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1 **Tissue localization of *Coxiella*-like endosymbionts in three European tick**
2 **species through Fluorescence *In Situ* Hybridization**

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4

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13 **Abstract**

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

31 **Introduction**

32 Ticks harbour complex microbial communities that are largely dominated by non-pathogenic
33 microorganisms (Clay et al., 2008; Andreotti et al., 2011; Carpi et al., 2011; Lalzar et al.
34 2012; Azagi et al., 2017; Bonnet et al., 2017; Duron et al., 2018). Among these, intracellular
35 bacteria of the genus *Coxiella* (Legionellales: Coxiellaceae) are undoubtedly the most
36 widespread and diverse (Almeida et al., 2012; Clay et al., 2008; Duron et al., 2017; Duron et
37 al., 2015a, Duron et al., 2014; Lalzar et al., 2014; Machado-ferreira et al., 2011). The best
38 known member of *Coxiella* is *C. burnetii*, the causative agent of Q fever, but this pathogen is
39 only rarely found in ticks (Duron et al., 2015b). However, others *Coxiella* are present in ticks,
40 *Coxiella*-like endosymbionts (*Coxiella*-LE hereafter), that are closely related but genetically
41 distinct from *C. burnetii* (Duron et al., 2015a). Most tick species are infected by *Coxiella*-LE:
42 of 81 examined species from both Ixodidae (hard ticks) and Argasidae (soft ticks), over 60%
43 were infected by *Coxiella*-LE (Duron et al., 2017, Duron et al., 2015a).

44
45 *Coxiella*-LE are maternally inherited endosymbionts that typically reach infection frequencies
46 close to 100% in infected tick populations (Almeida et al., 2012; Clay et al., 2008; Duron et
47 al., 2017; Lalzar et al., 2012; Machado-Ferreira et al., 2011; Machado-Ferreira et al., 2016).
48 As the elimination of *Coxiella*-LE through antibiotic treatments was shown to negatively
49 impact tick fitness , it was suggested that these bacteria are obligate symbionts required for
50 tick survival and reproduction (Guizzo et al., 2017; Li et al., 2018; Zhang et al., 2017; Zhong
51 et al., 2007). *Coxiella*-LE genomes were further shown to encode pathways for the synthesis
52 of essential amino acids, major B vitamins and cofactors, suggesting that *Coxiella*-LE are
53 nutrient-providing symbionts (Gottlieb et al., 2015; Smith et al., 2015). The dependency of
54 ticks on *Coxiella*-LE is best exemplified within the *Rhipicephalus* genus: the acquisition of
55 *Coxiella*-LE by *Rhipicephalus* spp. is ancient (>14 Million years ago) and was followed by

56 codiversification resulting in deeply congruent *Rhipicephalus*–*Coxiella*-LE phylogenies
57 (Duron et al., 2017).
58
59 Although *Coxiella*-LE are common across tick taxa, their tissue tropism has only been
60 investigated in a few ixodid species, mainly species of the genus *Rhipicephalus* (listed in
61 Table 1). Together, these studies suggest a high degree of lifestyle specialisation towards
62 mutualism of *Coxiella*-LE in ticks. Indeed, *Coxiella*-LE infections were found primarily in
63 two organs, ovaries and Malpighian tubules (Table 1). The infection of the ovaries is
64 consistent with vertical transmission of *Coxiella*-LE into developing oocytes (Klyachko et al.,
65 2007; Lalzar et al., 2014; Machado-ferreira et al., 2011; Noda et al., 1997). A high density of
66 *Coxiella*-LE in Malpighian tubules supports a nutritional role for these symbionts, as
67 Malpighian tubules are involved in excretion and osmoregulation (Sonenshine, 2014). A
68 likely hypothesis is that *Coxiella*-LE use compounds from the hemolymph to synthesize B
69 vitamins (Klyachko et al., 2007; Lalzar et al., 2014; Machado-Ferreira et al., 2011). Other
70 organs, such as midgut and salivary glands, have also been found sporadically infected (Table
71 1), but the significance of these tissue tropism remains poorly understood.
72
73 Our current knowledge of tissue tropism in *Coxiella*-LE is based on a few (Asian/American)
74 tick species and remains to be investigated more broadly across the tick phylogeny, including
75 species of the genera *Ornithodoros* (Argasidae) and *Ixodes* (Ixodidae) (Table 1). In the
76 present study, we have thus investigated the tissue localization of *Coxiella*-LE in three
77 widespread European tick species: *Ornithodoros maritimus* (Argasidae), *Dermacentor*
78 *marginatus* (Ixodidae) and *Ixodes hexagonus* (Ixodidae). These three species have been
79 previously found infected by *Coxiella*-LE (Duron et al., 2015a; Duron et al., 2017), but no
80 data are yet available on tissue tropism. To visualize *Coxiella*-LE, we used a fluorescence *in*

