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Original article

Tissue tropism and metabolic pathways of *Midichloria mitochondrii* suggest tissue-specific functions in the symbiosis with *Ixodes ricinus*

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ABSTRACT

A wide range of arthropod species harbour bacterial endosymbionts in various tissues, many of them playing important roles in the fitness and biology of their hosts. In several cases, many different symbionts have been reported to coexist simultaneously within the same host and synergistic or antagonistic interactions can occur between them. While the associations with endosymbiotic bacteria have been widely studied in many insect species, in ticks such interactions are less investigated.

The females and immatures of *Ixodes ricinus* (Ixodidae), the most common hard tick in Europe, harbour the intracellular endosymbiont "*Candidatus* Midichloria mitochondrii" with a prevalence up to 100%, suggesting a mutualistic relationship. Considering that the tissue distribution of a symbiont might be indicative of its functional role in the physiology of the host, we investigated *M. mitochondrii* specific localization pattern and the corresponding abundance in selected organs of *I. ricinus* females. We paired these experiments with *in silico* analysis of the metabolic pathways of *M. mitochondrii*, inferred from the available genome sequence, and additionally compared the presence of these pathways in seven other symbionts commonly harboured by ticks to try to obtain a comparative understanding of their biological effects on the tick hosts.

M. mitochondrii was found to be abundant in ovaries and tracheae of unfed *I. ricinus*, and in ovaries, Malpighian tubules and salivary glands of semi-engorged females. These results, together with the *in silico* metabolic reconstruction allow to hypothesize that the bacterium could play multiple tissue-specific roles in the host, both enhancing the host fitness (supplying essential nutrients, enhancing the reproductive fitness, helping in the anti-oxidative defence, in the energy production and in the maintenance of homeostasis and water balance) and/or for ensuring its presence in the host population (nutrients acquisition, vertical and horizontal transmission). The ability of *M. mitochondrii* to colonize different tissues allows to speculate that distinctive subpopulations may display different specializations in accordance with tissue tropism. Our hypotheses should be corroborated with future nutritional and physiological experiments for a better understanding of the mechanisms underlying this symbiotic interaction.

1. Introduction

Symbioses between bacteria and arthropods have been extensively studied and are considered a relevant driver of the evolution of both partners (Dale and Moran, 2006; McCutcheon and Moran, 2011; Moran, 2006; Moya et al., 2008). In the last years, the advancement of genomic approaches, together with the application of advanced microscopy techniques, extensively contributed to unveil the symbiotic interactions between bacteria and their invertebrate hosts.

Arthropods harbour a wide array of intracellular bacterial endosymbionts, which are in several cases transmitted to the progeny via the maternal route, and exhibit varying degrees of interaction with

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their hosts (Dale and Moran, 2006). Some have established ancient obligate mutualistic relationships, playing essential roles for host reproduction, development and survival, mainly providing supporting nutrients to complement the unbalanced diet of their hosts, such as amino acids or vitamins, or helping in the metabolism of nitrogen. These are often referred to as primary endosymbionts (Bonnet et al., 2017; Moran et al., 2008; Wernegreen, 2012). Other symbionts, not obligatory associated with the host and likely more recently acquired, still play significant roles in the host biology. These, usually referred to as secondary symbionts, can be involved in a wide range of phenomena and functions, such as manipulation of the host reproduction, resistance to xenobiotic compounds, heat tolerance, and protection against pathogens (Bonnet et al., 2017; Ferrari and Vavre, 2011; Moya et al., 2008). Intracellular symbionts can display different tissue tropism within the host body. The tissue localization can vary according to species, sex and life stages, and can be an important indicator of the specific role of a symbiont in the host's biology.

In ticks, symbionts belonging to at least 10 bacterial genera have been reported to be associated with different host species (Duron et al., 2017). Despite this high symbiont diversity, genomic studies show that most of these bacterial symbionts encode for *de novo* synthesis of B Vitamins, suggesting a crucial role in supplementing the deficiencies of a diet solely based on vertebrate blood. Horizontal acquisition of different symbionts in ticks is probably promoted by the feeding on shared vertebrate hosts, which act as an 'arena for the exchange of symbionts' (Duron et al., 2017). If different symbionts provide the same benefit for the host, competition among them may occur and the switches in symbiotic associations may result in the replacement of the existing symbiont, or alternatively metabolic complementarity between co-occurring symbionts can be established (Duron et al., 2017; Sudakaran et al., 2017). Current evidence indicates that the most widespread tick symbionts belong to the genus *Coxiella*, reported at high prevalence in several species of medical interest, such as *Rhipicephalus sanguineus* sensu lato and *Rhipicephalus turanicus* (Lalzar et al., 2014). Instead, the main Lyme borreliosis vector in Europe, *Ixodes ricinus*, harbours another endosymbiont, "*Candidatus* Midichloria mitochondrii" (hereafter *M. mitochondrii*), one of the most recently described symbionts of ticks. This symbiont, belonging to the order *Rickettsiales* and the family "*Candidatus* Midichloriaceae" (hereafter *Midichloriaceae*), has been reported to be present in females and immatures at 100% prevalence, while is presence in males is limited to around half of the individuals, likely due to a secondary loss during their development (Lo et al., 2006). *M. mitochondrii* exhibits a strong preferential localization in the host ovary, as seen through fluorescence *in situ*hybridization (FISH) and transmission electron microscopy (TEM) (Beninati et al., 2004; Sacchi et al., 2004). The use of more sensitive techniques, namely real-time PCR, mass spectrometry and immunofluorescence, revealed the presence of *M. mitochondrii* also in the salivary glands of *I. ricinus* (Epis et al., 2013; Di Venere et al., 2015; Mariconti et al., 2012a), but no studies have so far investigated whether *M. mitochondrii* colonizes other tick organs/tissues. At the intracellular level, *M. mitochondrii* presents an unusual lifestyle, indeed not only it resides in the cytoplasm of tick cells, but it is able to survive inside the inter-membrane space of host mitochondria (Beninati et al., 2004; Lo et al., 2006; Sacchi et al., 2004). A previous study investigated the symbiont load in whole *I. ricinus* ticks during the life cycle, showing an increase following the engorgement of each stage and a subsequent decrease after the molting (Sassera et al., 2008). This study however did not evaluate the specific tissue tropism of *M. mitochondrii*.

