Original article

Tissue localization of *Coxiella*-like endosymbionts in three European tick species through fluorescence *in situ* hybridization

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ARTICLE INFO

ABSTRACT

Ticks are commonly infected by *Coxiella*-like endosymbionts (*Coxiella*-LE) which are thought to supply missing B vitamin nutrients required for blood digestion. While this nutritional symbiosis is essential for the survival and reproduction of infected tick species, our knowledge of where *Coxiella*-LE is localized in tick tissues is partial at best since previous studies have focused on a limited number of Asian or American tick species. To fill this gap, we investigated the tissue localization of *Coxiella*-LE in three European tick species, *Ornithodoros maritimus*, *Dermacentor marginatus* and *Ixodes hexagonus*, using a diagnostic fluorescence *in situ* hybridization (FISH) assay, combined with PCR-based detection. Specific fluorescent foci were observed in several tick tissues. We visualized a pronounced tissue tropism of *Coxiella*-LE for tick ovaries and Malpighian tubules, a pattern suggestive of a high degree of lifestyle specialization toward mutualism: infection of the ovaries is indicative of transovarial transmission, whereas infection of the Malpighian tubules suggests a nutritional function. We postulate that Malpighian tubules are key organs for the nutritional symbiosis, notably the synthesis of B vitamins by *Coxiella*-LE, whereas the infection of the ovaries ensures vertical transmission of the symbionts to future generations. We also detected occasional infections in other organs, such as salivary glands and the midgut. Finally, we discuss the potential significance of the different tissue tropism for tick biology.

1. Introduction

Ticks harbour complex microbial communities that are largely dominated by non-pathogenic microorganisms (Clay et al., 2008; Andreotti et al., 2011; Carpi et al., 2011; Lalzar et al., 2012; Azagi et al., 2017; Bonnet et al., 2017; Duron et al., 2018). Among these, intracellular bacteria of the genus *Coxiella* (Legionellales: Coxiellaceae) are undoubtedly the most widespread and diverse (Almeida et al., 2012; Clay et al., 2008; Duron et al., 2017, 2015a, 2014; Lalzar et al., 2014; Machado-Ferreira et al., 2011). The best known member of *Coxiella* is *C. burnetii*, the causative agent of Q fever, but this pathogen is only rarely found in ticks (Duron et al., 2015b). However, other *Coxiella* are present in ticks, *Coxiella*-like endosymbionts (*Coxiella*-LE hereafter), that are closely related but genetically distinct from *C. burnetii* (Duron et al., 2015a). Many tick species are infected by *Coxiella*-LE: of 81 examined species from both Ixodidae (hard ticks) and Argasidae (soft ticks), over 60% were infected by *Coxiella*-LE (Duron et al., 2017, 2015a).

Coxiella-LE are maternally inherited endosymbionts that typically reach infection frequencies close to 100% in infected tick populations (Almeida et al., 2012; Clay et al., 2008; Duron et al., 2017; Lalzar et al., 2012; Machado-Ferreira et al., 2011, 2016). As the elimination of *Coxiella*-LE through antibiotic treatments was shown to negatively impact tick fitness, it was suggested that these bacteria are obligate symbionts required for tick survival and reproduction (Guizzo et al., 2017; Li et al., 2018; Zhang et al., 2017; Zhong et al., 2007). *Coxiella*-LE genomes were further shown to encode pathways for the synthesis of essential amino acids, major B vitamins and cofactors, suggesting that *Coxiella*-LE are nutrient-providing symbionts (Gottlieb et al., 2015; Smith et al., 2015). The dependency of ticks on *Coxiella*-LE is best exemplified within the *Rhipicephalus* genus: the acquisition of *Coxiella*-LE by *Rhipicephalus* spp. is ancient (> 14 Million years ago) and was followed by codiversification resulting in deeply congruent *Rhipicephalus–Coxiella*-LE phylogenies (Duron et al., 2017).


Table 1
Studies investigating tissue tropism of *Coxiella*-LE in ticks. +: presence of *Coxiella*-LE; −: absence of *Coxiella*-LE; NT: the presence of *Coxiella*-LE was not tested.