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

86

87 **Methods**

88 *Tick collection*

89 Adult females of three tick species, *O. maritimus*, *D. marginatus* and *I. hexagonus*, were
90 sampled from the field, either from nests (seabird nests for *O. maritimus*) or directly from
91 their mammal hosts (horses for *D. marginatus* and hedgehogs for *I. hexagonus*) in France
92 (2016-2017). Engorged females were maintained under laboratory conditions for several days
93 post-collection to allow blood digestion and ovary maturation before being used for
94 experiments. Part of our specimens were dissected in physiological serum in a Petri dish using
95 fine pins to remove specific organs. The upper cuticle was first removed to access organs, the
96 midgut, gonads, Malpighian tubules and salivary glands were then collected, individually
97 fixed with a 3.7% paraformaldehyde (PFA) solution for a week at 4°C, and then stored at 4°C
98 until hybridization. Carcasses were preserved in a 70% ethanol solution for further molecular
99 typing. The remaining specimens were treated for histological examination of whole body
100 sections (see below).

101

102 *Molecular typing of Coxiella-LE*

103 DNA was extracted from tick carcasses with DNeasy Blood & Tissue Kit (QIAGEN). The
104 infection status of ticks was verified by nested PCR amplifications using specific primers

105 targeting the *Coxiella*-LE *rpoB* gene, according to Duron et al. (2015a). The first PCR round
106 mix (10 μ L final volume) contained 1 μ L of PCR Buffer 10X (Roche Diagnostics), 1.25 mM
107 of each dNTP (Thermo Scientific), 7.5 mM of MgCl₂ (Promega), 3 μ M of each external
108 primer (Cox_rpoB_F2: GGGCGNCA YGGWAAYAAAGGSGT/Cox_rpoB_R1:
109 CACCRAAHCGTTGACCRCCAAATTG; 610pb), 0.5 U of Taq DNA polymerase (Roche
110 Diagnostics) and 1 μ L of genomic DNA. A 1- μ L aliquot of the PCR product from the first
111 reaction was then used as a template for the second round of amplification. The second PCR
112 round mix (25 μ L final volume) contained 2.5 μ L of PCR Buffer 10X (Roche Diagnostics),
113 3.125 mM of each dNTP (Thermo Scientific), 18.75 mM of MgCl₂ (Promega), 7.5 μ M of
114 each internal primer (Cox_rpoB_F3: TCGAAGAYATGCCYTATTTAGAAG
115 /Cox_rpoB_R3: AGCTTTMCCACCSARGGGTTGCTG; 542pb), 1.25 U of Taq DNA
116 polymerase (Roche Diagnostics). PCR amplifications were performed under the following
117 conditions: an initial denaturation at 95°C for 15 min, 35 cycles of denaturation (94°C, 30s),
118 binding (T_m = 56°C, 30s), extension (72°C, 1 min 20s) and a final extension at 72°C for 5
119 min. Control DNA samples, including samples positive and negative for *Coxiella*-LE, were
120 included in each PCR assay. PCR products were visualized through electrophoresis in a 1.5%
121 agarose gel. All positive *rpoB* PCR products were purified and sequenced in both directions
122 (Eurofins). Sequence chromatograms were manually cleaned with CHROMAS LITE
123 (http://www.technelysium.com.au/chromas_lite.html), and alignments were performed using
124 CLUSTALW, implemented in the MEGA software (Kumar et al., 2004). All sequences have
125 been deposited in GenBank (accession numbers MK248730-MK248732).

