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Original article

Midichloria mitochondrii, endosymbiont of *Ixodes ricinus*: evidence for the transmission to the vertebrate host during the tick blood meal

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ABSTRACT

Ticks are important vectors of a variety of pathogens affecting humans and other animals, but they also harbor numerous microorganisms whose role is still limitedly investigated. *Ixodes ricinus* harbors the endosymbiont *Midichloria mitochondrii*, which is localized in ovaries and in salivary glands. The bacterium is vertically transmitted and is present in 100% of wild adult females, while prevalence values drop after some generations under laboratory conditions. Molecular and serological evidences showed that *M. mitochondrii* molecules are transmitted to the vertebrate hosts by *I. ricinus* during the blood meal. Our work was focused on monitoring *M. mitochondrii* antigens and DNA in a vertebrate model after infestation with *I. ricinus* for a time-span of four months. Two groups of rabbits were infested with *I. ricinus* females, respectively from the wild (naturally infected with the symbiont) and laboratory strain (lab; considered devoid of *M. mitochondrii* after quantitative PCR investigations) and screened using molecular and serological assays at nine time points. *M. mitochondrii* presence was detected in rabbits infested with wild *I. ricinus* ticks, but surprisingly also in those infested with lab ticks, albeit at later time points. This result prompted a more sensitive molecular screening of lab ticks, which were found to harbor very low symbiont loads.

Our results indicate that transmission of the bacterium occurs even at low bacterial loads, and that antibody response against *M. mitochondrii* antigens begins within one week post-infestation with wild *I. ricinus*. Circulating DNA was detected in the blood of rabbits belonging to both groups up to the end of the experiment, suggesting a replication of the symbiont inside the vertebrate host.

1. Introduction

Ticks are hematophagous ectoparasites infesting mammals, birds, and reptiles (Klompen et al., 1996). They are globally distributed from the tropics to the polar regions and are currently considered among the most important pathogen vectors worldwide, second only to mosquitoes (Vu Hai et al., 2014; Dantas-Torres et al., 2012). During blood feeding, ticks can transmit pathogens (tick-borne pathogens; TBPs) of human and veterinary importance (Parola and Raoult, 2001; Vu Hai et al., 2013), including a variety of widely studied viruses, bacteria and protozoa (*e.g. Anaplasma* spp., *Ehrlichia* spp., *Borrelia* spp., tick-borne encephalitis virus, *Babesia* spp.; Medlock et al., 2013). Ticks, however, can also harbor other microorganisms, some of which are closely related to pathogens, whose role in tick biology and as pathogens to vertebrates still has to be clarified (Bonnet et al., 2017; Gerhart et al., 2016). *Midichloria mitochondrii*, a vertically transmitted bacterium present in *Ixodes ricinus* throughout its geographical distribution (Sassera et al., 2006), is an example of such an incompletely characterized symbiont. This bacterium can colonize multiple tick tissues, predominantly the ovary, but also salivary glands (Epis et al., 2013). *M. mitochondrii* has been observed inside the cells of the ovarian tissue and, within oocytes, it has been shown to localize not only in the cytoplasm, but also inside the mitochondria (Sacchi et al., 2004). Prevalence of *M. mitochondrii* in adult males and bacterial load in immature stages is highly variable (Sassera et al., 2008), whereas the endosymbiont is abundantly present in 100% of wild *I. ricinus* adult females (Lo et al.,

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2006; Sassera et al., 2008). On the contrary, laboratory-reared *I. ricinus* females show a reduced presence of the symbiont, with different lab populations exhibiting prevalence ranging from 44% to 18% (Lo et al., 2006) up to the 7th lab generation. The 100% prevalence of *M. mitochondrii* in wild *I. ricinus* specimens and its vertical transmission have led to speculations about a possible mutualistic role, helping in metabolic/nutritional functions (Sassera et al., 2011; Duron et al., 2017) or in molting processes (as suggested by the endosymbiont blooming after *I. ricinus* blood meal; Sassera et al., 2008; Pistone et al., 2011), but its actual function in the tick is currently unknown. Additionally, previous studies highlighted that *M. mitochondrii* molecules can be inoculated into the vertebrate host during the tick bite. Humans and other animals (*i.e.* dogs, roe deer), were found seropositive against the flagellar protein FliD of this endosymbiont (Mariconti et al., 2012a; Bazzocchi et al., 2013; Serra et al., 2018). In addition, circulating *M. mitochondrii* DNA was detected in the blood of various animals parasitized by *I. ricinus* (Skarphédinsson et al., 2005; Bazzocchi et al., 2013; Serra et al., 2018).