What is currently known thus opens the questions: what is the role of *M. mitochondrii* in the tick host? Is it similar to other symbionts or at least partially different?

Aiming to answer such questions, and considering that the tissue distribution of a symbiont can be indicative of its functional role in the physiology of the host, we investigated the specific localization pattern of *M. mitochondrii* and the corresponding abundance in selected organs in feeding and questing adult females of *I. ricinus.* We paired these experiments with *in silico* analyses of the metabolic pathways present in the genome of *M. mitochondrii* and compared the pathways presence in seven other symbionts commonly harboured in ticks, with the goal to obtain a better understanding of their biological effects on the tick hosts.

2. Materials and methods

2.1. Ticks

Ten semi-engorged adult females of *Ixodes ricinus* were collected from freshly killed wild roe deer (*Capreolus capreolus*) legally shot by hunters during the depopulation management plan launched in the Como province (Northern Italy). Thus, no animals were killed specifically for this study. Thirteen questing unfed specimens of *I. ricinus* were obtained by dragging a woolen blanket (100 × 90 cm) over the vegetation in the same area. Three semi-engorged females of *Ixodes hexagonus*, a tick species reported not to harbour *M. mitochondrii* (Epis et al., 2008), were collected from cats in a kennel located in the same province in Northern Italy. All collected ticks were kept alive and morphologically identified using standard taxonomic keys (Manilla, 1998). To confirm the morphological identification a specific PCR was performed from a leg of each tick to amplify a fragment of the 12S mitochondrial rRNA gene as previously described (Epis et al., 2008).

2.2. Dissection of organs

Specific organs (salivary glands, gut, ovary, rostrum, tracheae and Malpighian tubules) were dissected from 13 semi-engorged and ten unfed *I. ricinus* and from three semi-engorged *I. hexagonus* for qPCR analysis, whereas organs for indirect immunofluorescence assay (salivary glands, ovary, Malpighian tubules and tracheae) were collected only from semi-engorged and unfed *I. ricinus*. Before the dissection, ticks were individually surface-sterilized by immersion for 5 min in 70% ethanol solution and rinsed in sterile distilled water. The samples were dissected as previously described (Edwards et al., 2009) inside a droplet of sterile PBS 1X, removing the upper cuticle using a scissor; the specific organs were subsequently removed and placed in sterile PBS in a new Petri dish in order to wash them (this step was repeated twice). The rostrum was dissected taking the capitulum of the ticks with chelicerae and hypostome, and thus the pharynx, excluding palps. Organs were than individually stored at −80 °C or fixed for staining.

2.3. DNA extraction and quantitative PCR

DNA purification was performed from dissected organs of seven specimens of unfed *I. ricinus*, ten semi-engorged *I. ricinus* and three semi-engorged *I. hexagonus*. Organs were incubated in 30 µl of TRIS − HCl 10 mM, pH 8.0 at 95 °C for 5 min, then 5 µL of Proteinase K (20 mg/mL) were added to each sample, and incubated at 56 °C with rotation/shaking for 2 h. The mixture was inactivated at 90 °C for 5 min and centrifuged at max speed for 5 min. DNA quality and concentration were determined using a NanoDrop ND-1000 (Thermo Scientific, Delaware, USA).

Quantitative PCR (qPCR) specific for the *M. mitochondrii* gene coding for DNA gyrase B (*gyrB*) and for the host nuclear gene coding for calreticulin (*cal*) were used to measure symbiont loads in various stages of the host life as previously described (Sassera et al., 2008). qPCR results are expressed as gyrB/cal ratios, calculated from the absolute quantification of *gyrB* (*i.e.* the *M. mitochondrii* gene) and of *cal* (the *I. ricinus* gene) obtained by interpolating the threshold cycle (Ct) values of each sample on the equation calculated from serial dilutions of samples containing known copy numbers of each gene fragment, cloned in a plasmid vector.