126

127 Phylogenetic analyses were based on *rpoB* sequence alignments. The GBLOCKS program,
128 with default parameters, was used to remove poorly aligned positions and to obtain
129 unambiguous sequence alignments. Closely related organisms, including other *Coxiella*-LE

130 and *C. burnetii* obtained from GenBank, were also included in the analyses. The evolutionary
131 models that best fitted the sequence data were determined using the Akaike Information
132 Criterion (AIC) with the program MEGA. Tree-based phylogenetic analyses were performed
133 using maximum-likelihood (ML) analyses. ML heuristic searches were conducted in MEGA
134 using a starting tree obtained by neighbour joining. Clade robustness was assessed by
135 bootstrap analysis using 1,000 replicates.

136

137 *Histology*

138 To examine thin longitudinal sections of specimens for the presence of *Coxiella*-LE, whole
139 tick bodies were fixed with a 3.7% PFA solution for 4 days and then dehydrated in a series of
140 sucrose solutions (20%, 50%, 70%, until samples sank). All manipulations were performed at
141 4°C. After being incorporated into tissue-tek resin (OCT COMPOUND 4583 SAKURA), the
142 whole tick bodies were individually frozen in isopentane cooled with liquid nitrogen and were
143 then stored at -80°C. Cryosectioning was performed using a cryostat (CM1520) and thin
144 sections (15-20 µm) were collected on Superfrost®Plus slides (Thermo Scientific). Slides
145 were stored at -80°C until hybridization.

146

147 *Fluorescence in situ hybridization*

148 FISH probes were designed using the *Coxiella*-LE *rpoB* sequences that we obtained from our
149 *O. maritimus*, *D. marginatus* and *I. hexagonus* specimens. All probes were labelled with a
150 CY5 dye at the 5' end (Table 2) and used in hybridization assays. Whole-organs and body
151 sections (resin-embedded 15-20 µM sections) were submitted to the same hybridization
152 protocol, with only the volumes changed. The protocol was adapted from Lalar et al. (2014)
153 with the following modifications: samples were preincubated with 500 µL hybridization

154 buffer (HB) (SSC Buffer 20X/Denhardt's solution 50X) for 15 min and then placed in 200 μ L
155 of HB containing the appropriate probe (25 ng/ μ L) for a 16 h-incubation time at 46°C.
156 Samples were next washed twice with pre-warmed washing buffer (20 mM TrisHCl pH 8.0,
157 80 mM NaCl, 50 mM EDTA, 0.01% SDS) for 30 min at 48°C and then with 1 mL of PBS for
158 15 min at room temperature. In order to label nuclear DNA, samples were incubated with
159 4',6-diamidino-2-phenylindole (DAPI; 0.1mg/ μ l in PBS 1X) for 8 min in darkness and any
160 excess DAPI was removed by a PBS 1X washing solution. Samples were placed on a
161 microscope slide (RS France) containing Vectashield antifade mounting medium (Vector
162 Laboratories), topped with a cover glass and stored in the dark at 4°C. Fluorescent signals
163 were observed under an epifluorescent microscope (Zeiss Axioimager Z1). Negative-controls
164 were obtained by incubating organs without *Coxiella*-specific probes and by checking for
165 tissue auto-fluorescence. Great care was taken to perform microscopic studies with identical
166 settings.