Tick species	Geographic origin	Tick tissues				Detection method	Reference
		Salivary glands	Ovaries	Midgut	Malpighian tubules		
Ixodidae (hard ticks)							
Amblyomma americanum	USA, Southern Indiana	+	+	ND	+	FISH	Klyachko et al., 2007
Amblyomma cajennense sensu lato	Brazil, state of Rio de Janeiro and state of Minas Gerais and USA, South Texas (San Antonio, Houston, Austin)	+ ND	+ +	+ +	ND ND	PCR FISH	Machado-Ferreira et al., 2011
Dermacentor silvarum	China, Xiaowutai National Natural Reserve Area	−	+	−	+	PCR	Liu et al., 2013
	China, Xiaowutai National Natural Reserve Area	ND	+	ND	+	qPCR	Liu et al., 2015
	Inner Mongolia, Jiagedaqi forest	−	+	−	+	FISH	Wang et al., 2018
Haemaphysalis flava	Japan, Shizuoka Prefecture	+	ND	ND	ND	DNA barcoding	Qiu et al., 2014
Haemaphysalis tibetensis	China, Tibet Autonomous Region (Damxung County)	−	+	−	+	qPCR	Wang et al., 2017
Haemaphysalis longicornis	Laboratory colony	−	+	−	+	PCR	Noda et al., 1997
	China, Yunan Province	−	+	−	+	FISH	Wang et al., 2018
Ixodes ovatus	Japan, Shizuoka Prefecture	+	ND	ND	ND	DNA barcoding	Qiu et al., 2014
Ixodes persulcatus	Japan, Shizuoka Prefecture	+	ND	ND	ND	DNA barcoding	Qiu et al., 2014
Rhipicephalus bursa	Italy, Foggia	+	ND	ND	ND	FISH	Raele et al., 2015
Rhipicephalus haemaphysaloides	China, Yunan Province	−	+	+	+	FISH	Wang et al., 2018
Rhipicephalus microplus	Porto Alegre strain (maintained under laboratory conditions)	+	+	+	+	qPCR	Guizzo et al., 2017
Rhipicephalus sanguineus sensu lato	Laboratory colony	−	+	−	+	PCR	Noda et al., 1997
	Israel, Kibbutz Hulda & Caesarea & Rehovot	ND	+	ND	+	FISH	Lalzar et al., 2014
Rhipicephalus turanicus	Israel, Kibbutz Hulda & Caesarea & Rehovot	+ ND	+ +	+ ND	+ +	qPCR FISH	Lalzar et al., 2014

Table 2
List of probes used in this study.

