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1 **A dual endosymbiosis supports nutritional adaptation to**
2 **hematophagy in the invasive tick *Hyalomma marginatum***

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27 **Abstract**

28 Many animals are dependent on microbial partners that provide essential nutrients lacking
29 from their diet. Ticks, whose diet consists exclusively on vertebrate blood, rely on maternally
30 inherited bacterial symbionts to supply B vitamins. While previously studied tick species
31 consistently harbor a single lineage of those nutritional symbionts, we evidence here that the
32 invasive tick *Hyalomma marginatum* harbors a unique dual-partner nutritional system
33 between an ancestral symbiont, *Francisella*, and a more recently acquired symbiont,
34 *Midichloria*. Using metagenomics, we show that *Francisella* exhibits extensive genome
35 erosion that endangers the nutritional symbiotic interactions: Its genome includes folate and
36 riboflavin biosynthesis pathways but deprived functional biotin biosynthesis on account of
37 massive pseudogenization. Co-symbiosis compensates this deficiency since the *Midichloria*
38 genome encompasses an intact biotin operon, which was primarily acquired via lateral gene
39 transfer from unrelated intracellular bacteria commonly infecting arthropods. Thus, in *H.*
40 *marginatum*, a mosaic of co-evolved symbionts incorporating gene combinations of distant
41 phylogenetic origins emerged to prevent the collapse of an ancestral nutritional symbiosis.
42 Such dual endosymbiosis was never reported in other blood feeders but was recently
43 documented in agricultural pests feeding on plant sap, suggesting that it may be a key
44 mechanism for advanced adaptation of arthropods to specialized diets.

45

46 **Keywords**

47 Endosymbiosis, hematophagy, *Francisella*, *Midichloria*, *Hyalomma*

48 **Introduction**

49 Ticks evolved to hematophagy through the acquisition of key genomic adaptations (Gulia-
50 Nuss et al., 2016; Jia et al., 2020) and the evolution of mutualistic interactions with microbial
51 symbionts (Buysse and Duron, 2021; Duron and Gottlieb, 2020). Ticks rely on vertebrate
52 blood as their sole food source throughout their development, although it is nutritionally
53 unbalanced: Blood contains high levels of proteins, iron, and salts, but low levels of
54 carbohydrates, lipids, and vitamins. To overcome these dietary challenges, tick genomes
55 present large repertoires of genes related to vitamin and lipid shortages, iron management,
56 and osmotic homeostasis (Gulia-Nuss et al., 2016; Jia et al., 2020). Yet, tick genomes do not
57 contain genes for synthesis of some essential vitamins, thus vitamin-provisioning pathways
58 have indirectly arisen from symbiotic associations with transovarially transmitted intracellular
59 bacteria (Ben-Yosef et al., 2020; Duron et al., 2018; Duron and Gottlieb, 2020; Gerhart et al.,
60 2016; Gottlieb et al., 2015; Guizzo et al., 2017; Smith et al., 2015). This partnership allowed
61 the long-term acquisition of heritable biochemical functions by ticks and favored their
62 radiation into the nearly 900 tick species that currently exist (Duron and Gottlieb, 2020). This
63 co-evolutionary process is reflected by microbial communities dominated by tick nutritional
64 symbionts (Bonnet and Pollet, 2020; Narasimhan and Fikrig, 2015).

65

66 Most tick species typically harbor a single lineage of nutritional symbiont, but multiple
67 symbiont lineages exist in different tick species (Duron and Gottlieb, 2020). The most
68 common lineages belong to the *Coxiella*-like endosymbiont group and to the *Francisella*-like
69 endosymbiont group (CLE and FLE, hereafter) (Azagi et al., 2017; Binetruy et al., 2020;
70 Duron et al., 2017; Machado-Ferreira et al., 2016), but a few tick species may rely on other
71 symbionts (Duron et al., 2017), such as the *Rickettsia*-like endosymbionts (Narasimhan et al.,
72 2021) and the intramitochondrial bacterium *Midichloria* (Sassera et al., 2006). Once deprived

73 of their nutritional symbionts, ticks show a complete stop of growth and moulting, as well as
74 lethal physical abnormalities (Ben-Yosef et al., 2020; Duron et al., 2018; Guizzo et al., 2017;
75 Zhong et al., 2007), which can be fully restored with an artificial supplement of B vitamins
76 (Duron et al., 2018). Nutritional symbionts of ticks exhibits a high degree of lifestyle
77 specialization toward mutualism. They have been found primarily in two tick organs, the
78 ovaries and Malpighian tubules, while other organs have only been found sporadically
79 infected (Buysse et al., 2019; Duron and Gottlieb, 2020). Ovary infection is consistent with
80 vertical transmission of nutritional symbionts in developing oocytes. A high density of
81 nutritional symbionts in Malpighian tubules supports a nutritional role for these symbionts, as
82 Malpighian tubules are involved in excretion and osmoregulation. A likely hypothesis is that
83 nutritional symbionts use tick hemolymph compounds to synthesize *de novo* B vitamins
84 (Duron and Gottlieb, 2020).

85

86 Genomes of CLE and FLE consistently contain complete biosynthesis pathways of the same
87 three B vitamins: biotin (vitamin B₇), riboflavin (B₂), and folate (B₉) (Duron et al., 2018;
88 Gerhart et al., 2018, 2016; Gottlieb et al., 2015; Guizzo et al., 2017; Nardi et al., 2021; Smith
89 et al., 2015). These three pathways form a set of core symbiotic genes essential for ticks
90 (Duron and Gottlieb, 2020) while other functions are disposable: As such, the genomes of
91 CLE, FLE and *Midichloria* underwent massive reduction as observed in other nutritional
92 endosymbionts of arthropods with a specialized diet (Duron et al., 2018; Gerhart et al., 2018,
93 2016; Gottlieb et al., 2015; Nardi et al., 2021; Sassera et al., 2011; Smith et al., 2015). This
94 reduction process is primarily initiated as a consequence of relaxed selection on multiple
95 genes which are not essential within the host stable and nutrient-rich cellular environment
96 (Bennett and Moran, 2015; McCutcheon et al., 2019). Over time, genes encoding for
97 functions such as motility, regulation, secondary-metabolite biosynthesis, and defense are

98 pseudogenized and then definitively jettisoned (Bennett and Moran, 2015; McCutcheon et al.,
99 2019).