M. mitochondrii belongs to the order *Rickettsiales* (fam. *Midichloriaceae*), a group known to be composed exclusively of intracellular bacteria, including several important pathogens (Raoult and Roux, 1997; Montagna et al., 2013). Some authors suggested that the current behavior of *M. mitochondrii* could have emerged from an ancestral state of vertebrate pathogen, later followed by an adaptation to the tick environment (Narasimhan and Fikrig, 2015). Other maternally-inherited tick symbionts (such as *Arsenophonus, Coxiella*-like and *Francisella*-like endosymbionts) indeed present transmission mechanisms other than the transovarial way. These additional mechanisms allow them to spread also horizontally and potentially represent an infection risk to vertebrates (Bonnet et al., 2017). For example, tick-transmitted *Coxiella*-like endosymbionts have been reported to cause mild infections in patients presenting eschars at the site of tick bite (Angelakis et al., 2016; Guimard et al., 2017).

Based on the current state of knowledge, several questions still need to be addressed: does the tick transmit to the vertebrate host viable *M. mitochondrii* cells during the blood meal, or it simply inoculates bacterial molecules (antigens and DNA)? If the bacterium is transmitted, is it able to replicate in the vertebrate host, and potentially cause pathogenic effects? No studies have been performed thus far about the timing and kinetic of seroconversion against *M. mitochondrii* or about the timing in which *M. mitochondrii* DNA becomes detectable in vertebrate blood, and for how long it remains detectable in the bloodstream. The transmission of arthropod symbionts to the arthropod-parasitized host has been experimentally investigated in detail only on plants (Chrostek et al., 2017). Vertically transmitted bacteria such as *Rickettsia, Wolbachia, Cardinium*, and the bacterial parasite of the leafhopper *Euscelidius variegatus* (BEV), are transmitted from their phytophagous arthropod hosts to plants, then localize into plant tissues, and are transmitted back to naïve arthropod hosts feeding on the plant (Purcell et al., 1994; Gottlieb et al., 2006; Caspi-Fluger et al., 2012; Gonella et al., 2015; Chrostek et al., 2017; Li et al., 2017). These studies show how plant-mediated horizontal transmission could explain the occurrence of phylogenetically related symbionts among unrelated phytophagous insect species, with plants serving as a reservoir/way for horizontal transmission (Caspi-Fluger et al., 2012). Whether some of these symbionts cause any pathological alteration in the plant is still unknown.

Our work was focused on the evaluation of the onset of the antibody response against *M. mitochondrii* flagellar protein FliD in a vertebrate model (rabbits) after infestation with *I. ricinus*, and its kinetic during a time-span of four months. Furthermore, the circulation of *M. mitochondrii* DNA in blood was evaluated by PCR for the duration of the experiment.

2. Materials & methods

2.1. I. ricinus ticks collection

For the experimental infestation, a total of 60 wild unengorged *I. ricinus* adult females and 60 males were used. These ticks were obtained from fully engorged nymphs sampled on roe deer in the Chizé forest (Western France 46° 7′ 18.89″ N, 0° 25′ 3.72″ W) and maintained at the ONIRIS-INRA laboratory under controlled conditions. Positivity of this tick population to *M. mitochondrii* had been already assessed in a previous study (Di Venere et al., 2015).

Sixty unengorged laboratory strain (hereafter “lab”) *I. ricinus* adult females and 60 males from the same colony were used as *M. mitochondrii*-negative control for the experimental infestation. Lab *I. ricinus* ticks used in this work have been maintained under laboratory conditions at the ONIRIS-INRA laboratory in Nantes (France) for over 15 years (> 10 generations) and were found to be free of common tick-borne bacteria such as *Borrelia* spp. and *Anaplasma* spp. (data not shown).