2.4. Statistical analysis

We analysed the variation in *M. mitochondrii* load in diverse organs of unfed and semi-engorged ticks, as inferred from qPCR results. The *gyrB/cal* ratio of absolute copy number was log-transformed to meet the assumption of normality (Shapiro-Wilk test: $p > 0.9$) and were thus analysed through Generalised Linear Mixed Models (GLMMs) with Gaussian error structure. We tested the effect of organ, engorgement status, their second order interaction, and included tick ID as random factor to account for repeated measures. Final significant results of factors with more than two levels were interpreted through pair-wise t-tests of Differences of Least Square Means (DLSM), applying Tukey correction for multiple comparisons. Statistical analyses were carried out using PROC GLIMMIX in SAS/STAT 9.4 software (Copyright © 2012, SAS Institute Inc., Cary, NC, USA).

2.5. Indirect immunofluorescence assay

Organs from three semi-engorged and three unfed *I. ricinus* were fixed in 4% (w/v) paraformaldehyde in PBS for 20 min at 4 °C, permeabilized with 0.5% (v/v) Triton X-100 (10 min at room temperature), and then washed three times in PBS as previously described (Mariconti et al., 2012b). Organs were individually incubated with anti-rFliD antibodies (targeting the flagellar cap protein FliD of *M. mitochondrii*; Mariconti et al., 2012b) at 1:5000 dilution for 1 h at 37 °C, washed twice in PBS and incubated with anti-rabbit secondary antibodies conjugated with FITC (Sigma Aldrich) at 1:40 dilution plus 6 mM TOTO-3 for nucleus detection for 30 min. Samples were mounted with glycerol on glass slides. Observations were performed using a laser-scanning confocal microscope (Leica, Wetzlar, Germany).

2.6. Analysis of the metabolic pathways of M. mitochondrii and comparison with selected tick endosymbionts

The presence of metabolic pathways potentially involved in host interaction in the genome of *M. mitochondrii* (NC_015722) was preliminarily investigated employing the Biocyc and KEGG reference databases (Caspi et al., 2016; Kanehisa et al., 2016). Then, the presence/absence of each gene was verified by manually curated blastp searches against the predicted *M. mitochondrii* proteins using as queries suitable sequences from closely related and/or reference (*i.e. Escherichia coli*) organisms.

Homologs of the previously evaluated genes of *M. mitochondrii* were searched in the genomes of selected endosymbionts of other tick species: three *Coxiella*-like endosymbionts (CERM, CLAA, CRt), two *Francisella*-like endosymbionts (FLE-Am and F-Om), *Rickettsia buchneri* and *Rickettsia peacockii* (Duron et al., 2018; Felsheim et al., 2009; Gerhart et al., 2016; Gottlieb et al., 2015; Guizzo et al., 2017; Kurtti et al., 2015; Smith et al., 2015,) (Supplementary Table S1). *M. mitochondrii* genes were queried with blastp on the proteins of each tick endosymbiont, and the results were manually evaluated to identify homologs. In details, for all blastp hits with evalue ≤1e-4, the length of query, subject and alignment were compared, and the identity percentage inspected. In addition, the annotation of the subject was verified by online blastp.

3. Results

3.1. Quantification of M. mitochondrii in tick organs

M. mitochondrii amount was determined by specific qPCR in salivary glands, gut, ovary, rostrum, tracheae, and Malpighian tubules of semi-engorged and unfed females of *I. ricinus* (Table 1) and semi-engorged *I. hexagonus* (included as a *M. mitochondrii* negative control).

M. mitochondrii DNA presence was detected in all of the above body parts of *I. ricinus* and was quantified normalizing the gene copy numbers with the quantification of the *I. ricinus* nuclear gene calreticulin (Table 1, *i.e gyrB/cal* as in Sassera et al., 2008). *M. mitochondrii* was never detected in *I. hexagonus* organs. Symbiont concentrations in *I. ricinus* varied significantly depending on the organ and the engorgement status of the tick ($F_{5,\ 65} = 4.38$, $p = 0.002$).

In semi-engorged ticks, *M. mitochondrii* was mostly localized in the ovary, showing a significantly higher concentration than in the other organs (all pair-wise comparisons $p < 0.0001$), that instead harbored amounts of the bacterium that do not differ significantly. In unfed ticks the symbiont was more abundant in the ovary and the tracheae, both organs harboring a higher concentration than the rostrum, salivary glands, Malpighian tubules and intestine (all $p < 0.03$). Comparisons between organs before and after the blood meal showed a significant increase in *M. mitochondrii* abundance in the ovary (estimate: $+3.9 \pm 1.1$; $t_{65} = 3.5$, $p = 0.032$) and a significant decrease in the tracheae (estimate: -2.7 ± 1; $t_{65} = -2.6$, $p = 0.011$). Variations between load before and after the blood meal in other organs were not statistically significant.

3.2. Localization of M. mitochondrii in organs

The spatial distribution of *M. mitochondrii* in the tick tissues was also investigated using indirect immunofluorescence, through specific anti-rFliD antibodies in the salivary glands, ovary, Malpighian tubules and tracheae dissected from semi-engorged and from unfed *I. ricinus*.