100

101 Coevolution of hosts and their nutritional symbionts over millions of years is expected to
102 result in stable co-cladogenesis (Bennett and Moran, 2015). However, in sap-feeding insects
103 where symbionts compensate for nutritional deficiencies of the sap diet, cases of unstable
104 interactions have been observed (Bennett and Moran, 2015; McCutcheon et al., 2019).

105 Indeed, as the symbiont genome decays, repair mechanisms can be lost, leading to faster
106 pseudogenization, which can affect genes in all functional categories including those involved
107 in nutritional supplementation (Bennett and Moran, 2015; McCutcheon et al., 2019). This
108 may ultimately lead to overly reduced and maladapted genomes, limiting beneficial
109 contributions to hosts and opening the road to wholesale replacement by new symbiont(s)
110 (Bennett and Moran, 2015; McCutcheon et al., 2019; Sudakaran et al., 2017). This

111 degeneration–replacement model has been proposed in sap-feeding insects (Campbell et al.,
112 2015; Łukasik et al., 2017; Manzano-Marín et al., 2020; Matsuura et al., 2018), but is difficult
113 to document as such replacements are expected to be transient (McCutcheon et al., 2019). In
114 parallel, other forms of multi-partite interactions have been reported, where a novel bacterium
115 with similar function to a pre-existing symbiont enters a bipartite system (Husnik and
116 Mccutcheon, 2016; Santos-Garcia et al., 2018; Takeshita et al., 2019). This can lead to
117 relaxation of conservative selection pressure on the original symbionts, which can lose genes
118 that were previously fundamental, ultimately leading to a tripartite stable symbiosis (Husnik
119 and Mccutcheon, 2016; Takeshita et al., 2019).

120

121 In this study we document a complex nutritional endosymbiosis in the invasive bont-legged
122 tick *Hyalomma marginatum* characterized by a maladapted ancestral symbiont genome

123 compensated by gene functions of a more recently acquired symbiont. This large tick species
124 usually feeds on humans and a diversity of mammals and birds in the Mediterranean climatic
125 zone but has recently expanded its range to the north, invading Western Europe (Vial et al.,
126 2016). *H. marginatum* can transmit Crimean-Congo hemorrhagic fever virus, an emerging
127 disease affecting humans in the northern Mediterranean Basin, as well as protozoan parasites
128 and intracellular bacterial pathogens such as *Rickettsia aeschlimannii* (Vial et al., 2016).
129 Previous studies detected the presence of diverse highly prevalent bacterial symbionts across
130 *H. marginatum* populations (Di Lecce et al., 2018), but their contribution to tick nutrition was
131 not studied. A previous study showed that high densities of FLE can be observed in
132 Malpighian tubules and ovaries of *H. marginatum*, while infections in other organs are
133 scattered (Azagi et al., 2017). Here we sequenced three microbial metagenomes of *H.*
134 *marginatum* in three distinct locations, and determined the nutritional interactions in this
135 unique symbiosis system.

136

137 **Results**

138 **Microbial metagenomes**

139 We sequenced three microbial metagenomes from *H. marginatum* specimens collected in
140 Spain (i.e., the Hmar-ES metagenome), Italy (Hmar-IT), and Israel (Hmar-IL). We obtained
141 57,592,510 paired-end reads from the Hmar-ES metagenome, 68,515,944 paired-end reads
142 from Hmar-IT, and 10,807,740 paired-end reads from Hmar-IL. We assembled one FLE
143 genome with >99% estimated completeness from each metagenome (FLE-Hmar-ES, FLE-
144 Hmar-IT, and FLE-Hmar-IL; also designated as FLE-Hmar genomes; Table S1). In parallel,
145 we assembled one genome with >95% estimated completeness from each metagenome for the
146 bacterium *Midichloria*, and thus obtained three additional complete genomes (Midi-Hmar-ES,
147 Midi-Hmar-IT, and Midi-Hmar-IL; also designated as Midi-Hmar genomes; Table S1). In

148 addition, we detected a third bacterium, the tick-borne pathogen *Rickettsia aeschlimannii*, in
149 the Hmar-IT and Hmar-IL, but not Hmar-ES, metagenomes, and further assembled one
150 genome with 94% estimated completeness (RAES-Hmar-IT) and one partial (RAES-Hmar-
151 IL) genome (Table S1; Figure 1).

152

153 The assemblies of the three FLE draft genomes (13 contigs for FLE Hmar-IT, 17 for FLE
154 Hmar-ES, and 25 FLE Hmar-IL) were very similar in size (1.51 to 1.536 Mb), average G+C
155 content (31.11 to 31.23%), number of predicted protein-coding genes (937 to 961), and
156 protein-coding sequences with inactivating mutations (pseudogenes, 789 to 798), no
157 prophages, and 2 identical insertion sequences (Supplementary Table S1). Phylogenomic
158 analysis based on 436 single-copy orthologous *Francisella* genes showed that FLE-Hmar-ES,
159 FLE-Hmar-IT, and FLE-Hmar-IL cluster together in a robust clade within other FLE of ticks
160 (Figure 2A). The closest relative of this clade is the FLE of another *Hyalomma* species,
161 *Hyalomma asiaticum* from China, while FLEs of other tick genera (*Ornithodoros*, *Argas*, and
162 *Amblyomma*) are more distantly related. The FLE clade is embedded in the *Francisella* genus
163 and shares a common origin with pathogenic species, including the tularemia agent, *F.*
164 *tularensis*, and an opportunistic human pathogen, *F. opportunistica* (Figure 2A). The FLE-
165 Hmar genomes are similar in organization and content overall, apart from a 127,168 bp
166 inversion in FLE-Hmar-ES and a 4,165 bp inversion in FLE-Hmar-IL (Figure 2B). Compared
167 to other FLE genomes (i.e. FLEOm and FLEAm), they are slightly reduced in size and exhibit
168 substantial rearrangements (Figure 2B, Tables S1 and S2). Based on gene orthologs, the core-
169 genome of these FLE shares 743 genes, while the core genome of the FLE of *H. marginatum*
170 contains 796 genes because the FLE-Hmar genomes share 53 genes that are not present in
171 other FLE genomes (Figure 2C). Interestingly, pseudogenes are highly abundant in the three
172 genomes of FLE of *H. marginatum*, compared with other FLE genomes (Figure 2D), and