167

168 **Results**

169 We examined tissue tropism of *Coxiella*-LE in eight gravid females (*O. maritimus* - $n=3$, *D.*
170 *marginatus* - $n=3$, and *I. hexagonus* - $n=2$). We found that all specimens were infected by
171 *Coxiella*-LE through positive *rpoB* PCR amplifications. The *rpoB* sequences were easily
172 readable without double peaks, indicating that there was no co-infection of *Coxiella* strains in
173 any specimen. The *Coxiella*-LE *rpoB* sequences were strictly identical among specimens from
174 the same species, but varied between the three tick species: three distinct *rpoB* alleles were
175 identified with a moderate level of nucleotide identity (72.1–73.3% pairwise nucleotide
176 identity). The *Coxiella*-LE *rpoB* sequences from *O. maritimus* were 100% identical to a
177 sequence previously obtained from the same tick species (GenBank accession number
178 KP985278.1). Similar results were obtained with *Coxiella*-LE *rpoB* sequences from *D.*

179 *marginatus* and *I. hexagonus* (100% identical with sequences from the same two tick species;
180 GenBank accession numbers KP985306.1 and KP985318.1, respectively). None of the
181 *Coxiella*-LE strains found in *O. maritimus*, *D. marginatus* and *I. hexagonus* were identical to
182 *C. burnetii*, as shown by a moderate level of *rpoB* nucleotide identity (73.7– 95.5% pairwise
183 nucleotide identity). On the basis of *rpoB* sequences, the *Coxiella*-LE strains found in *O.*
184 *maritimus*, *D. marginatus* and *I. hexagonus* clearly fall within the *Coxiella* genus, closely
185 related to other *Coxiella*-LE and to *C. burnetii* (Fig. 1). However, the *Coxiella*-LE of *O.*
186 *maritimus*, *D. marginatus* and *I. hexagonus* have different evolutionary origins: these
187 *Coxiella*-LE do not cluster in the same monophyletic group and belong to three distinct
188 lineages within the *Coxiella* genus (Fig. 1), as previously observed by Duron et al. (2015a).

189

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

203 major advantage of using whole organs for FISH is that an organ, or part of an organ, can be
204 rapidly diagnosed as infected.

205

206 Using observations on body sections and whole organs, we searched for the presence of
207 *Coxiella*-LE in four organs of the *O. maritimus*, *I. hexagonus* and *D. marginatus* individuals:
208 ovaries, Malpighian tubules, salivary glands and midgut (Fig. 2A-F, 3A-D, 4A-B and 5A-B).
209 Ovaries were always infected by *Coxiella*-LE with higher signal intensity in mature oocytes
210 compared to primary oocytes (Fig. 2E-F). Malpighian tubules also always contained
211 *Coxiella*-LE with high concentrations (Fig. 3). *Coxiella*-LE were not observed in salivary
212 glands, except for some salivary gland acini of one *D. marginatus* individual (Fig. 4).
213 Similarly, *Coxiella*-LE were not observed in the midguts of ticks, but we did detect an
214 infection signal in multiple parts of the midgut diverticula of one *D. marginatus* individual
215 (Fig. 5). Interestingly, the *Coxiella*-LE positive salivary glands and midgut are not from the
216 same *D. marginatus* tick individual: *Coxiella*-LE was never found present in all organs of a *D.*
217 *marginatus* individual. No autofluorescence was observed in controls (Fig. 2A, 3A and 4A),
218 demonstrating that the fluorescence seen in tick samples is due to the reactivity of specific
219 probes with *Coxiella*-LE.

220

221 **Discussion**

222 In this study, we investigated the presence of *Coxiella*-LE in three European tick species, *D.*
223 *marginatus*, *I. hexagonus* and *O. maritimus*, which had never before been examined for tissue
224 tropism. We found that these three species are infected by different *Coxiella*-LE symbionts
225 and that these symbionts differ from the Q fever pathogen, *C. burnetii*, as previously observed
226 (Duron et al., 2017, Duron et al., 2015a). We further showed that *Coxiella*-LE's tissue tropism

227 in these three species is clearly shifted towards ovaries and Malpighian tubules, but that other
228 organs, such as midgut and salivary glands, can occasionally be infected.