In accordance with qPCR results, specific green-stained bodies revealed the presence of *M. mitochondrii* in the enlarged ovaries of semi-engorged ticks, indicating high abundance of bacteria, which resulted disseminated in the cytoplasm of most of the immature oocytes, and clustered at the periphery of mature oocytes that contained fully grown yolk granules (Fig. 1A–C, red arrows). Bright fluorescence was also reported in the anterior two thirds of the Malpighian tubules (Fig. 1D, E), but the posterior parts connected to the rectal bladder were free of bacteria (data not shown). A strong fluorescence was also observed in the salivary glands, revealing a clustered distribution pattern inside the cytoplasm of the acini (Fig. 1F, G). *M. mitocondrii* was also present in the epithelial cells of the tracheae in unfed *I. ricinus* (Fig. 1H, I).

3.3. Analysis of the metabolic pathways of M. mitochondrii and comparison with selected tick endosymbionts

The *in silico* metabolic reconstruction from the *M. mitochondrii* genome reveals the presence of several genes potentially involved in the interaction with the host *I. ricinus* (Fig. 2). Complete pathways for the biosynthesis of B vitamins, in particular folate (B9 vitamin) and biotin (B7 vitamin), suggest a nutrient-provisioning role of the symbiont. Six out of the eight other analyzed symbiont genomes show the same capability, whereas *Rickettsia peacockii* presents only the B9 genes (Supplementary Table S1).

The genome of *M. mitochondrii* also contains genes encoding for proteins which could exert a potential beneficial role for the host in the response to oxidative stress and in the energy metabolism, including a superoxide dismutase (SOD), a ferrochelatase (FECH), a protoeme ferro-lyase, heme exporter proteins, the pathway for the production of lipoic acid, the subunits of a cytochrome *cbb3* oxidase, and two nucleotide *tlc* translocases. In addition, the presence of a wide set of genes potentially involved in the transport of ions indicates a possible role of the symbiont in the maintenance of homeostasis. The capabilities of the other symbionts in these regards are variable: in particular none of them possesses *ccb3*, and only *Rickettsia* spp. have *tlc* nucleotide translocases. Overall, *Rickettsia* spp. repertoires are, as expected due to phylogenetic relatedness, the most similar to *M. mitochondrii* (Supplementary Table S1).

Table 1
Quantification of *gyrB* and *cal*: copy numbers and of *gyrB/cal* ratio, indicative of *M. mitochondrii* abundance in host tissues.

	Sp	Dissected organs (n of samples)	gyrB copies/μl		cal copies/μl		gyrB/cal ratio	
			Mean	SD	Mean	SD	Mean	SD
Unfed	I. ricinus	Salivary glands (7)	7.46	10.75	3133.23	1395.49	0.002	0.004
		Gut (7)	44.61	59.2	2520.20	1674.48	0.02	0.003
		Ovary (7)	54,085.32	90,713.58	37,076.98	79,795.15	8.08	14.35
		Rostrum (7)	25.56	41,26	1971.63	911.53	0.016	0.03
		Tracheae (7)	22,045.18	54314.54	3473.82	2184.32	3.67	8.48
		Malpighian tubules (4)	15.76	12.28	1240.27	625.02	0.01	0.01
Semi engorged	I. ricinus	Salivary glands (10)	81.75	111.38	4389.68	4018.61	0.02	0.017
		Gut (10)	66.80	116.36	7154.69	8138.76	0.06	0.14
		Ovary (10)	1,525,686.49	2,092,974.62	5520.07	7713.58	580.42	737.10
		Rostrum (10)	6.11	6.11	2410.19	2737.66	0.002	0.04
		Tracheae (10)	61.53	66.11	5130.63	3436.71	0.01	0.01
		Malpighian tubules (10)	2040.25	2426.84	34,568.99	17,227.26	0.07	0.096

SD: standard deviation.

4. Discussion

This study was aimed at inferring a functional role of the endosymbiont *M. mitochondrii* in the tick host *I. ricinus*, by combining experimental quantification of its abundance in different tissues of unfed and semi-engorged ticks with the genomic inference on its metabolic repertoire.

4.1. *M. mitochondrii* as energy provider

The highest concentration of *M. mitochondrii* was detected in the ovary of semi-engorged ticks, confirming previous results (Epis et al., 2013; Sassera et al., 2008). The presence of *M. mitochondrii* in the ovary is not surprising, since all major symbionts of ticks are present in this organ to ensure maternal transmission and to improve the fitness of the