173 most functional categories are impacted by this advanced pseudogenization process. Most of
174 the genes associated with virulence in pathogenic *Francisella* species, including the
175 *Francisella* Pathogenicity Island (FPI) and secretion system, are pseudogenized or completely
176 missing in FLE-Hmar genomes, consistent with previous observations in other FLEs (Figure
177 S1). Furthermore, FLE-Hmar genomes contain fewer genes involved in replication,
178 recombination, and repair than any other FLEs analyzed (Figure 2C). The nucleotide excision
179 repair system (*uvrABCD*) in FLE-Hmar genomes is conserved, however the DNA mismatch
180 repair system (*mutSL*) is fully pseudogenized. In other FLEs, these systems are conserved,
181 with the exception of the *mutSL* system in the FLE of *Ornithodoros moubata* and *H.*
182 *asiaticum* genomes (Figure S2).

183

184 The *Midichloria* genomes, Midi-Hmar-ES, Midi-Hmar-IT, and Midi-Hmar-IL, were
185 assembled in 179, 359 and 123 contigs respectively, and their genomes reach 1.13 to 1.16 Mb,
186 with consistent G+C content (35.03-35.23%), number of predicted protein-coding genes
187 (1,032 to 1,071), pseudogenes (190 to 192), no prophages, and between 3 and 10 insertion
188 sequences (Table S1). Remarkably, their average coding density (76%) is higher than those of
189 FLEs of *H. marginatum* (54%), and furthermore, those of other FLE representatives (range
190 from 57 to 69%). Phylogenomic analysis based on 242 single-copy orthologous *Rickettsiales*
191 genes shows that Midi-Hmar-ES, Midi-Hmar-IT, and Midi-Hmar-IL cluster in a highly
192 supported monophyletic sister group of the single other sequenced *Midichloria* genome,
193 *Candidatus M. mitochondrii* strain IricVA (*M. mitochondrii* hereafter), the intra-
194 mitochondrial endosymbiont of the European sheep tick, *Ixodes ricinus*. Altogether, they
195 delineate a monophyletic clade within the *Midichloriaceae* family (Figure 3A). The high
196 number of Midi-Hmar contigs precluded comparison of their genome structures. However,
197 gene contents of the Midi-Hmar genomes are highly similar, differing substantially from *M.*

198 *mitochondrii* (Tables S1 and S2). Based on gene orthologs, the pan-genome of *Midichloria*
199 shared 745 genes, and Midi-Hmar genomes shared 181 genes that are not present in *M.*
200 *mitochondrii* (Figure 3B). Pseudogenization only impacted a few functional categories (e.g.,
201 genes of chromatin structure and dynamics [category B] and genes of the mobilome
202 [prophages and transposons, category X]), across Midi-Hmar genomes (Figure 3C). By
203 contrast to FLE genomes, the DNA mismatch repair system (*mutSL*) and the nucleotide
204 excision repair (*uvrABCD*) are not pseudogenized in Midi-Hmar genomes and are thus
205 potentially functional (Figure S2).

206

207 In two metagenomes, we also detected contigs belonging to *R. aeschlimannii*. We recovered
208 one possibly complete chromosome (1.35 Mb for 165 contigs, with 1,122 genes, 475
209 pseudogenes and 32.32% G+C content) for RAES-Hmar-IT (Table S1), whereas for RAES-
210 Hmar-IL only a partial genome (0.215 Mb) was obtained. No *Rickettsia* reads were detected
211 in the Hmar-ES metagenome. This may be due to the absence of the bacterium in the Hmar-
212 ES microbiome, or, possibly, to experimental artefact: This metagenome was obtained by
213 sequencing DNA extracted from the dissected ovaries of 10 females, whereas the Hmar-IT
214 metagenome was sequenced from DNA extracted from a whole female, suggesting that
215 *Rickettsia* may be absent or at low density in tick ovaries. This pattern of tissue localization
216 may be corroborated by the Hmar-IL metagenome, sequenced from a pool of dissected
217 ovaries of 10 ticks, but for which we obtained only a few *Rickettsia* reads. This reflects either
218 the absence of *Rickettsia* in the dissected ticks (*Rickettsia aeschlimannii* usually reaches low-
219 to-medium prevalence in field populations of *H. marginatum*; Azagi et al., 2017), or a lower
220 abundance of *Rickettsia* in ovaries. Phylogenetic analysis based on 324 single-copy
221 orthologous genes present in *Rickettsia* genomes showed that RAES-Hmar-IT clusters with
222 the tick-associated human pathogen *R. aeschlimannii* (including the strain MC16 previously

223 isolated from *H. marginatum*) and altogether delineate a robust clade with the Spotted Fever
224 *Rickettsia* group (Figure S3). Overall, the low abundance of *Rickettsia* in the Hmar-IL
225 metagenome (8x average genome coverage), its absence in the Hmar-ES metagenome, as well
226 as the genetic proximity with *R. aeschlimannii*, are all indicative of a tick-borne pathogen,
227 rather than a nutritional endosymbiont.