Probe	Targeted gene	Tick species	5'-3' sequence	Dye
CoxOrn	*rpoB*	*Ornithodoros maritimus*	ATTTCCTCGCCTGTATTCGACG	CY5
CoxDerm	*rpoB*	*Dermacentor marginatus*	TGGTGAGTTGATCGATCAAGGT	CY5
CoxIxo	*rpoB*	*Ixodes hexagonus*	GATGCATGCGCGTTCAACGGG	CY5
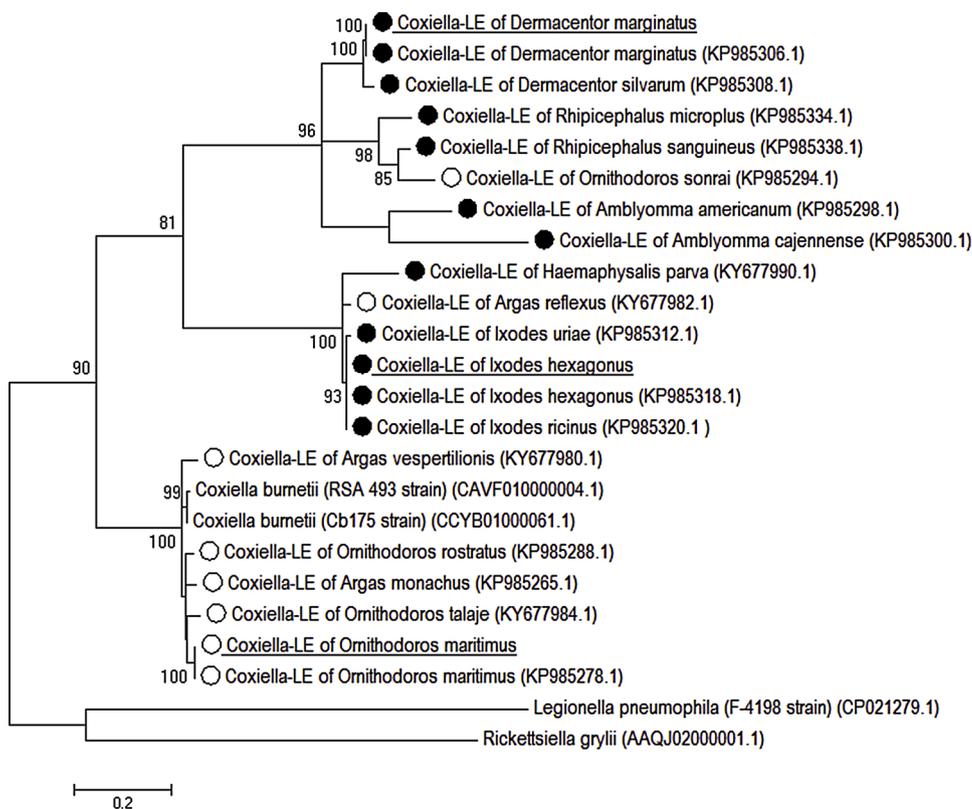
Fig. 1. *Coxiella*-LE phylogeny constructed using maximum-likelihood (ML) estimations based on *rpoB* gene sequences (472 unambiguously aligned bp). *Coxiella*-LE *rpoB* gene sequences from the tick DNA templates examined in this study are underlined. Sequences from other *Coxiella*-LE and others members of Legionellales (*Rickettsiella grylii* and *Legionella pneumophila*) available in GenBank were added to the analysis. Black and white circles indicate *Coxiella*-LE found in hard ticks (Ixodidae) and soft ticks (Argasidae), respectively. Bacterial name, host species and GenBank accession number are shown on the tree. Branch numbers indicate percentage bootstrap support for major branches (1000 replicates; only bootstrap values > 70% are shown). The scale bar is in units of substitution/site.
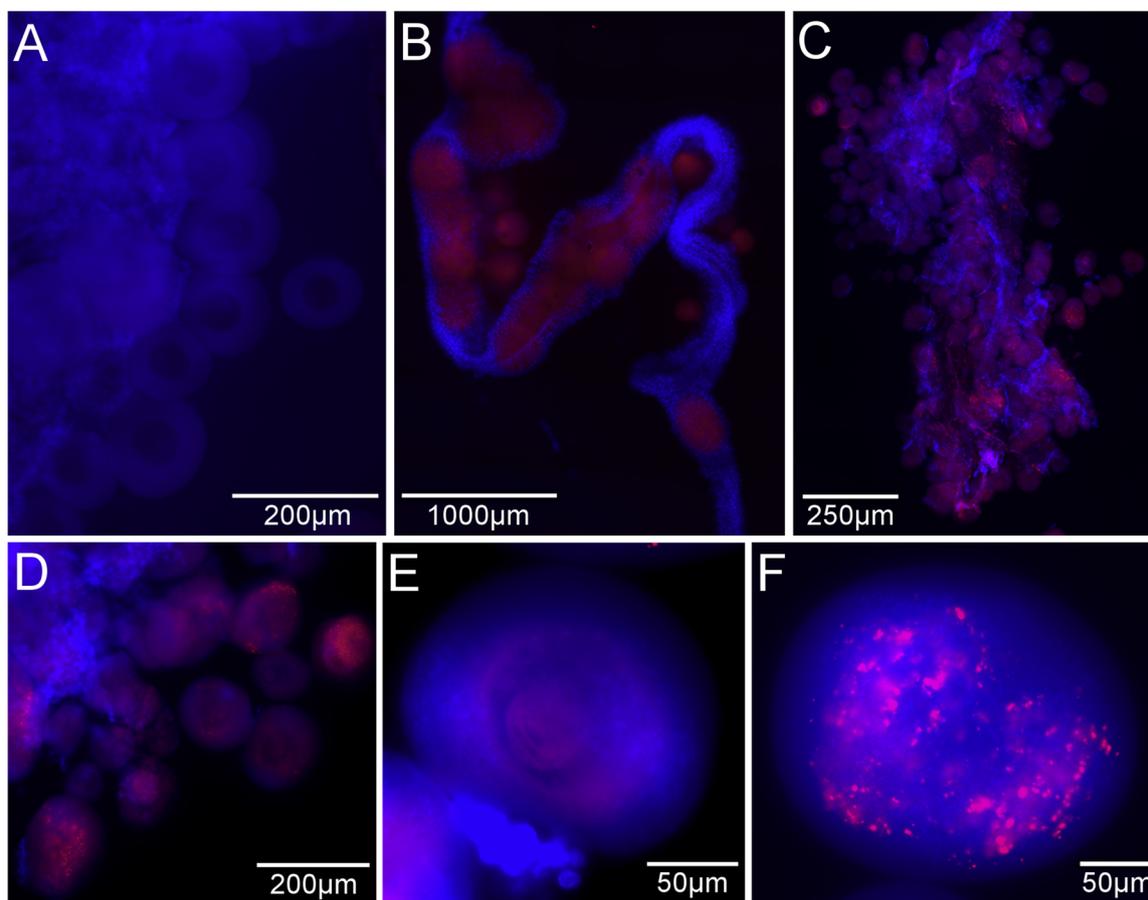
Fig. 2. *Coxiella*-LE detection using epifluorescent microscopy within individual (ie. dissected) ovaries of *O. maritimus* (A and C–F) and *D. marginatus* (B). DAPI signal (blue) and *Coxiella*-LE probes signals (red) are merged to localize *Coxiella*-LE within tick tissues (B–F). A: negative control obtained by incubating an ovary of *O. maritimus* without *Coxiella*-LE specific probe; B: oviduct of a *Coxiella*-LE infected female *D. marginatus*; C: ovary of a *Coxiella*-LE infected female *O. maritimus*; D: close-up view of *Coxiella*-LE infected oocyts of *O. maritimus*; E: a primary oocyt infected by *Coxiella*-LE showing a weak *Coxiella*-LE probe signal; F: a mature oocyt infected by *Coxiella*-LE showing a bright *Coxiella*-LE probes signal (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Although *Coxiella*-LE are common across tick taxa, their tissue tropism has only been investigated in a few ixodid species, mainly species of the genus *Rhipicephalus* (listed in Table 1). Together, these studies suggest a high degree of lifestyle specialisation towards mutualism of *Coxiella*-LE in ticks. Indeed, *Coxiella*-LE infections were found primarily in two organs, ovaries and Malpighian tubules (Table 1). The infection of the ovaries is consistent with vertical transmission of *Coxiella*-LE into developing oocytes (Klyachko et al., 2007; Lalzar et al., 2014; Machado-Ferreira et al., 2011; Noda et al., 1997). A high density of *Coxiella*-LE in Malpighian tubules supports a nutritional role for these symbionts, as Malpighian tubules are involved in excretion and osmoregulation (Sonenshine, 2014). A likely hypothesis is that *Coxiella*-LE use compounds from the hemolymph to synthesize B vitamins (Klyachko et al., 2007; Lalzar et al., 2014; Machado-Ferreira et al., 2011). Other organs, such as midgut and salivary glands, have also been found sporadically infected (Table 1), but the significance of this tissue tropism remains poorly understood.