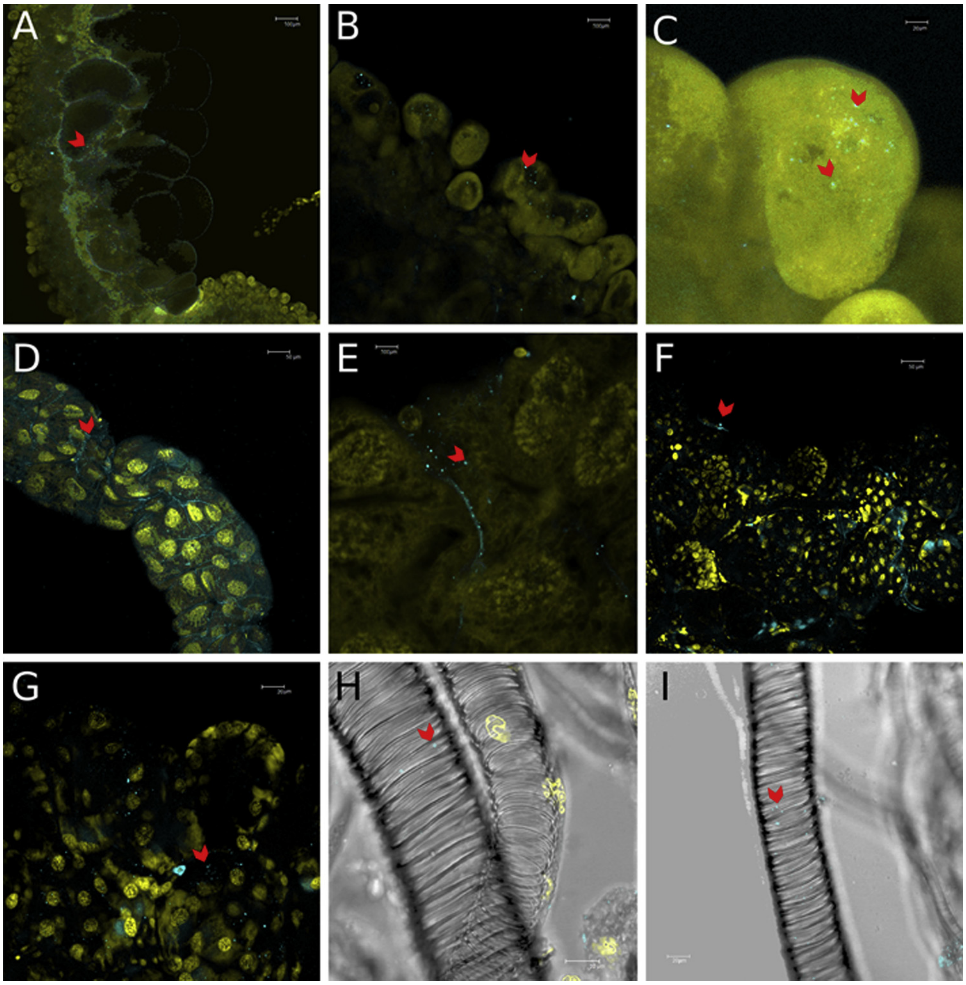

Fig. 1. Indirect immunofluorescence assay (FITC-conjugated secondary antibodies) in different tissues of wild semi-engorged (A–G) and unfed (H, I) *I. ricinus* ovary (A), close-up view of the young oocytes (B, C); Malpighian tubules (D), close-up view of Malpighian tubules (E); salivary glands (F), close-up view of salivary glands (G) and tracheae (H, I). The light blue spots (red arrows) indicate *M. mitochondrii* and the yellow spots show the cellular nuclei (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