228

229 **Complementation in nutritional endosymbiosis**

230 While all previously sequenced FLE genomes contain complete pathways for biotin,
231 riboflavin, and folate, the FLE-Hmar genomes only retain intact pathways for riboflavin and
232 folate (Figure 4A). The biosynthesis pathway for biotin is non-functional, as from six genes of
233 the complete pathway, five (*bioA*, *bioD*, *bioC*, *bioF*, and *bioB*) are pseudogenized
234 (frameshifted) in the FLE-Hmar genomes (only *bioH* is not pseudogenized; Figure 4A).
235 Pseudogenization are due to indels or mutations that induce premature codon stops
236 interrupting the coding sequences, indicating that FLE-Hmar cannot produce biotin (Figure
237 S4). While *bioA*, *bioD*, *bioC*, and *bioF* contains multiple indels or mutations (introducing
238 premature stop codon, or missing start codon), *bioB* shows a low level of pseudogenization
239 with a unique indel: A stop codon comes from a reading frame shift due to the insertion of
240 one adenine base on a poly(A) zone (Figure S4). Since infidelity at poly(A) tracks can rescue
241 pseudogenes in endosymbionts (Tamas et al., 2008), the *bioB* gene could be thus functional.
242 Partial biosynthesis pathways of four other B vitamins (pantothenic acid: B₅; nicotinic acid:
243 B₃; pyridoxine: B₆, and thiamine: B₁), each containing one to four pseudo/missing genes, are
244 also present in the FLE-Hmar genomes, a pattern consistent with other FLE genomes (Figure
245 S5).

246

247 The B-vitamin pathways in Midi-Hmar genomes are similar to those of *M. mitochondrii*. A
248 complete pathway of biotin was consistently recovered in *Midichloria* genomes (Figure 4A).
249 For folate, five of the six biosynthesis genes (*folE*, *folB*, *folKP*, *folC*, and *folD*) were present
250 but the sixth gene, *folA* (encoding for a dihydrofolate reductase), was not. However, we
251 detected an alternative dihydrofolate reductase gene, *folM* (also known as *ydgB*), that was
252 shown in other bacteria to provide the same metabolic function than *folA* (Giladi et al., 2003)
253 suggesting that the folate pathway is functional in Midi-Hmar genomes. Only partial
254 pathways were detected for riboflavin and pantothenic acid with three and four missing genes,
255 respectively (Figures 4A and S5).

256

257 No B vitamin pathway seems complete in the RAES-Hmar genomes. In the RAES-Hmar-IT
258 genome, partial pathways of biotin and folate were detected with each three missing genes
259 (Figure 4A). For pantothenic acid, only one of the five biosynthesis genes is present (Figure
260 S5). No genes for other B vitamin pathways were found in the RAES-Hmar-IT genome. No
261 additional B vitamin gene was observed in the other *Rickettsia* genome, RAES-Hmar-IL,
262 which is incomplete. There is thus no obvious evidence that presume that RAES-Hmar are B-
263 vitamin provisioning endosymbionts in *H. marginatum*.

264

265 We further searched for B vitamin genes of other origins than FLE, *Midichloria* and
266 *Rickettsia* genomes in tick metagenomes. In the Hmar-ES and Hmar-IL metagenomes
267 (obtained from ovary samples), we found no significant hits, indicating that neither an
268 additional B vitamin provisioning microorganism was present, nor did the tick genome itself
269 encodes B vitamin pathways. In the Hmar-IT metagenome (obtained from a whole tick), we
270 found four biotin genes (*bioA*, *bioD*, *bioF*, and *bioB*) in low abundance contigs of
271 *Staphylococcus* bacteria. Previous studies report the presence of *Staphylococcus*, a common

272 skin-associated bacteria, on tick exoskeletons and midguts and hypothesize this to be due to
273 contamination, or acquisition during feeding (Greay et al., 2018).
274
275 Ticks differ from other eukaryotes in that they lack most of the genes encoding proteins
276 required to produce and degrade heme (Gulia-Nuss et al., 2016; Jia et al., 2020). We thus
277 searched for relevant pathways in the symbiont genomes and found that the FLE-Hmar and
278 Midi-Hmar genomes contain genes involved in heme and iron homeostasis. The heme
279 biosynthetic pathway was consistently detected, as well as a gene encoding heme oxygenase
280 that catalyzes the degradation of heme and the subsequent release of iron (Figure 4B). In these
281 genomes we detected similar genetic patterns as those observed in the other FLE and
282 *Midichloria* genomes. However, the heme pathway is partially degraded in FLE-Hmar
283 genomes since their *hemN* gene is pseudogenized, a pattern not observed in other FLE
284 genomes, suggesting an early stage of degradation for the heme pathway. This gene is present
285 and not pseudogenized in the Midi-Hmar genomes (Figure 4B), suggesting these symbionts
286 may compensate for the FLE heme pathway. In addition, three other genes, *hemL*, *hemY*, and
287 *hemG* were not found in *Midichloria* genomes, but were present in FLE genomes (Figure 4B).
288 As a result, a combination of FLE and *Midichloria* genes may be required to obtain a
289 complete heme pathway.

290

291 **Phylogenetic origins of B vitamin biosynthetic pathways**

292 Our analysis shows that the B vitamin biosynthesis genes in the FLE-Hmar genomes have all
293 been vertically inherited from a *Francisella* ancestor as their B vitamin genes are always more
294 closely related to *Francisella* orthologous genes than to genes of more distantly related
295 bacteria (examples are shown in Figures S6A-B). Similarly, folate and riboflavin biosynthesis
296 genes in the Midi-Hmar genomes are of *Midichloria* origin as these genes are more closely

297 related to orthologous genes present in *M. mitochondrii* and other members of the
298 *Midichloriaceae* family than to genes of other bacteria (Figures S6A-B).
299
300 By contrast, the six biotin genes present in the Midi-Hmar genomes did not originate from the
301 *Midichloriaceae* family, but from an exogenous origin. The biotin synthesis genes *bioD*,
302 *bioC*, *bioH*, *bioF*, and *bioB* formed a compact operon in the Midi-Hmar genomes while the
303 *bioA* gene is more distantly located along the bacterial chromosome (Figure 5). A similar
304 operon structure is also present on the *M. mitochondrii* genome. In contrast, an arrangement
305 in which the *bioA* is gene physically associated with the remainder of the biosynthetic operon
306 was identified on the genomes of multiple intracellular bacteria that are all distantly related to
307 *Midichloria*, including the *Cardinium* endosymbionts (Bacteroidetes: Flexibacteraceae) of
308 parasitoid wasps and leaf-hoppers; the *Wolbachia* endosymbionts (Alphaproteobacteria:
309 Anaplasmataceae) of bed bugs, bees, planthoppers, and fleas; the *Legionella* endosymbiont
310 (Gammaproteobacteria: Legionellaceae) of the rodent lice; *Rickettsia buchneri*
311 (Alphaproteobacteria: Rickettsiaceae) symbiont of the blacklegged tick *Ixodes scapularis*; and
312 diverse intracellular pathogens of vertebrates, such as *Lawsonia* (Deltaproteobacteria:
313 Desulfovibrionaceae) and *Neorickettsia* (Alphaproteobacteria: Anaplasmataceae) (Figure 5
314 and Table S3). In most cases, the biotin genes form a compact and streamlined operon of 5-7
315 kb without major structural variation among bacteria. Phylogenetic analyses of the six biotin
316 synthesis genes consistently exhibited similar evolutionary patterns (Figure 5). These patterns
317 suggest that the biotin synthesis genes were acquired as a whole operon by an ancestor of
318 *Midichloria* from an unrelated intracellular bacterium. In some cases, this streamlined biotin
319 operon is also associated with nutritional symbionts of obligate blood feeders, such as
320 *Wolbachia* of bedbugs, *Legionella* of rodent lice, and *R. buchneri* in the blacklegged tick, *I.*
321 *scapularis* (Figure 5).