229

230 Our observations of *Coxiella*-LE tissue tropism for ovaries and Malpighian tubules is
231 consistent with those of other tick genera, such as *Rhipicephalus* and *Amblyomma* (Table 1),
232 suggesting that the main biological feature of the *Coxiella*-LE endosymbiosis is similar
233 among tick species. The presence of *Coxiella*-LE in the ovaries is indicative of vertical
234 (transovarial) transmission and explains why most of the progeny of an infected tick female
235 are also infected (Almeida et al., 2012; Duron et al., 2015a; Klyachko et al., 2007; Lalar et
236 al., 2014; Machado-Ferreira et al., 2011). The presence of *Coxiella*-LE in Malpighian tubules
237 may be pivotal for nutritional interactions with ticks; vitamin-providing symbionts of
238 arthropods, including those of blood feeders such as lice or bedbugs, are typically found in
239 symbiotic organs (bacteriomes) containing specialized cells (bacteriocytes) and surrounding
240 cells (Gottlieb et al., 2006; Hosokawa et al., 2010). The tropism of *Coxiella*-LE for
241 Malpighian tubules suggests that one of the functions of this organ may act as the equivalent
242 of the insect bacteriome in ticks. Malpighian tubules are extremely long in ticks and loop
243 around the internal organs extending into most parts of the body cavity (Sonenshine, 2014).
244 Since Malpighian tubules are primarily involved in excretion and osmoregulation with the
245 surrounding hemolymph (Sonenshine, 2014), a likely hypothesis is that *Coxiella*-LE recycle
246 nitrogenous wastes to synthesize novel compounds such as B vitamins. Furthermore, tick
247 species that do not harbour *Coxiella*-LE always harbour other obligate endosymbionts, such
248 as *Francisella*-like endosymbionts (*Francisella*-LE) (Thiotrichales: Francisellaceae), that are
249 also found in the Malpighian tubules (Duron et al., 2017; Gerhart et al., 2016). *Coxiella*-LE
250 and *Francisella*-LE are unrelated endosymbionts but share important similarities, including
251 the ability to synthesize major B vitamins and a clear tissue tropism for the Malpighian

252 tubules (for *Francisella*, see Duron et al., 2018). This suggests that Malpighian tubules have a
253 propensity to host B-vitamin provisioning endosymbionts and supports the hypothesis that
254 this organ can be considered as a bacteriome that functions in bacterial-based B vitamin
255 synthesis. Similar cases have also been observed in other blood feeding arthropods: the
256 localization of B vitamin-providing endosymbionts has been consistently observed in
257 bacteriomes close to the midgut in bat flies (Hosokawa et al., 2012), bedbugs (Hosokawa et
258 al., 2010) and lice (Perotti et al., 2007).

259

260 If we consider organs other than ovaries and Malpighian tubules, substantial variation is
261 apparent among tick species. Indeed, while *Coxiella*-LE tend to be absent from organs other
262 than ovaries and Malpighian tubules in most tick species (this study, see also Table 1), we
263 observed *Coxiella*-LE in the midgut and salivary glands of specimens of *D. marginatus*, a
264 feature also described from specimens of *Amblyomma* and *Rhipicephalus* (Table 1). While the
265 presence of *Coxiella*-LE in the midgut suggests a nutritive function, its presence in salivary
266 glands is more equivocal. One possibility is that *Coxiella*-LE may be an opportunistic tick-
267 borne pathogen that is inoculated into vertebrates with the tick saliva. A few studies have
268 indeed reported occasional infections by *Coxiella*-LE in vertebrates: these infections were
269 associated with scalp eschar and neck lymphadenopathy in humans (Angelakis et al., 2016),
270 and progressive degenerative health leading to death in pet birds (Shivaprasad et al., 2008;
271 Woc-Colburn et al., 2008). While many tick species harbour *Coxiella*-LE at high prevalence,
272 the rarity of cases in vertebrates strongly suggests that infectious transmission is sporadic
273 (Bonnet et al., 2017; Duron et al., 2015b). A second possibility is that *Coxiella*-LE may not be
274 injected with the saliva. Indeed, Guizzo et al. (2017) detected *Coxiella*-LE in the salivary
275 glands but not in the saliva of the cattle tick *R. microplus*. In this context, it has been assumed
276 that *Coxiella*-LE may facilitate tick feeding through the production of critical molecules with