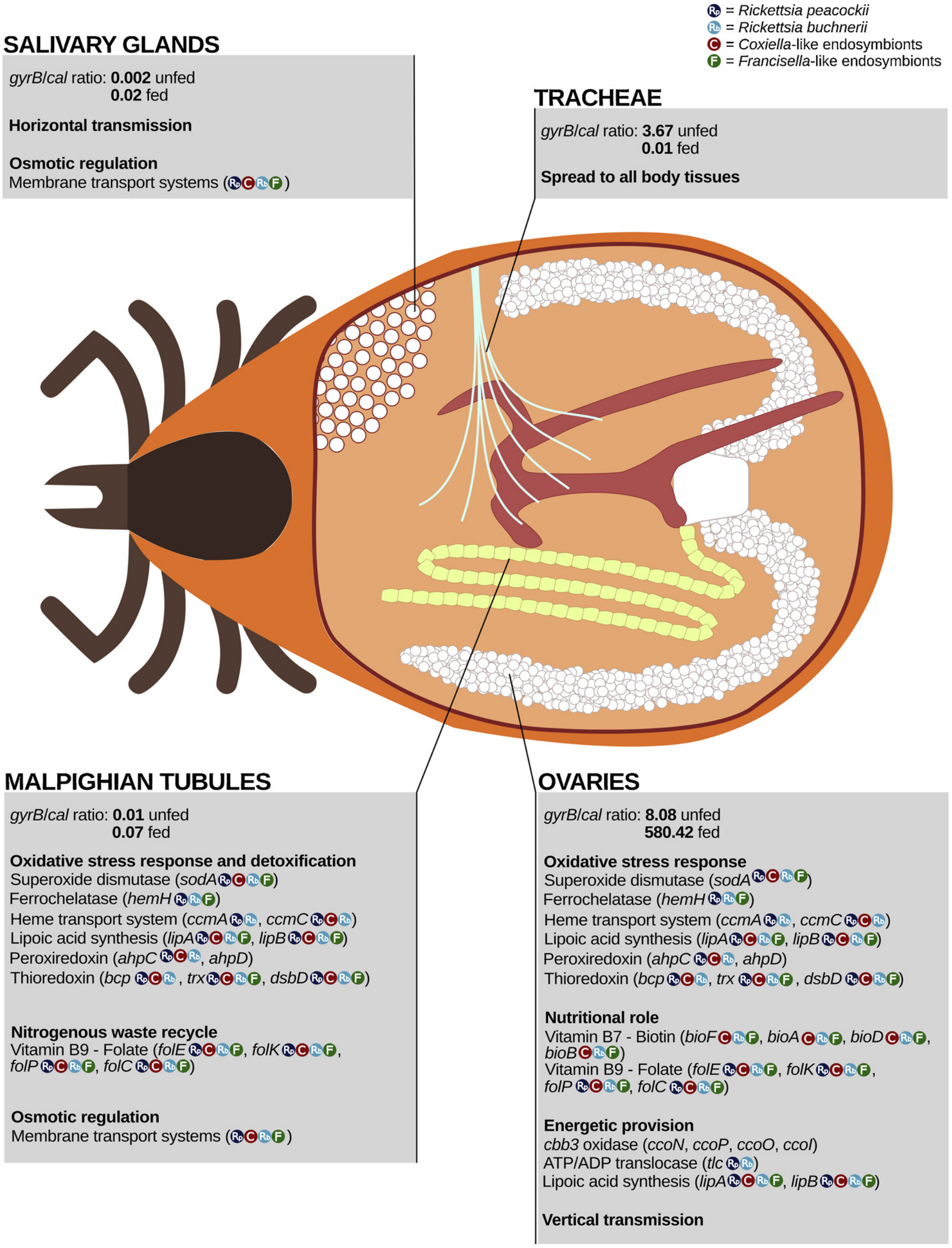

Fig. 2. Schematic overview of the tissues localization of *M. mitochondrii* within its tick hosts. Using comparative genomic data we speculate on the biological roles of *M. mitochondrii* and the other symbionts of ticks. For each organ, the gyrB/cal ratios in unfed and partially engorged females (representing the abundance of *M. mitochondrii*) are reportedtogether with the inferred functions of the symbiont, the putative involved genes and whether homologs of the genes were found in other tick symbionts.

next generation (Duron et al., 2017). Interestingly, recent studies linked this tissue tropism also to a role played in the host's biology, specifically in the reproductive fitness: for instance, reduction of the *Coxiella*-like endosymbiont load was shown to correlate with negative effects in fecundity, showing prolonged time to oviposition and hatching and lower numbers of eggs and larvae (Guizzo et al., 2017; Zhang et al., 2017; Zhong et al., 2007).

Indeed, the oogenesis represents a critical stage in the tick life cycle, characterized by the increase of metabolic and biosynthetic activity mainly linked to the synthesis and uptake of proteins, carbohydrates, and lipids by oocytes for the vitellogenesis, activities that require a high rate of O_2 consumption and energy (Aboul-Nasr and Bassal, 1972). However, the blood meal has been hypothesized to reduce the availability of oxygen, and thus hamper the energetic metabolism, in particular ATP production (Ferreira et al., 2018; Sassera et al., 2011).

The contribution of *M. mitochondrii* in this stage could be providing an additional ATP source to the host. The presence of genes encoding for a cytochrome *cbb3* oxidase supports this hypothesis, as this complex allows ATP production at low oxygen concentrations (Sassera et al., 2011). The presence of this complex is a conserved and distinctive trait of *Midichloriaceae* hosted by multiple different eukaryotes (Schulz et al., 2016; Wang and Wu, 2014), and none of the other known tick symbionts retain genes encoding for the cytochrome *cbb3* oxidase. Thus, the presence of this complex could have represented a peculiar adaptive trait in the evolution of the association between *I. ricinus* and *M. mitochondrii*, and possibly also between ticks and *Midichloria* spp. in general. Molecular, genomic and functional studies on other members of the genus *Midichloria*, harboured by other tick species at varying prevalence and load (Cafiso et al., 2016; Buysse and Duron, 2018), could allow to test this hypothesis. For example *Midichloria* bacteria present in tick species at 100% prevalence, such as the symbiont of *Ixodes aulacodi* (Cafiso et al., 2016) and of *Ixodes holocyclus* (Beninati et al., 2009) could share genomic traits and localization with *M. mitochondrii* of *I. ricinus*, while other *Midichloria,* present at lower prevalence, may have different tissue tropisms and gene repertoires, indicative of other functions.