322

323 **Co-infections of nutritional endosymbionts in tick ovaries**

324 We next assessed the abundance of FLE and *Midichloria* in *H. marginatum* ovaries, which are
325 the key organ where vertical transmission of nutritional symbionts to developing oocytes
326 occurs (Azagi et al., 2017). Real-time qPCR assays on 10 field *H. marginatum* adult females
327 indicated that FLE and *Midichloria* are both abundant in tick ovaries, with FLE and
328 *Midichloria* detected in 10 and 9 ovarian samples, respectively. High densities were always
329 observed for FLE (686.09 ± 206.89 FLE *rpoB* gene copies per tick *cal* gene copies) and for
330 *Midichloria* (88.72 ± 28.41 *Midichloria gyrB* gene copies per tick *cal* gene copies) (Figure
331 6A). FLE were consistently most abundant with densities 5.8 to $21.4 \times$ higher than
332 *Midichloria* densities (paired t test, $p = 0.013$; one outlier was removed from analysis, $n = 8$).
333 The FLE and *Midichloria* densities covaried positively in individual ticks ($\chi^2 = 23.937$, $p =$
334 0.003 ; Figure 6B).

335

336 **Evolutionary history of endosymbiosis in the *Hyalomma* genus**

337 We further examined the evolutionary history of *Hyalomma* species and their associations
338 with FLE and *Midichloria* through a bibliographic survey. Of 10 *Hyalomma* species with
339 sufficient published data, all were found infected by FLE, and 9 by *Midichloria* (Table S4).
340 We next reconstructed *Hyalomma* phylogeny using mitochondrial DNA sequences of the
341 cytochrome c oxidase I (*cox1*) gene available on Genbank (Figure S7). Mapping of FLE and
342 *Midichloria* onto the *Hyalomma* phylogenetic tree showed that co-infection by FLE and
343 *Midichloria* is widespread across all *Hyalomma* lineages (Figure S8). However, FLE and
344 *Midichloria* co-infection was not detected in one species, *H. asiaticum*, nested within the
345 *Hyalomma* phylogeny among co-infected species.

346

347 **Discussion**

348 In this study, we show that the tick *H. marginatum* is a complex symbiotic system, combining
349 genes of diverse phylogenetic origins to generate nutritional adaptations to obligate
350 hematophagy. This tick harbors a microbiome functionally analogous to those observed in
351 other tick species, dominated by intracellular bacterial symbionts producing the core set of B
352 vitamins (biotin, riboflavin, and folate) essential for tick growth and survival. However, while
353 in other tick species, as in other obligate blood feeders, production of all three core B vitamins
354 is performed by a single nutritional symbiont (Binetruy et al., 2020; Duron et al., 2017; Duron
355 and Gottlieb, 2020), *H. marginatum* harbors two bacterial partners whose core B vitamins
356 biosynthetic capacities depend on a combination of genes, with indigenous and exogenous
357 phylogenetic origins. This multi-partner nutritional symbiosis is stably maintained at least at
358 the tick species level, as supported by our founding of genetically similar FLE and
359 *Midichloria* present in *H. marginatum* populations from geographically distant regions.

360

361 Genome reconstructions confirmed the key role of symbionts in *H. marginatum* nutrition, but
362 contrary to other tick species, no single symbiont does compensate for all nutritional needs.
363 The FLE-Hmar genomes contain the genes to synthesize folate and riboflavin but its biotin
364 synthesis pathway is degraded, and thus non-functional. Genome reduction is a conserved
365 trait in all FLEs, but this process has extended in the FLE of *H. marginatum*, with an
366 additional loss of genes in most COG functional categories. This process could have been
367 triggered by the pseudogenization of the mismatch repair system (*mutSL*), leaving the FLE of
368 *H. marginatum* with only one DNA repair system, nucleotide excision repair (*uvrABCD*).
369 Absence of the *mutSL* system, coupled with strict clonality and a small population size during
370 transovarial transmission, have probably led the FLE- Hmar genomes to accumulate

371 deleterious mutations, that resulted in maladaptation, and ultimately limited their beneficial
372 contributions to ticks.

373

374 In the context of FLE genome degradation, co-infection with *Midichloria* prevents the
375 breakdown and collapse of nutritional symbiosis in *H. marginatum*; i.e., the *Midichloria*
376 genome contains an intact biotin biosynthesis operon, and thus compensates for genome
377 decay of FLE. Inversely, *Midichloria* has only partial pathways for riboflavin and folate and
378 hence cannot meet the nutritional needs of *H. marginatum* for these B vitamins.