277 cytolytic, vasodilator, anticoagulant, anti-inflammatory, or immunosuppressive activity
278 (Guizzo et al., 2017), although formal evidence is lacking. A third possibility is that *Coxiella*-
279 LE in salivary glands may protect ticks from pathogens, effectively blocking the transmission
280 of tick-borne pathogens. This has never been demonstrated but, in other arthropods, a number
281 of maternally inherited bacteria, such as *Wolbachia* in mosquitoes, limit the proliferation of
282 pathogenic infectious agents within arthropod tissues (Bian et al., 2013; Hughes et al., 2011;
283 Moreira et al., 2009), and *Coxiella*-LE may behave similarly in ticks (Bonnet et al., 2017).
284 Each of these possibilities now remains to be formally tested, if we are to understand the
285 potential impact of *Coxiella*-LE in salivary glands.

286

287 In conclusion, evidence is now accumulating in support of the hypothesis that Malpighian
288 tubules are key organs for nutritional symbiosis between ticks and B vitamin-providing
289 bacteria, including *Coxiella*-LE. Slight variations in tissue tropism among tick species are also
290 frequently observed, resulting in presence of *Coxiella*-LE outside the Malpighian tubules and
291 ovaries. Future investigations should thus focus on explaining variation in *Coxiella*-LE tissue
292 tropism and its consequences for tick biology and tick-borne diseases.

293

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

Tick species	Geographic origin	Tick tissues				Detection method	Reference
		Salivary glands	Ovaries	Midgut	Malpighian tubules		
Ixodidae (hard ticks)							
<i>Amblyomma americanum</i>	USA, Southern Indiana	+	+	ND	+	FISH	Klyachko et al. 2007
<i>Amblyomma cajennense sensu lato</i>	Brazil, state of Rio de Janeiro and state of Minas Gerais and USA, South Texas (San Antonio, Houston, Austin)	+	+	+	ND	PCR	Machado-Ferreira et al. 2011
		ND	+	+	ND	FISH	
<i>Dermacentor silvarum</i>	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
<i>Haemaphysalis flava</i>	Japan, Shizuoka Prefecture	+	ND	ND	ND	DNA barcoding	Qiu et al. 2014
<i>Haemaphysalis tibetensis</i>	China, Tibet Autonomous Region (Damxung County)	-	+	-	+	qPCR	Wang et al. 2017
<i>Haemaphysalis longicornis</i>	Laboratory colony	-	+	-	+	PCR	Noda et al. 1997
	China, Yunan Province	-	+	-	+	FISH	Wang et al. 2018
<i>Ixodes ovatus</i>	Japan, Shizuoka Prefecture	+	ND	ND	ND	DNA barcoding	Qiu et al. 2014
<i>Ixodes persulcatus</i>	Japan, Shizuoka Prefecture	+	ND	ND	ND	DNA barcoding	Qiu et al. 2014
<i>Rhipicephalus bursa</i>	Italy, Foggia	+	ND	ND	ND	FISH	Raele et al. 2015
<i>Rhipicephalus haemaphysaloides</i>	China, Yunan Province	-	+	+	+	FISH	Wang et al. 2018

<i>Rhipicephalus microplus</i>	Porto Alegre strain (maintained under laboratory conditions)	+	+	+	+	qPCR	Guizzo et al. 2017
<i>Rhipicephalus sanguineus sensu lato</i>	Laboratory colony	-	+	-	+	PCR	Noda et al. 1997
	Israel, Kibbutz Hulda & Caesarea & Rehovot	ND	+	ND	+	FISH	Lalzar et al. 2014
<i>Rhipicephalus turanicus</i>	Israel, Kibbutz Hulda & Caesarea & Rehovot	+	+	+	+	qPCR	Lalzar et al. 2014
		ND	+	ND	+	FISH	

451 **Table 2.** List of probes used in this study.