Another potentially 'energy-related' function of *M. mitochondrii* could be the capacity of synthetizing lipoic acid, a cofactor that is part of several multi-enzyme complexes of the electron transport chains located in the mitochondria (Packer et al., 1995). Almost all analysed tick symbionts have genes encoding for the synthesis of lipoic acid. The ATP produced by *M. mitochondrii* could then be exported to the host through the *tlc* nucleotide translocase. This capability could be also hypothesized for *Rickettsia* spp., which present numerous nucleotide translocase genes homologous to those of *M. mitochondrii.*

4.2. *M. mitochondrii* as B vitamins producer

In parallel, several lines of evidence indicate that the supportive contribution of the symbionts during the oogenesis could be additionally related to the provision of specific nutrients during this period of increased biosynthetic demands. Indeed, ticks lack the genetic capacity to synthesise *de novo* several vitamins and other cofactors required for their growth and survival, and they feed exclusively on vertebrate blood, which is rich in some nutrients, such as proteins and iron, but it is deficient in essential micronutrients, such as B vitamins and cofactors in general (Hosokawa et al., 2010). Indeed, the possibility that symbionts provide B vitamins to the tick host has recently been supported by a study on *Ornithodoros moubata* and its *Francisella* symbiont: the negative effects due to the elimination of the symbiont were fully restored with an oral supplement of B vitamins (Duron et al., 2018). The presence in the *M. mitochondrii* genome of genes coding for proteins involved in the biosynthesis of vitamin B7 and B9 suggest its role could be as well of supplying these essential nutrients in the unbalanced diet of ticks. While the nutritional role of the symbionts has been linked up to now only to their tropism for the ovary, the ability to synthesize B vitamins could also explain the detected high numbers of *M. mitochondrii* in Malpighian tubules during the engorgement of *I. ricinus*. One of the main functions of Malpighian tubules is to act as excretory organs, thus nitrogenous waste is accumulated here and then removed as excreta consisting of guanine (rather than uric acid as in insects), other purines, and large amounts of heme and haemoglobin derived from the ingested blood meal. It was suggested that Malpighian tubules could represent a nutritive environment for symbionts (Gottlieb et al., 2015; Hamdy, 1977; Martin et al., 2018; Sonenshine and Roe, 2014). Indeed, metabolic pathway analysis suggests that the presence of *M. mitochondrii* could enable this bacterium to get guanine, possibly thanks to the *tlc* nucleotide translocases. The bacterium is unable to produce this compound, which is necessary for the synthesis of folate (vitamin B9). In summary, guanine abundance in the Malpighian tubules could favour B9 vitamin production by *M. mitochondrii*, then, potentially, the supply of this vitamin to the tick host.

4.3. *M. mitochondrii* role in detoxification

Since the Malpighian tubules play a key role in detoxification, the tropism of *M. mitochondrii* for this tissue could be also related to anti-oxidative and detoxification processes. In these regards, for adult females, a critical step is represented by the ingestion of huge amounts of vertebrate blood within few days (Balashov, 1972), which also has toxic effects caused by the high quantities of pro-oxidant molecules that expose ticks to strong oxidative stress. The digestion of blood hemoglobin results in the release of large amounts of free heme, consisting in a porphyrin ring with a highly reactive iron atom. These chemicals are able to promote the production of hydroxyl radicals, reactive oxygen species (ROS), eventually causing extensive oxidative damage including lipid peroxidation. Although ticks have developed various protective mechanisms against the damage of oxidative stress (anti-oxidant enzymes, hemosomes and heme-binding storage proteins), a contribution from a symbiont could increase the oxidative stress tolerance of the host (Galay et al., 2015; Richier et al., 2005).

Interestingly, *M. mitochondrii* possesses genes for the biosynthesis of several enzymes and co-factors potentially involved in the anti-oxidative response, such as the superoxide dismutase (SOD), peroxiredoxins (Prxs), thioredoxins, protoheme ferro-lyase, heme transporter proteins and lipoic acid. SOD is an enzyme, also having a counterpart in the tick, involved in the first line of protection against oxidative stress, catalyzing the conversion of the highly reactive superoxide anion (O_2^-) into hydrogen peroxide (H_2O_2), subsequently scavenged by a set of antioxidant enzymes (McCord and Fridovich, 1969; Perner et al., 2016). These scavengers, including the Prxs, functionally complement the anti-oxidative activity of the SOD, catalyzing the reduction of H_2O_2 into water (H_2O), with thioredoxin as hydrogen or thiol donor (Choi et al., 1998; Kusakisako et al., 2016). Regarding the lipoic acid, it is also able to scavenge several types of free radicals such as hydroxyl radicals, and singlet oxygen, due to its unique sulfur-containing structure (Packer et al., 1995). The protoheme ferro-lyase, a ferrochelatase that catalyzes the insertion of ferrous iron into protoporphyrin IX in the heme biosynthesis pathway, can limit the excess of free iron acquired with the diet. Concerning the heme transporter proteins, *M. mitochondrii* can bind free heme released from the blood meal limiting its toxicity. While we hypothesize it in the Malpighian tubules, the support in the anti-oxidative activity may occur also in the ovary, in order to protect the developing oocytes from the oxidative damage. Indeed, the regulation of H_2O_2 concentrations is considered important for the success of the reproduction (Kusakisako et al., 2016).