Our current knowledge of tissue tropism in *Coxiella*-LE is based on a few (Asian/American) tick species and remains to be investigated more broadly across the tick phylogeny, including species of the genera *Ornithodoros* (Argasidae) and *Ixodes* (Ixodidae) (Table 1). In the present study, we have thus investigated the tissue localization of *Coxiella*-LE in three widespread European tick species: *Ornithodoros maritimus* (Argasidae), *Dermacentor marginatus* (Ixodidae) and *Ixodes hexagonus* (Ixodidae). These three species have been previously found infected by *Coxiella*-LE (Duron et al., 2015a, 2017), but no data are yet available on

tissue tropism. To visualize *Coxiella*-LE, we used a fluorescence *in situ* hybridization (FISH) protocol on both whole-organs and body-sections of ticks. This molecular cytogenetic technique uses fluorescent probes to bind a target DNA sequence with a high degree of sequence specificity. These analyses were then verified by organ-specific PCR detection. We compared the distribution of the symbiont among tick species and tested if tissue tropism differs among organs and tick species.

2. Methods

2.1. Tick collection

Adult females of three tick species, *O. maritimus*, *D. marginatus* and *I. hexagonus*, were sampled from the field, either from nests (seabird nests for *O. maritimus*) or directly from their mammal hosts (horses for *D. marginatus* and hedgehogs for *I. hexagonus*) in France (2016–2017). Engorged females were maintained under laboratory conditions for several days post-collection to allow blood digestion and ovary maturation before being used for experiments. A proportion of our specimens were dissected in physiological serum in a Petri dish using fine pins to remove specific organs. The upper cuticle