Thirty unengorged lab *I. ricinus* females, randomly selected from the laboratory colony, were sampled for further molecular investigations. DNA extraction was performed using the DNeasy Blood and Tissue Kit (Qiagen) following manufacturer instructions. Extracted DNA samples were quantified and stored at −80 °C for subsequent analyses.

One hundred semi-engorged wild *I. ricinus* females, sampled from roe deer in the Chizé forest, and 50 semi-engorged lab specimens, obtained after blood feeding on a pathogen-free New Zealand White (NZW) rabbit (Charles River Laboratories Int.), were also collected for salivary glands (SG) dissection. Semi-engorged ticks were manually detached when they reached the size of interest (median blood meal duration of 9 days; Vu Hai et al., 2013), and maintained under controlled conditions until dissection.

2.2. Experimental infestation

Six pathogen-free NZW rabbits (Charles River Laboratories International) were selected for the experimental infestation with *I. ricinus* (Sonenshine and Roe, 2014). Rabbits were divided into two groups: three individuals (R1, R2 and R3) were infested with 20 wild *I. ricinus* females and 20 wild *I. ricinus* males each (hereafter “group W”); the remaining three individuals (R4, R5 and R6) were infested with 20 lab *I. ricinus* females and 20 lab *I.ricinus* males each (hereafter “group L”). Three days before tick infestation, the individual ticks used for the experimental infestation (*i.e.* 60 lab females, 60 lab males, 60 wild females, 60 wild males) were gathered in six groups of 20 females and 20 males each from the respective population (wild or lab), to promote mating and to maximize the chance to obtain complete repletion. Each of those groups of 40 ticks (20 females and 20 males) was then placed in a single ear bag for each rabbit, and ticks were allowed to feed to repletion. During the infestation, the six rabbits were kept in single separated cages and fed *ad libitum*. Physiological health parameters of rabbits were constantly monitored (data not shown).

A total of nine blood samplings per rabbit were performed to collect serum for serology and EDTA-treated blood for molecular analyses. Blood withdrawals were performed on the ear free from ticks, starting from one week before exposure to ticks (T_0) and at week 1 (T_1), week 2 (T_2), week 3 (T_3), week 4 (T_4), week 6 (T_5), week 8 (T_6), week 12 (T_7) and week 16 ($T_{8;}$ end of the experiment) post-infestation. A workflow of the entire experiment is shown in Supplementary Fig. S1.

DNA was extracted from EDTA-preserved blood samples using QIAamp DNA Blood Midi Kit (Qiagen) following manufacturer instructions and then quantified. Quality of the extracted DNA was checked by amplifying a fragment of the *GAPDH* rabbit gene as described in Peng et al. (2012). DNA and serum samples were stored at −80 °C until use.

2.3. Salivary glands dissection and crude protein extract production

The semi-engorged females collected were individually dissected under a stereomicroscope (Leica) to isolate SG. To avoid possible contamination, SG from wild and lab *I. ricinus* females were processed at different times. SG were pooled in groups of ten specimens each in a final volume of 100 µl 1X PBS and 1 µl of 100X protease inhibitor (Sigma-Aldrich). Each SG pool was subjected to manual disruption with a sterile pestle and 80 µl were subjected to sonication with Digital Sonifier 450 (Branson Ultrasonic Corporation), with three five-second treatments in ice. Each sample was then centrifuged at maximum speed for 10 min at 4 °C and supernatants were recovered, quantified (Nanodrop 1000 Spectrophotometer) and stored at −80 °C until use. DNA was extracted from the remaining 20 µl of disrupted tissues using DNeasy Blood and Tissue Kit (Qiagen) following manufacturer instructions. The extracted DNA was quantified and stored at −80 °C until use.

2.4. Molecular analyses

A previously published quantitative PCR (qPCR) protocol, based on the amplification of *M. mitochondrii gyrB* gene fragment and *I. ricinus cal* gene fragment (Sassera et al., 2008), was performed on the extracted DNA samples obtained from: i) 30 representative lab *I. ricinus* adult females, ii) wild and lab SG pools, iii) rabbits blood samples.