Probe	Targeted gene	Tick species	5'-3' sequence	Dye
CoxOrn	<i>rpoB</i>	<i>Ornithodoros maritimus</i>	ATTCCTCGCCTGTATTCGACG	CY5
CoxDerm	<i>rpoB</i>	<i>Dermacentor marginatus</i>	TGGTGAGTTGATCGATCAAGGT	CY5
CoxIxo	<i>rpoB</i>	<i>Ixodes hexagonus</i>	GATGCATGCGCGTTCAACGGG	CY5

452

453 **Figure legend**

454 **Figure 1.** *Coxiella*-LE phylogeny constructed using maximum-likelihood (ML) estimations
455 based on *rpoB* gene sequences (472 unambiguously aligned bp). *Coxiella*-LE *rpoB* gene
456 sequences from the tick DNA templates examined in this study are underlined. Sequences
457 from other *Coxiella*-LE and others members of Legionellales (*Rickettsiella gryllii* and
458 *Legionella pneumophila*) available in GenBank were added to the analysis. Black and white
459 circles indicate *Coxiella*-LE found in hard ticks (Ixodidae) and soft ticks (Argasidae),
460 respectively. Bacterial name, host species and GenBank accession number are shown on the
461 tree. Branch numbers indicate percentage bootstrap support for major branches (1000
462 replicates; only bootstrap values >70% are shown). The scale bar is in units of
463 substitution/site.

464

465 **Figure 2.** *Coxiella*-LE detection using epifluorescent microscopy within individual (ie.
466 dissected) ovaries of *O. maritimus* (A and C-F) and *D. marginatus* (B). DAPI signal (blue)
467 and *Coxiella*-LE specific probes signals (red) are merged to localize *Coxiella*-LE within tick
468 tissues (B-F). A: negative-control obtained by incubating an ovary of *O. maritimus* without
469 *Coxiella*-LE specific probe; B: oviduct of a *Coxiella*-LE infected female *D. marginatus*; C:
470 ovary of a *Coxiella*-LE infected female *O. maritimus*; D: close-up view of *Coxiella*-LE
471 infected oocytes of *O. maritimus*; E: a primary oocyte infected by *Coxiella*-LE showing a weak
472 *Coxiella*-LE specific probe signal; F: a mature oocyte infected by *Coxiella*-LE showing a
473 bright *Coxiella*-LE specific probes signal.

474

475 **Figure 3.** *Coxiella*-LE detection using epifluorescent microscopy within individual (ie.
476 dissected) Malpighian tubules of *O. maritimus* (A–C) and *I. hexagonus* (D). DAPI signal
477 (blue) and *Coxiella*-LE specific probes signals (red) are merged to localize *Coxiella*-LE

478 within tick tissues (B-D). A: negative-control obtained by incubating a Malpighian tubule of
479 *O. maritimus* without *Coxiella*-LE specific probe; B: Malpighian tubules of a *Coxiella*-LE
480 infected female *O. maritimus*; C: close-up view of *Coxiella*-LE infected Malpighian tubules
481 of *O. maritimus*; D: close-up view of *Coxiella*-LE infected Malpighian tubules of *I.*
482 *hexagonus*.

483

484 **Figure 4.** *Coxiella*-LE detection using epifluorescent microscopy within *D. marginatus*
485 salivary glands (A–B) from cross body section. DAPI signal (blue) and *Coxiella*-LE specific
486 probes signals (red) are merged to localize *Coxiella*-LE within tick tissues (B). A: negative-
487 control obtained by incubating a salivary gland of *O. maritimus* without *Coxiella*-LE specific
488 probe; B: close-up view of salivary glands of a *Coxiella*-LE infected female *D. marginatus*.
489 Ac: acini.

490

491 **Figure 5.** *Coxiella*-LE detection using epifluorescent microscopy within individual (ie.
492 dissected) *D. marginatus* midgut (A-B). DAPI signal (blue) and *Coxiella*-LE specific probes
493 signals (red) are merged to localize *Coxiella*-LE within tick tissues (A-B). A: midgut of a
494 *Coxiella*-LE infected female *D. marginatus*; B: close-up view of a *Coxiella*-LE infected
495 midgut diverticulum of *D. marginatus*.