was first removed to access organs, the midgut, gonads, Malpighian tubules and salivary glands were then collected, individually fixed with a 3.7% paraformaldehyde (PFA) solution for a week at 4 °C, and then stored at 4 °C until hybridization. Carcasses were preserved in a 70% ethanol solution for further molecular typing. The remaining specimens were treated for
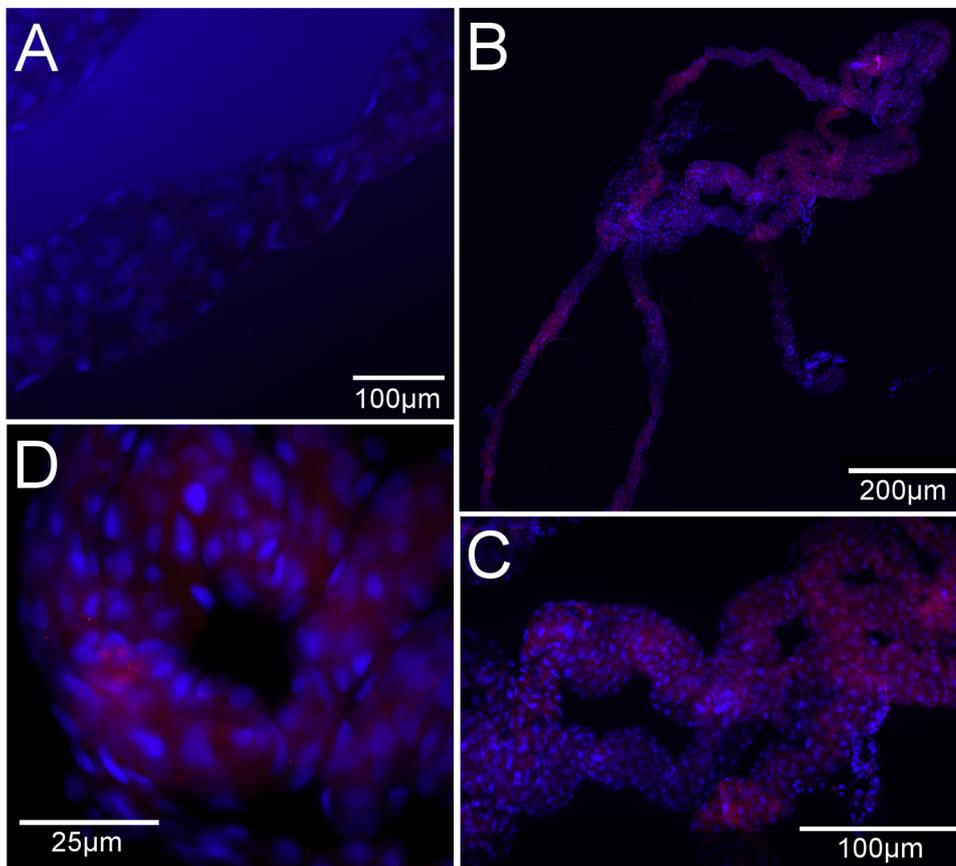
Fig. 3. *Coxiella*-LE detection using epifluorescent microscopy within individual (ie. dissected) Malpighian tubules of *O. maritimus* (A–C) and *I. hexagonus* (D). DAPI signal (blue) and *Coxiella*-LE probes signals (red) are merged to localize *Coxiella*-LE within tick tissues (B–D). A: negative control obtained by incubating a Malpighian tubule of *O. maritimus* without *Coxiella*-LE specific probe; B: Malpighian tubules of a *Coxiella*-LE infected female *O. maritimus*; C: close-up view of *Coxiella*-LE infected Malpighian tubules of *O. maritimus*; D: close-up view of *Coxiella*-LE infected Malpighian tubules of *I. hexagonus* (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
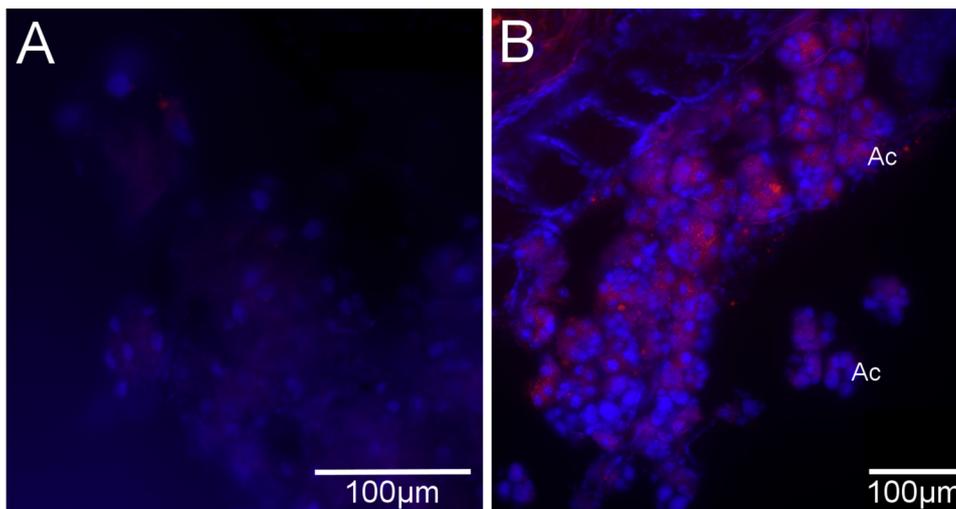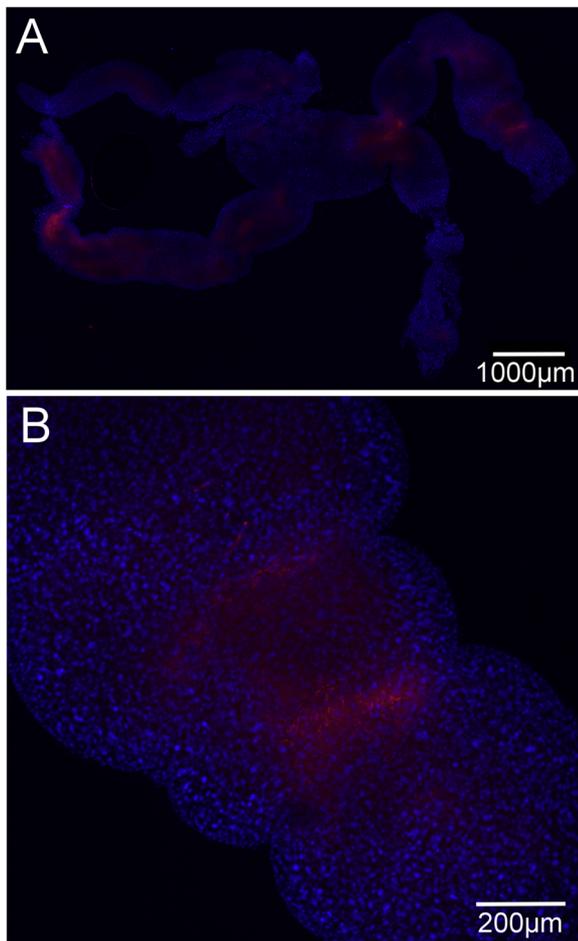

Fig. 4. *Coxiella*-LE detection using epifluorescent microscopy within *D. marginatus* salivary glands (A–B) from whole body longitudinal section. DAPI signal (blue) and *Coxiella*-LE probes signals (red) are merged to localize *Coxiella*-LE within tick tissues (B). A: negative control obtained by incubating a salivary gland of *O. maritimus* without *Coxiella*-LE specific probe; B: close-up view of salivary glands of a *Coxiella*-LE infected female *D. marginatus*. Ac: acini (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

histological examination of whole body sections (see below).