In order to achieve a higher sensitivity in the detection of the symbiont, a nested qualitative PCR approach on the *gyrB* gene was also performed, following the protocol described by Serra et al. (2018). To determine the sensitivity of the nested PCR, a 776 bp *M. mitochondrii gyrB* gene fragment, obtained with the external primers of the nested PCR assay, was cloned in the pGEM-T Easy Vector (Promega). A clone was purified, the plasmid DNA was quantified and sequenced, diluted from 10 copies/µL to 1 copy/µL and then subjected to the nested PCR protocol.

To avoid any contamination during the nested PCR protocol, wild and lab *I. ricinus* DNA samples, as well as blood samples from group W and group L rabbits, were performed in one single run, using novel reagents, in a laboratory where *M. mitochondrii* DNA had never been processed.

The presence/absence of common tick-borne pathogens (TBPs), *i.e. Borrelia* spp., *Anaplasma* spp., *Ehrlichia* spp., *Rickettsia* spp., *Babesia* spp. and *Bartonella* spp., in wild-SG pools was also assessed using previously published protocols (Hilpertshauser et al., 2006; Cotté et al., 2009; Pesquera et al., 2015) to exclude possible cross-reactions during further serological analyses.

A representative number of the obtained PCR products were loaded on agarose gel, excised, purified and Sanger sequenced to confirm the specificity of the amplification.

2.5. Enzyme-Linked Immunosorbent Assay (ELISA) on rabbits sera

ELISA assays were performed on the obtained rabbit sera using the following substrates: i) wild ticks-SG crude protein extract, ii) lab ticks-SG crude protein extract, iii) *M. mitochondrii* recombinant FliD protein (rFliD; Mariconti et al., 2012b).

SG crude protein extracts (1 µg/well of wild- and lab-SG) were diluted in 100 µl carbonate-bicarbonate buffer (pH 9.6) and the 96-well microplates were incubated overnight at 4 °C. Plates were subjected to three 5-minutes washes (wash solution: PBS and 0.1% Tween-20) and then treated with 100 µl of blocking solution (3% BSA in 1X PBS) for 1 h at 37 °C. After further rinsing of the wells, each serum sample (tested in duplicate) was diluted 1:100 (dilution buffer: 1% BSA in 1X PBS) and plates were incubated for 1 h at 37 °C. After the washing steps, peroxidase-conjugated Goat anti-Rabbit IgG secondary antibody (Sigma-Aldrich) diluted 1:2000 in dilution buffer was added and plates were incubated for 45 min at 37 °C. One hundred µl/well of orthophenylenediamine (Sigma-Aldrich) with hydrogen peroxide was added to each well for the development of the reaction. The O.D. values were measured without stop solution at 450 nm with a SpectraMAX 340PC (Molecular Devices Corporation) spectrophotometer. Threshold values (cut-off) for the assays were established as mean $O.D._{450\ nm}$ values + 3 standard deviations of the T_0 sera from the six infested rabbits. Values greater than or equal to the established thresholds were considered as positive.

ELISA using rFliD substrate (iii) was performed following the protocol conditions described above; the 96-well microplates were coated with 100 ng of rFliD in carbonate-bicarbonate buffer (pH 9.6).

2.6. Statistical analysis

Variation in the antibody response of rabbits was analyzed through General Linear Mixed Models (GLMM), using O.D. values as the response variable and rabbit IDs as repeated measures. We tested the effect of group (*i.e.* wild or lab) and substrate (*i.e.* wild-SG, lab-SG or rFLiD), including time post-infestation as a covariate. All second-order interactions were included in the model. Interpretation of significant factors with more than two levels was based on pair-wise t-tests of Differences of Least Square Means (DLSM), applying Tukey correction for multiple comparisons. Normality of residuals was visually assessed, and the alpha level was set at 0.05. All the analyses were carried out using SAS® 9.4 Software (Copyright © 2012 SAS Institute Inc.).