Several other symbiotic bacteria massively colonize the tick Malpighian tubules, including *Coxiella*-like in *Amblyomma americanum, Haemaphysalis longicornis, Rhipicephalus haemaphysaloides, R. microplus, R. sanguineus* sensu lato*, R. turanicus, Dermacentor silvarum* (Guizzo et al., 2017; Klyachko et al., 2007; Lalzar et al., 2014; Wang et al., 2018; Zhang et al., 2017); *Rickettsia* spp. in *H. longicornis, D. silvarum* (Wang et al., 2018) and *Francisella*-like in *Hyalomma marginatum* and *Ornithodoros moubata* (Azagi et al., 2017; Duron et al., 2018). These symbionts could share, at least partially, similar functions to those here hypothesized for *M. mitochondrii*, as most possess the ability to synthesise B vitamins. The combination of *tlc* translocases and folate is shared instead only with *Rickettsia* spp., while most of the anti-oxidant cofactors and enzymes are also encoded by the genome of *Coxiella*-LE, *Francisella* spp. and *Rickettsia* spp., reinforcing the hypothesis of a key role of the symbionts in the anti-oxidative defence of the hosts. Specifically, all of them have retained SOD and lipoic acid, whereas in *Coxiella* spp. the protoheme ferro-lyase is absent and in *Francisella* spp. genes encoding for heme exporter proteins are absent and for Prxs resulted scarce (*i.e.* 2 genes in one genome and 1 gene in the second, see Table S1). The composition of the analysed gene set resulted more closely related between *Rickettsia* spp. and *M. mitochondrii.*

4.4. Protection against osmotic stress

Another potential role of *M. mitochondrii* could be protection against osmotic stress in engorged ticks. Indeed, during feeding, ticks intake an enormous water load, that represents a potential source of osmotic stress. The Malpighian tubules and the salivary glands, two organs in which the symbiont resides, play an important role in balancing the uptake and the excretion of ions and water. The genome of *M. mitochondrii* encodes for a set of proteins that are likely involved in the transport of ions and fluids, suggesting a potential role of the symbiont in the tolerance to water stress and in the osmoregulation during this critical stage. On the other hand, these same proteins, such as the members of the major facilitator superfamily (MFS) and the drug/metabolite transporter superfamily (DMT) are involved in the uptake of various classes of organic compounds, such as amino acids, and could alternatively be used by *M. mitochondrii* to obtain nutrients, especially in the Malpighian tubules, which are rich of nutritive products for symbionts (Chen et al., 2008). Genes involved in the production of transporters are commonly found also in the other tick symbiont genomes.

4.5. Horizontal transmission and systemic diffusion

The presence of *M. mitochondrii* in the tracheae, salivary glands and rostrum suggests that the bacterium could spread into different tick organs and tissues during the tick life/development, and to the vertebrate host during feeding. Evidence that *M. mitochondrii* can undergo horizontal transmission was provided by the finding of antibody response against a specific antigen in vertebrate hosts exposed to tick bite as well as the detection of *M. mitochondrii* DNA in the blood of experimentally infested rabbits, four months after blood feeding, suggesting that *M. mitochondrii* can also replicate in vertebrate blood (Bazzocchi et al., 2013; Cafiso et al., 2019; Di Lecce et al., 2018; Serra et al., 2018; Skarphédinsson et al., 2005). Although several tick symbionts are reported to be transmitted to vertebrates during the blood meal the risk of infection for the vertebrate hosts is considered low due to the absence of virulence genes (Bonnet et al., 2017; Gottlieb et al., 2015; Olivieri et al., 2018). It is more likely that *M. mitochondrii* uses this mechanism to allow horizontal transmission to other ticks through co-feeding, as suggested by the presence of highly similar symbionts in different tick populations and species (Al-khafaji et al., 2019; Cafiso et al., 2016).

More novel is the detection of *M. mitochondrii* in the tracheae of unfed *I. ricinus*. The tracheal system penetrates into the arthropod tissues and allows the exchange of respiratory gases throughout the body (Gullan and Cranston, 2014). As previously suggested for *Rickettsia monacensis* in *Ixodes scapularis*, the tracheal system can play a pivotal role in the dissemination of symbionts into the tick tissues and organs (Baldridge et al., 2007). However, this hypothesis remains to be critically demonstrated, since clean dissection of tracheal tissue can be quite challenging for the close connection of the tracheal system with the internal organs, that can give rise to a cross-contamination potentially influencing the finding of *M. mitochondrii* in this tissue.

5. Conclusions

Summing up, the presence of *M. mitochondrii* in different tissues inside the tick hosts allows to speculate that distinctive sub-populations may play different metabolic roles in accordance with the specific tissue tropism. Overall our findings indicate that *M. mitochondrii* could be “the right bacterium in the right place”, being localized in key tissues in different phases of the host life cycle and being equipped with the metabolic potential for playing several important roles, contributing to the fitness of its host. Our results and hypotheses should be corroborated with future nutritional and physiological experiments, combined with the analysis of gene expression of the symbiont in relation with the localization within the host body.

Conflict of interest

The authors have nothing to disclose.

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Appendix A. Supplementary Data

Supplementary table S1

Appendix B. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2019.05.019.

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