2.2. Molecular typing of Coxiella-LE

DNA was extracted from tick carcasses with DNeasy Blood & Tissue Kit (QIAGEN). The infection status of ticks was verified by nested PCR amplifications using specific primers targeting the *Coxiella*-LE *rpoB* gene, according to Duron et al. (2015a). The first PCR round mix (10 μL final volume) contained 1 μL of PCR Buffer 10X (Roche Diagnostics), 1.25 mM of each dNTP (Thermo Scientific), 7.5 mM of $MgCl_2$ (Promega), 3 μM of each external primer (Cox_rpoB_F2: GGGCGNCAYGGWAAYAAAGGSGT/ Cox_rpoB_R1: CACCRAAHCGTTGACCRCCAAATTG; 610 pb), 0.5 U of Taq DNA polymerase (Roche Diagnostics) and 1 μL of genomic DNA. A 1-μL aliquot of the PCR product from the first reaction was then used as a template for the second round of amplification. The second PCR round mix (25 μL final volume) contained 2.5 μL of PCR Buffer 10X (Roche Diagnostics), 3.125 mM of each dNTP (Thermo Scientific), 18.75 mM of $MgCl_2$ (Promega), 7.5 μM of each internal primer (Cox_rpoB_F3: TCGAAGAYA-TGCCYTATTTAGAAG /Cox_rpoB_R3: AGCTTTMCCACCSARGGGTTGCTG; 542 pb), 1.25 U of Taq DNA polymerase (Roche Diagnostics). PCR amplifications were performed under the following conditions: an initial denaturation at 95 °C for 15 min, 35 cycles of denaturation (94 °C, 30 s), binding (Tm = 56 °C, 30 s), extension (72 °C, 1 min 20 s) and a final extension at 72 °C for 5 min. Control DNA samples, including samples positive and negative for *Coxiella*-LE, were included in each PCR assay. PCR products were visualized through electrophoresis in a 1.5% agarose gel.

Fig. 5. *Coxiella*-LE detection using epifluorescent microscopy within individual (ie. dissected) *D. marginatus* midgut (A–B). DAPI signal (blue) and *Coxiella*-LE probes signals (red) are merged to localize *Coxiella*-LE within tick tissues (A–B). A: midgut of a *Coxiella*-LE infected female *D. marginatus*; B: close-up view of a *Coxiella*-LE infected midgut diverticulum of *D. marginatus*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

All positive *rpoB* PCR products were purified and sequenced in both directions (Eurofins). Sequence chromatograms were manually cleaned with CHROMAS LITE (http://www.technelysium.com.au/chromas lite.html), and alignments were performed using CLUSTALW, implemented in the MEGA software (Kumar et al., 2004). All sequences have been deposited in GenBank (accession numbers MK248730-MK248732).

Phylogenetic analyses were based on *rpoB* sequence alignments. The GBLOCKS program, with default parameters, was used to remove poorly aligned positions and to obtain unambiguous sequence alignments. Closely related organisms, including other *Coxiella*-LE and *C. burnetii* obtained from GenBank, were also included in the analyses. The evolutionary models that best fitted the sequence data were determined using the Akaike Information Criterion (AIC) with the program MEGA. Tree-based phylogenetic analyses were performed using maximum-likelihood (ML) analyses. ML heuristic searches were conducted in MEGA using a starting tree obtained by neighbour joining. Clade robustness was assessed by bootstrap analysis using 1000 replicates.

2.3. Histology

To examine thin longitudinal sections of specimens for the presence of *Coxiella*-LE, whole tick bodies were fixed with a 3.7% PFA solution for 4 days and then dehydrated in a series of sucrose solutions (20%, 50%, 70%, until samples sank). All manipulations were performed at

4 °C. After being embedded in Tissue-Tek O.C.T. Compound (Sakura), the whole tick bodies were individually frozen in isopentane cooled with liquid nitrogen and were then stored at −80 °C. Cryosectioning was performed using a cryostat (CM1520) and thin sections (15–20 μm) were collected on Superfrost®Plus slides (Thermo Scientific). Slides were stored at −80 °C until hybridization.

2.4. Fluorescence in situ hybridization

FISH probes were designed using the *Coxiella*-LE *rpoB* sequences that we obtained from our *O. maritimus*, *D. marginatus* and *I. hexagonus* specimens. All probes were labelled with a CY5 dye at the 5′ end (Table 2) and used in hybridization assays. Whole-organs and body sections (resin-embedded 15–20 μm sections) were submitted to the same hybridization protocol, with only the volumes changed. The protocol was adapted from Lalzar et al. (2014) with the following modifications: samples were preincubated with 500 μL hybridization buffer (HB) (SSC Buffer 20X/Denhardt's solution 50X) for 15 min and then placed in 200 μL of HB containing the appropriate probe (25 ng/μL) for a 16 h-incubation time at 46 °C. Samples were next washed twice with pre-warmed washing buffer (20 mM TrisHCl pH 8.0, 80 mM NaCl, 50 mM EDTA, 0.01% SDS) for 30 min at 48 °C and then with 1 mL of PBS for 15 min at room temperature. In order to label nuclear DNA, samples were incubated with 4′,6-diamidino-2-phenylindole (DAPI; 0.1 mg/μl in PBS 1X) for 8 min in darkness and any excess DAPI was removed by a PBS 1X washing solution. Samples were placed on a microscope slide (RS France) containing Vectashield antifade mounting medium (Vector Laboratories), topped with a cover glass and stored in the dark at 4 °C. Fluorescent signals were observed under an epifluorescent microscope (Zeiss Axioimager Z1). Negative-controls were obtained by incubating organs without *Coxiella*-specific probes and by checking for tissue auto-fluorescence. Great care was taken to perform microscopic studies with identical settings.

