, the absence of the *Borrelia* genus in our data contrasts with its molecular detection (by PCR with 99% sequence identity with *Borrelia turicatae* an agent of relapsing fever) in *O. maritimus* parasitising the Mediterranean storm petrel (*Hydrobates pelagicus*) from Espartar Island (Balearic archipelago, Spain) [57]. This difference suggests microbial variation according to the considered vertebrate host and/or geographical region where ticks are sampled.

Our study demonstrates that bacterial diversity increases throughout tick development. This pattern is best explained by the acquisition of bacterial taxa through environmental contamination and/or infection during successive blood meals. These results contrast with studies reporting low bacterial diversity in adult hard tick species compared with larvae or nymphs [31, 38, 95, 99]; such reports may result from technical issues associated with relative abundance of different bacterial species (see below) and/or host factors such as the nature of bloodmeal species [31, 38, 99].

Our data also show that bacterial diversity in *O. maritimus* differs according to sex, with females displaying lower bacterial diversity than males. These results are consistent with

studies carried out on hard ticks [40, 44] and a recent study on the soft tick *Argas japonicus* [20] suggesting that this is a general trend in ticks. Treuren et al. [40] proposed that the low bacterial diversity detected in female *Ixodes* ticks results from the relative high abundance of *Rickettsia* that hinders the detection of rare bacterial taxa. Indeed, both Thapa et al. [39] and Brinkerhoff et al. [100] found similar microbial diversities in adult male and female *Ixodes scapularis* after the *in silico* removal of *Rickettsia* sequences from bacterial communities. In our study, we found that *Rickettsia* are abundant in both males and females (females 17.0% of reads, range 0.0–40.4%; males 3.2% of reads, range 0.0–15.7%) and that *Coxiella* are significantly more abundant in females than in males (females 36.0% of reads, range 21.3–59.1%; males 26.4% of reads, range 14.6–49.3%). The high abundance of *Coxiella* in females is consistent with previous reports for some hard tick species [33, 37, 95]. In our study, the *in silico* removal of *Coxiella* and/or *Rickettsia* sequences did not alter the relative patterns of diversity; males continued to show higher alpha diversity compared with females.

The construction of bacterial networks allowed us to characterise major bacterial interactions in *O. maritimus*. The generated networks indicated that co-occurrence (positive correlation) was more frequent than co-exclusion (negative correlation). Bacterial interactions also appeared to be distinct across developmental stages, with the number of non-random interactions increasing through tick development. The increase in network complexity over development correlates with increasing bacterial diversity. However, additional investigations are now required to obtain comparable data for each life stage (larva, nymph and adult) from distinct geographic locations in order to strengthen our conclusions and test the link between bacterial diversity and network complexity. In addition, as tick's microbiota also depend on the environment [17] and bloodmeals [7], future work should be conducted under laboratory conditions [95] in order to control for confounding factors which may blur our understanding of microbial interactions.

Despite developmental changes, our analyses show that some bacterial interactions are stable throughout the life of a tick, regardless of its sex. For example, positive interactions between *Brevibacterium* and *Staphylococcus* and between *Coxiella* and *Arsenophonus* were found in almost all bacterial networks. In keeping with the concept of a core microbiome, we propose here that there are also core microbial interactions that are maintained throughout development with potentially important biological functions. The case of *Coxiella* and *Arsenophonus* is particularly illustrative: these two symbionts are vertically transmitted from mother to offspring through the egg cytoplasm [58, 87]. This co-inheritance suggests that their long-term and stable coexistence in ticks is probable and may be essential for tick survival.

In the present study, we investigated the bacterial diversity harboured by *O. maritimus* hosted by a single seabird species (*L. michahellis*) in one colony (Carteau Islet). However, *O. maritimus* and other *Ornithodoros* species can parasitise a large range of seabird species [53, 101]. These ticks depend completely on their vertebrate hosts for dispersal among colonies, and even potentially among nests within a colony [101, 102]. For these reasons, *Ornithodoros* ticks constitute a relevant model for future studies to explore the effect of the vertebrate host species and sampling region on the composition of tick microbiota. In the present study, bacterial diversity was characterized with Illumina sequencing of the bacterial 16S rRNA V3–V4 hypervariable region. Even if this technology allows us to have an overview of the majority of bacterial taxa evolving in ticks, it may still underestimate total diversity due to differences in detectability among bacterial groups due to primer choice [103, 104] or taxonomic annotation errors linked to the chosen reference database [105]. Thus, future studies in this system should both compare alternative protocols at the within-population level to account for potential detection biases and apply the same technique across geographic locations and host species to ensure robust comparisons of overall bacterial diversity and the role of different factors in shaping microbial interactions over a tick's lifetime.

It is important to emphasize that we investigated bacterial interactions from NGS reads and microbial network inferences. Despite recent technological advances, understanding complex microbial relationships remain challenging and microbial network inferences present some computational limitations [74, 106–108]. In addition, microbial networks do not provide information on the biological nature of interactions. Thus, experimental investigations are now required to specifically test the biological basis of the positive and negative interactions between taxa that we found here. Hypotheses on the nature of these interactions could be provided by conducting descriptive analyses on different tick tissues [29, 109]. Indeed, the detection of bacterial taxa in the same tissue(s) (e.g. midguts, salivary glands, reproductive tissues, etc.) could be indicative of direct interaction. Alternatively, the use of fluorescent *in situ* hybridisation (FISH) [110, 111] would allow us to localise bacterial taxa within tick organs and would hence provide relevant information regarding co-occurrence or co-exclusion [112]. In ticks, the best-documented examples for co-exclusion involve *Coxiella* and *Francisella*, both of which have a pronounced tropism for ovaries and Malpighian tubules [18, 113, 114]. This type of tropism is considered indicative of a high level of lifestyle specialisation towards mutualism; infection of the ovaries suggests transovarian transmission, whereas infection of the Malpighian tubules suggests a nutritional function [18, 113, 114]. In this way, experimental studies could illuminate the mechanisms and biological significance of the bacterial interactions that we uncovered in the present study.

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**Data Accessibility** The bacterial sequence reads produced in this study have been deposited in NCBI Sequence Reads Archive (SRA) (<https://www.ncbi.nlm.nih.gov/sra>) under the accession number PRJNA590124.

**Author Contributions** Karen D. McCoy and Pablo Tortosa contributed equally to this work. Karen D. McCoy and Pablo Tortosa designed the study. Marion Vittecoq, Thomas Blanchon, Karen D. McCoy and Céline Toty organised fieldwork and collected the samples. Céline Toty performed DNA extractions. Yann Gomard and Olivier Flores analysed the data. Yann Gomard, Olivier Flores, Karen D. McCoy, Pablo Tortosa, Olivier Duron and Patrick Mavingui wrote the paper. All the authors read and approved the final manuscript.

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## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

**Ethics Approval** Permission for tick collection was provided by DDTM 13/Service Mer Eau Environnement/Pôle Nature et Territoires n°13 2018 02 22 003 and under the approval of the Grand Port Maritime de Marseille within the context of the project ESPEVEC (ANR 13 BSV7 0018).

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