379 Consequently, the co-infection of FLE with *Midichloria* forms a cooperative system essential
380 for the nutritional symbiosis in *H. marginatum*. In addition, complementary heme synthesis
381 genes are present in the FLE and *Midichloria* genomes, and these symbionts may be an
382 additional source of heme, perhaps conferring a fitness advantage to *H. marginatum* during
383 periods of starvation. In animals, heme is essential for the function of all aerobic cells, playing
384 an important role in controlling protein synthesis and cell differentiation. Complete
385 biosynthetic heme gene pathways exist in most animals, but not in ticks which need an
386 exogenous source of heme (Gulia-Nuss et al., 2016; Jia et al., 2020). Vertebrate blood is
387 usually considered to be their unique source for this essential cofactor (Perner et al., 2016),
388 however ticks spend most of their life off host, and thus the presence of heme genes in FLE
389 and *Midichloria* genomes may also indicate symbiont provisioning of heme in *H.*
390 *marginatum*.

391

392 The co-localization of FLE and *Midichloria* in tick ovaries suggests that vertical transmission
393 has led to their stable co-inheritance through tick generations, linking their evolutionary fate,
394 as observed in other symbiotic systems of arthropods (McCutcheon et al., 2019; Vautrin and
395 Vavre, 2009). This co-transmission fidelity creates the conditions under which cooperative

396 interactions can emerge: The stable coexistence of FLE and *Midichloria* genomes makes
397 various genes redundant, as we observed for B vitamin and heme synthesis pathways.
398 Furthermore, the covariance of FLE and *Midichloria* densities in ticks is positive, suggesting
399 that these two symbionts are not in direct competition but rather cooperative. However, FLE
400 is about 100-times more abundant than *Midichloria*, which can be suggestive of competition.
401 Indeed, negative interactions among these endosymbionts can occur because their tick host
402 provides a limited amount of resources and space. Under this hypothesis, the density of
403 *Midichloria* can be depressed by FLE, which could be more competitive. Regardless of the
404 issue of this interaction, FLE and *Midichloria* depend on each other since their host tick
405 would not survive without their combined production of B vitamins. The asymmetrical
406 abundance of FLE and *Midichloria* may have further implications in nutritional
407 endosymbiosis. First, it suggests that, at lower abundance, *Midichloria* can fully bridge the
408 biotin requirements for *H. marginatum*. Second, it could imply that biotin (produced by
409 *Midichloria*) is required at much lower abundance than riboflavin (FLE) and folate (FLE and
410 *Midichloria*), or that *Midichloria* could over-express its biotin genes, possibly thanks to the
411 streamlined structure of the biotin operon, and finally produce biotin at the same abundance
412 than riboflavin and folate.

413

414 While the nutritional association of FLE with *Hyalomma* species has persisted for millions of
415 years (Azagi et al., 2017), the nutritional role of *Midichloria* seems more recent in this tick
416 genus. Co-infection with *Midichloria* was reported in at least nine *Hyalomma* species (cf.
417 Table S4), but in most species, such as *H. excavatum* and *H. impeltatum* (Azagi et al., 2017;
418 Selmi et al., 2019), *Midichloria* is present at much lower frequencies than would be expected
419 for an obligate nutritional mutualist, suggesting that it is instead a facultative (i.e., not
420 essential) association. Interestingly, the *Hyalomma* species apparently devoid of *Midichloria*,

421 *H. asiaticum*, harbors a FLE with intact biotin, folate, and riboflavin synthesis pathways
422 (Buysse and Duron, 2021). In *H. marginatum*, the decay of the FLE genome may have been
423 accelerated by the presence of *Midichloria* and the redundancy of biotin biosynthesis, a
424 process also reported in sap-feeding insects (Husnik and Mccutcheon, 2016; Manzano-Marín
425 et al., 2020). While it is clear that two events have led to the current status (loss of biotin
426 genes and acquisition of *Midichloria*), the order by which these happened is unclear.
427 *Midichloria* could have rescued a decaying symbiosis, or could have led, with its entrance in
428 the system, to relaxation of selective constraints on FLE. Based on the common presence of
429 *Midichloria* at lower frequencies in other *Hyalomma* species, a scenario of gradual parallel
430 occurrence of these two events cannot be ruled out.

431

432 Our analyses also indicated that the biotin operon detected in the *Midichloria* of *H.*
433 *marginatum* has an exogenous origin, being acquired following an ancient lateral gene
434 transfer pre-dating the divergence between the *Midichloria* of *H. marginatum* and the one of
435 *I. ricinus*. Accumulating genomic sequences indicate that this streamlined biotin operon
436 (denoted in some studies as the Biotin synthesis Operon of Obligate intracellular Microbes –
437 BOOM (Driscoll et al., 2020)) has experienced transfers between distantly related
438 endosymbionts inhabiting arthropod cells including *Wolbachia* (Driscoll et al., 2020; Gerth
439 and Bleidorn, 2016; Ju et al., 2020; Nikoh et al., 2014), *Rickettsia* (Gillespie et al., 2012),
440 *Cardinium* (Penz et al., 2012) and *Legionella* (Říhová et al., 2017). Remarkably, the invasive
441 nature of this streamlined biotin operon has contributed not only to the emergence of novel
442 nutritional symbioses in *H. marginatum* with *Midichloria*, but also in other blood feeders,
443 such as bedbugs with *Wolbachia* (Nikoh et al., 2014) and rodent lice with *Legionella* (Říhová
444 et al., 2017), or in sap feeders as white flies with *Cardinium* (Santos-Garcia et al., 2014) and
445 planthoppers with *Wolbachia* (Ju et al., 2020). Until recently, lateral gene transfer was

446 thought to be rare in intracellular symbionts, as they reside in confined and isolated
447 environments. However, co-infections of unrelated endosymbionts within the same host cell
448 have created freely recombining intracellular bacterial communities, a unique environment
449 previously defined as the ‘intracellular arena’ (Kent and Bordenstein, 2010). The detection of
450 the streamlined biotin operon in *Wolbachia*, *Rickettsia*, and *Cardinium* – three of the most
451 common intracellular symbionts of arthropods (Duron et al., 2008) – corroborates the role of
452 such ‘intracellular arenas’ in the evolutionary dynamics of nutritional symbioses, especially as
453 the biotin operon can be found on plasmids (Gillespie et al., 2012).