2.5. Ethical statement

All animals were handled in strict accordance with good animal practice as defined by the French code of practice for the care and use of animals for scientific purposes, established by articles R214-87 to R214-137 of the French rural code.

3. Results

We examined tissue tropism of *Coxiella*-LE in eight gravid females (*O. maritimus* - $n = 3$, *D. marginatus* - $n = 3$, and *I. hexagonus* - $n = 2$). We found that all specimens were infected by *Coxiella*-LE through positive *rpoB* PCR amplifications. The *rpoB* sequences were easily readable without double peaks, indicating that there was no co-infection of *Coxiella* strains in any specimen. The *Coxiella*-LE *rpoB* sequences were strictly identical among specimens from the same species, but varied between the three tick species: three distinct *rpoB* alleles were identified with a moderate level of nucleotide identity (72.1–73.3% pairwise nucleotide identity). The *Coxiella*-LE *rpoB* sequences from *O. maritimus* were 100% identical to a sequence previously obtained from the same tick species (GenBank accession number KP985278.1). Similar results were obtained with *Coxiella*-LE *rpoB* sequences from *D. marginatus* and *I. hexagonus* (100% identical with sequences from the same two tick species; GenBank accession numbers KP985306.1 and KP985318.1, respectively). None of the *Coxiella*-LE strains found in *O. maritimus*, *D. marginatus* and *I. hexagonus* were identical to *C. burnetii*, as shown by a moderate level of *rpoB* nucleotide identity (73.7–95.5% pairwise nucleotide identity). On the basis of *rpoB* sequences, the *Coxiella*-LE strains found in *O. maritimus*, *D. marginatus* and *I. hexagonus* clearly fall within the *Coxiella* genus, closely related to other *Coxiella*-LE and to *C. burnetii* (Fig. 1). However, the *Coxiella*-LE of *O. maritimus*, *D. marginatus* and *I. hexagonus* have different evolutionary origins: these *Coxiella*-LE do not

cluster in the same monophyletic group and belong to three distinct lineages within the *Coxiella* genus (Fig. 1), as previously observed by Duron et al. (2015a).

Since *Coxiella*-LE *rpoB* sequences of *O. maritimus*, *D. marginatus* and *I. hexagonus* were slightly divergent, we designed specific *Coxiella*-LE probes for each tick species (Table 2). Using these FISH probes, we investigated the tissue tropism of *Coxiella*-LE in the three tick species. We observed *Coxiella*-LE in all ticks tested in our study. The examination of tissue tropism showed substantial variation in detection sensitivity between whole-organ and body sections. Higher FISH resolution was obtained for body sections as these sections were thinner than whole-organs. Identifying the infected tissues within the body sections, however, remained difficult, especially in cross sections of threadlike organs such as Malpighian tubules. In addition, the hybridization process for body sections is very delicate and commonly results in the folding and overlaying of sections, preventing tissue identification. Lower FISH resolution was observed with whole-organ sections due to the difficulty of probes to enter into the cells and the three dimensional nature of the organs: the higher number of cellular layers in the organs slightly blurred the visualization of *Coxiella*-LE within the organs. However, the major advantage of using whole organs for FISH is that an organ, or part of an organ, can be rapidly diagnosed as infected.

Using observations on body sections and whole organs, we searched for the presence of *Coxiella*-LE in four organs of the *O. maritimus*, *I. hexagonus* and *D. marginatus* individuals: ovaries, Malpighian tubules, salivary glands and midgut (Figs. 2A–F, 3 A–D, 4 A–B and 5 A–B). Ovaries were always infected by *Coxiella*-LE with higher signal intensity in mature oocytes compared to primary oocytes (Fig. 2E–F). Malpighian tubules also always contained *Coxiella*–LE with high concentrations (Fig. 3). *Coxiella*-LE were not observed in salivary glands, except for some salivary gland acini of one *D. marginatus* individual (Fig. 4). Similarly, *Coxiella*-LE were not observed in the midguts of *O. maritimus* and *I. hexagonus* individuals, but we did detect an infection signal in multiple parts of the midgut diverticula of one (out of three) *D. marginatus* individual (Fig. 5). Interestingly, the *Coxiella*-LE positive salivary glands and midgut were not from the same individual *D. marginatus* tick: *Coxiella*-LE were not found to be present in all four organs of any of the *D. marginatus* individuals examined (as whole body sections, n = 1; as dissected organs, n = 2). No autofluorescence was observed in controls (Figs. 2A, 3 A and 4 A), demonstrating that the fluorescence seen in tick samples was due to the reactivity of specific probes with *Coxiella*-LE.

4. Discussion

In this study, we investigated the presence of *Coxiella*-LE in three European tick species, *D. marginatus, I. hexagonus* and *O. maritimus*, which had never before been examined for tissue tropism. We found that these three species are infected by different *Coxiella*-LE symbionts and that these symbionts differ from the Q fever pathogen, *C. burnetii*, as previously observed (Duron et al., 2017, 2015a). We further showed that *Coxiella*-LE tissue tropism in these three species is clearly shifted towards ovaries and Malpighian tubules, but that other organs, such as midgut and salivary glands, can occasionally be infected.

Our observations of *Coxiella*-LE tissue tropism for ovaries and Malpighian tubules is consistent with those of other tick genera, such as *Rhipicephalus* and *Amblyomma* (Table 1), suggesting that the main biological feature of the *Coxiella*-LE endosymbiosis is similar among tick species. The presence of *Coxiella*-LE in the ovaries is indicative of vertical (transovarial) transmission and explains why most of the progeny of an infected tick female are also infected (Almeida et al., 2012; Duron et al., 2015a; Klyachko et al., 2007; Lalzar et al., 2014; Machado-Ferreira et al., 2011). The presence of *Coxiella*-LE in Malpighian tubules may be pivotal for nutritional interactions with ticks; vitamin-providing symbionts of arthropods, including those of blood feeders such as lice or bedbugs, are typically found in symbiotic organs (bacteriomes)

containing specialized cells (bacteriocytes) and surrounding cells (Gottlieb et al., 2006; Hosokawa et al., 2010). The tropism of *Coxiella*-LE for Malpighian tubules suggests that one of the functions of this organ may be to act as the equivalent of the insect bacteriome in ticks. Malpighian tubules are extremely long in ticks and loop around the internal organs extending into most parts of the body cavity (Sonenshine, 2014). Since Malpighian tubules are primarily involved in excretion and osmoregulation with the surrounding hemolymph (Sonenshine, 2014), a likely hypothesis is that *Coxiella*-LE recycle nitrogenous wastes to synthesize novel compounds such as B vitamins. Furthermore, tick species that do not harbour *Coxiella*-LE usually harbour other obligate endosymbionts, such as *Francisella*-like endosymbionts (*Francisella*-LE) (Thiotrichales: Francisellaceae), that are also found in the Malpighian tubules (Duron et al., 2017; Gerhart et al., 2016). *Coxiella*-LE and *Francisella*-LE are unrelated endosymbionts but share important similarities, including the ability to synthesize major B vitamins and a clear tissue tropism for the Malpighian tubules (for *Francisella*, see Duron et al., 2018). This suggests that Malpighian tubules have a propensity to host B vitamin-providing endosymbionts and supports the hypothesis that this organ can be considered as a bacteriome that functions in bacterial-based B vitamin synthesis. Similar cases have also been observed in other blood-feeding arthropods: the localization of B vitamin-providing endosymbionts has been consistently observed in bacteriomes close to the midgut in bat flies (Hosokawa et al., 2012), bedbugs (Hosokawa et al., 2010) and lice (Perotti et al., 2007).

If we consider organs other than ovaries and Malpighian tubules, substantial variation is apparent among tick species. Indeed, while *Coxiella*-LE tend to be absent from organs other than ovaries and Malpighian tubules in most tick species (this study, see also Table 1), we observed *Coxiella*-LE in the midgut and salivary glands of specimens of *D. marginatus*, a feature also described from specimens of *Amblyomma* and *Rhipicephalus* (Table 1). While the presence of *Coxiella*-LE in the midgut suggests a nutritive function, its presence in salivary glands is more equivocal. One possibility is that *Coxiella*-LE may be opportunistic tick-borne pathogens that are inoculated into vertebrates with the tick saliva. A few studies have indeed reported occasional infections by *Coxiella*-LE in vertebrates: these infections were associated with scalp eschar and neck lymphadenopathy in humans (Angelakis et al., 2016), and progressive degenerative health leading to death in pet birds (Shivaprasad et al., 2008; Woc-Colburn et al., 2008). While many tick species harbour *Coxiella*-LE at high prevalence, the rarity of cases in vertebrates strongly suggests that infectious transmission is sporadic (Bonnet et al., 2017; Duron et al., 2015b). A second possibility is that *Coxiella*-LE may not be injected with the saliva. Indeed, Guizzo et al. (2017) detected *Coxiella*-LE in the salivary glands but not in the saliva of the cattle tick *R. microplus*. In this context, it has been assumed that *Coxiella*-LE may facilitate tick feeding through the production of critical molecules with cytolytic, vasodilator, anticoagulant, anti-inflammatory, or immunosuppressive activity (Guizzo et al., 2017), although formal evidence is lacking. A third possibility is that *Coxiella*-LE in salivary glands may protect ticks from pathogens, effectively blocking the transmission of tick-borne pathogens. This has never been demonstrated but, in other arthropods, a number of maternally inherited bacteria, such as *Wolbachia* in mosquitoes, limit the proliferation of pathogenic infectious agents within arthropod tissues (Bian et al., 2013; Hughes et al., 2011; Moreira et al., 2009), and *Coxiella*-LE may behave similarly in ticks (Bonnet et al., 2017). Each of these possibilities now remains to be formally tested, if we are to understand the potential impact of *Coxiella*-LE in salivary glands.

In conclusion, evidence is now accumulating in support of the hypothesis that Malpighian tubules are key organs for nutritional symbiosis between ticks and B vitamin-providing bacteria, including *Coxiella*-LE. Slight variations in tissue tropism among tick species are also frequently observed, resulting in presence of *Coxiella*-LE outside the Malpighian tubules and ovaries. Future investigations should thus

focus on explaining variation in *Coxiella*-LE tissue tropism and its consequences for tick biology and tick-borne diseases.

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