454

455 The macroevolutionary and ecological consequences of acquiring nutritional symbionts are
456 immense, ticks would simply not exist as strict blood feeders without such mutualistic
457 interactions (Duron and Gottlieb, 2020). Our investigations corroborate that nutritional
458 symbionts of ticks have all converged towards an analogous process of B vitamin
459 provisioning. While past investigations showed that this evolutionary convergence consists in
460 severe degeneration of symbiont genomes accompanied by preservation of biotin, folate, and
461 riboflavin biosynthesis pathways (Buisse and Duron, 2021; Duron and Gottlieb, 2020),
462 nutritional endosymbiosis in *H. marginatum* is influenced by a more complex web of
463 interactions resulting in a dynamic mosaic of symbiont combinations and offering novel
464 evolutionary possibilities.

465

466 **Materials and Methods**

467 **Tick collection, DNA extraction and molecular screening**

468 Adult females of *H. marginatum* were collected from domestic animals from three
469 geographical regions (Italy, Spain, and Israel) (Figure 1). Whole body of one female from
470 Italy was processed for metagenome sequencing, while ovaries from females collected in

471 Spain (n=10) and Israel (n=10) were used for metagenome sequencing (and for qPCR in the
472 case of samples from Spain). For each sample, total DNA was extracted using either the
473 DNeasy Blood and Tissue kit (QIAGEN) or the NucleoSpin Plant II kit (Macherey-Nagel).

474

475 A molecular screening was performed by qPCR (iQ5 system, Bio-Rad) on Spanish samples to
476 evaluate the bacterial load of FLE and *Midichloria* symbionts in ovaries. For each specimen,
477 three SYBR Green-based qPCR (Bio-Rad) assays were performed: (i) A specific
478 amplification for the *H. marginatum cal* gene, (ii) a specific amplification for the FLE *rpoB*
479 gene, and (iii) a specific amplification for the *Midichloria gyrB* gene. Thermal protocols and
480 primers are listed in Supplementary Table S5.

481

482 **Genome sequencing, assembly and annotation**

483 Metagenomes were sequenced on Illumina HiSeq 2500, after a library preparation performed
484 with Nextera XT. For the Italian and the Spanish samples, the raw paired-end reads quality
485 was evaluated with FastQC (Andrews, 2010). Paired-end reads were trimmed via
486 Trimmomatic (v0.40) (Bolger et al., 2014) and then assembled using SPAdes (v3.10.0)
487 (Bankevich et al., 2012) following the Blobology pipeline (Kumar et al., 2013) (thus
488 separating reads according to G+C content, coverage, and taxonomic annotation). Discarding
489 of contaminating sequences and refining of the assemblies were performed manually through
490 the analysis of the assemblies' graphs with Bandage (v0.8.1) (Wick et al., 2015). Bacterial
491 reads were separated from the tick reads, allowing the assembling of different bacterial
492 genomes ("filtered" assemblies). For the Israeli sample, reads were trimmed using Cutadapt
493 (v1.13) (Martin, 2011) and contaminants' reads were removed through a mapping against
494 Coliphage phi-X174 (GenBank: NC_001422) and *Staphylococcus aureus* (NC_010079)
495 genomes with BWA (v0.7.17-r1188) (option mem) and SAMtools (v1.10) (Li and Durbin,

496 2009). Remaining reads were further assembled using SPAdes (v3.11.1) (Bankevich et al.,
497 2012) with default parameters. These “raw” assembled scaffolds were compared to different
498 reference genomes separately (FLE of *O. moubata* FLEOm, *M. mitochondrii*, and *R.*
499 *aeschlimannii*; Table S2) to discard unspecific scaffolds using RaGOO (v1.02) (Alonge et al.,
500 2019), and, for each reference genome separately, the assignation of the remaining scaffolds
501 (“filtered” assembly) was confirmed using the online NCBI BLAST tool
502 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The quality assessment and the completeness of the
503 “filtered” assemblies from all *H. marginatum* metagenomes were estimated using QUAST
504 (v4.6.3) (Gurevich et al., 2013) and miComplete (v1.1.1) (Hugoson et al., 2020) with the --
505 hmms Bact105 setting. The newly obtained genomes were annotated using Prokka (v1.13.1)
506 (Seemann, 2014) with default parameters. The new *Midichloria* genomes were annotated
507 using a manually revised annotation of *M. mitochondrii* (Table S2) as a reference.

508

509 **Genomic content, structure, and metabolic pathways analyses**

510 The FLE and *Midichloria* whole-genome alignments were obtained separately using Mauve
511 (Darling et al., 2004). Previously, contigs from newly obtained genomes were reordered with
512 Mauve. Graphical representations of newly sequenced genomes were produced using
513 CGView (v1.5) (Stothard and Wishart, 2005). Genes involved in biosynthesis pathways (B
514 vitamins and heme), *Francisella* Pathogenicity Island (FPI), and mismatch repair systems
515 (*mutSL* and *uvrABCD*) were retrieved using BLASTn, BLASTp, and tBLASTx on both newly
516 obtained and reference FLE and *Midichloria* genomes.

517 To identify mobile elements we used ISEScan (Xie and Tang, 2017) to predict insertion
518 sequences and PhySpy (Akhter et al., 2012) for prophages detection. Pseudogenes prediction
519 was performed using Pseudofinder (version downloaded in date April 1st 2020) (Syberg-Olsen
520 et al., 2020) on each genome, including newly obtained and reference genomes of FLE and

521 *Midichloria* (Table S2). The FLE and *Midichloria* datasets were filtered excluding
522 pseudogenes from the subsequent analyses. Clusters of orthologous groups of proteins
523 (COGs) were predicted using the NCBI pipeline (Galperin et al., 2015) and COGs presence
524 was then plotted with an in-house R script. Then, two different datasets were created: (i) a
525 FLE dataset including the three newly sequenced FLE-Hmar genomes and other published
526 FLE genomes; and (ii) a *Midichloria* dataset including the three newly sequenced Midi-Hmar
527 genomes and *M. mitochondrii*. For each dataset, single-copy orthologs (SCO) were identified
528 using OrthoFinder (v2.3.12) (Emms and Kelly, 2019), then SCO lists were retrieved on a R
529 environment (v3.6.2) to build Venn diagrams using the “VennDiagram” R package (Chen and
530 Boutros, 2011).

531

532 To check if whether B-vitamin biosynthesis genes of other origin than FLE, *Midichloria*, and
533 *Rickettsia*, we used the following procedure: We predicted the genes present in the
534 preliminary assemblies of the 3 tick metagenomes using prodigal (using the setting "-p meta")
535 and annotated them with GhostKOALA (Kanehisa et al., 2016). Indeed, for biotin, we
536 checked for the presence of the *bioA*, *bioD*, *bioC*, *bioH*, *bioF*, and *bioB* genes searching for
537 "K00833, K01935, K02169, K02170, K00652, K01012" sequences in the annotations, setting
538 a cutoff to exclude contigs shorter than 500 bp or with a kmer coverage below 4. The hits
539 above cutoff were manually examined with BLAST for functional and taxonomic
540 classification.

541

542 **Phylogenomics**

543 The FLE, *Midichloria* and *Rickettsia* datasets were filtered excluding the pseudogene
544 sequences and were then used for the phylogenetic analyses. For each symbiont dataset,
545 single-copy orthologs (SCO) were identified using OrthoFinder (v2.3.12) (Emms and Kelly,

546 2019) and then aligned with MUSCLE (v3.8.31) (Edgar, 2004). Poorly aligned positions and
547 divergent regions were removed by Gblocks (v0.91b) (Castresana, 2000) prior to
548 concatenation of individual alignments using an in-house script. Finally, the most suitable
549 evolutionary model was determined using modeltest-ng (v0.1.5) (Darriba et al., 2020)
550 according to the Akaike information criterion and maximum likelihood (ML) trees were
551 inferred using RAxML (v8.2.9) (Stamatakis, 2014) with 1,000 bootstrap replicates.

552

553 **Biotin operon phylogeny and structure**

554 Nucleic and protein sequences of the six genes of the biotin biosynthesis pathway were
555 retrieved using BLASTn, BLASTp, and tBLASTx on all *Midichloria* genomes. Sequences
556 from described streamlined biotin operons and sequences from biotin genes from other
557 symbionts (retrieved from GenBank) were included in the dataset (details in Table S3). All
558 protein sequences of the six biotin genes were manually concatenated and aligned with
559 MAFFT (v7.450) (Kato et al., 2002) and ambiguous positions were removed using trimAl
560 (v1.2rev59) (Capella-Gutiérrez et al., 2009). The appropriate substitution model, and then
561 ML-tree, were determined as described above. Meanwhile, 16S rRNA sequences from all
562 organisms included in this dataset were retrieved from GenBank and aligned with MEGA
563 software. The Gblocks program with default parameters was used to obtain non-ambiguous
564 sequence alignments. Phylogenetic analyses were performed with MEGA software, i.e.
565 determination of the appropriate substitution model using Akaike information criterion, ML-
566 tree inference. Clade robustness was assessed by bootstrap analysis using 1,000 replicates.

567

568 Contigs carrying the streamlined biotin operon were retrieved from newly obtained and
569 reference genomes of different intracellular bacteria. Contigs were annotated using Prokka
570 (v1.13.1) (Seemann, 2014) with default parameters. Prokka GenBank outputs were imported

571 in R (v3.6.2) and analyzed using the “genoplotR” R package (Guy et al., 2010). Sequences of
572 *ribH* and *fold* genes from bacteria carrying the streamlined biotin operon and from other
573 bacteria were secondarily separately analyzed. Phylogenetic analyses on these two genes were
574 performed as described above for biotin genes.

575

576 **Bibliographic survey and phylogeny of *Hyalomma***

577 To access the associations between *Hyalomma* species with FLE and *Midichloria*, we
578 performed an extensive bibliographic survey of published studies. For *Hyalomma* species for
579 which presence/absence of FLE and *Midichloria* is documented, we retrieved cytochrome c
580 oxidase I (*coxI*) gene sequences from GenBank and further performed a ML phylogenetic
581 analysis with MEGA software as described above.

582

583 **Statistical analyses**

584 Statistical analyses were carried out using the R statistical package (v3.6.2) and the “lme4” R
585 package (Bates et al., 2015). The FLE load was analyzed using linear mixed-effects models
586 on a dataset containing loads measured in ovaries from adult engorged ticks. The normality of
587 the data distribution was tested through a Shapiro-Wilk test with a 95% confidence. Outliers
588 were removed from datasets. To build models, *Midichloria* load was fitted as a fixed
589 explanatory variable. The best-fitted model was determined with the following procedure: the
590 maximal model, including all higher-order interactions, was simplified by sequentially
591 eliminating interactions and non-significant terms to establish a minimal model. A likelihood
592 ratio test (LRT) (using a chi-square distribution and a p-value cut-off of 0.05) was performed
593 to establish the difference between sequential models. The comparison between the densities
594 of FLE and *Midichloria* of *H. marginatum* was tested by a paired t test.

595

596 **Data and code availability**

597 The bacterial genomes obtained in this study were deposited in GenBank under accession
598 numbers GCA_910592745 (FLE-Hmar-ES), GCA_910592815 (FLE-Hmar-IL),
599 GCA_910592705 (FLE-Hmar-IT), GCA_910592695 (Midi-Hmar-ES), GCA_910592725
600 (Midi-Hmar-IL), GCA_910592865 (Midi-Hmar-IT) and GCA_910592765 (RAES-Hmar-IT).
601 Metagenome reads were deposited under accession numbers ERR7255744 (Hmar-ES),
602 ERR7255745 (Hmar-IL) and ERR7255743 (Hmar-IT). Command lines used for the different
603 genomic analyses are available on GitHub (<https://github.com/mariebuysse/Hmar-2021>).

604

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616

617 **Author contributions**

618 D.S., Y.G. and O.D. designed the study. M.B., A.M.F., Y.G., D.S. and O.D. wrote the
619 manuscript with input from other authors. M.B., A.M.F., T.N. and F.C. performed the
620 genomic, phylogenomic and statistical analyses. F.C., E.O., A.G., A.P., B.M., C.B. and A.C.

621 participated to collection and identification of specimen collection, or molecular biology. All
622 authors agreed on the final version of the manuscript.

623

624 **Competing interests**

625 The authors declare no competing interests.

626

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