3. Results and discussion

3.1. Molecular analyses on I. ricinus ticks and salivary glands pools

Thirty unengorged lab *I. ricinus* females were screened by qPCR, routinely used to quantify *M. mitochondrii* in *I. ricinus* (Sassera et al., 2008), to assess the absence of *M. mitochondrii* bacteria from the colony maintained in the lab for over 10 generations. To date, molecular analyses have shown that *M. mitochondrii* load decreases after five/seven generations under laboratory conditions, but no data are available on the actual removal of the symbiont after > 10 generations (Lo et al., 2006). The qPCR assay highlighted the absence of the symbiont from all 30 tested individuals (data not shown). Based on the obtained result, lab ticks > 10 generations were chosen as *M. mitochondrii* negative control for the experimental infestation.

A total of ten wild-SG pools and five lab-SG pools were obtained after dissection of 100 wild and 50 lab semi-engorged females and subsequently subjected to qPCR both to test the presence/absence of *M. mitochondrii* and to evaluate the bacterial load. *M. mitochondrii* load in wild-SG pools showed *gyrB/cal* gene ratios between 10^1 and 10^3 (data not shown). On the contrary, the qPCR assay did not detect the presence of the symbiont in any of the five DNA samples extracted from lab-SG pools.

In parallel, wild-SG pools were tested for the presence of TBPs. Four out of 10 wild-SG pools were excluded from further analyses as they resulted positive to at least one of the tested TBPs (data not shown).

The crude protein extract of the wild-SG pool sample that resulted negative to TBPs and had the highest load of *M. mitochondrii* was selected as substrate for subsequent serological analyses, to maximize the amount of *M. mitochondrii* antigens.

3.2. Serological analyses

A total of three ELISA assays were carried out using in turn wild-SG crude protein extract, lab-SG crude protein extract and rFliD protein as substrates. This experimental design allowed us to evaluate the response to a specific *M. mitochondrii* antigen (rFliD), to tick antigens (lab-SG) and to a combination of *M. mitochondrii* and tick antigens (wild-SG).

Two groups of three rabbits each were infested with wild (R1, R2,

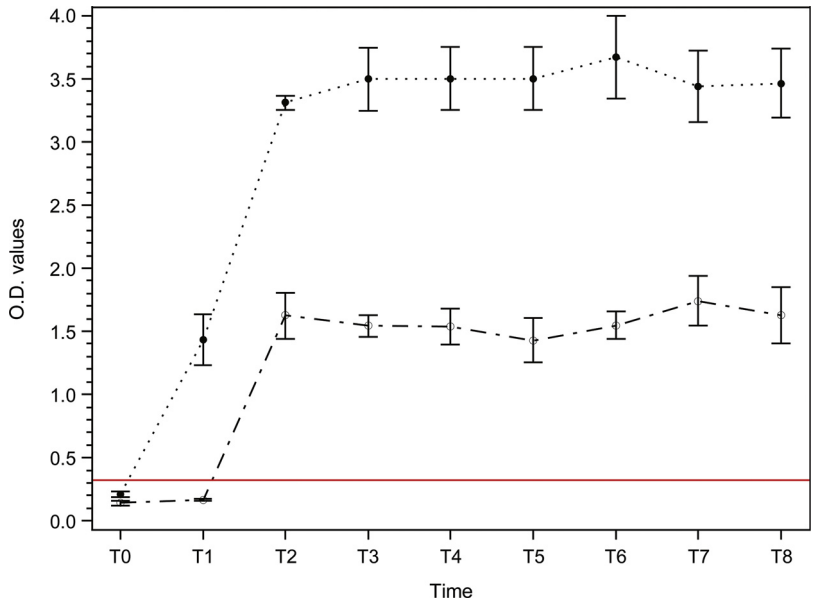

Fig. 1. Mean (± SE) antibody response against wild-SG crude protein extract in rabbits infested with wild (filled circles) and lab (blank circles) *I. ricinus* females. Line indicates the cut-off value (0.32).

and R3; group W) and lab ticks (R4, R5 and R6; group L), respectively. Sera and blood samples from rabbits were collected up to 16 weeks after the experimental infestation. None of the rabbits in this study showed any clinical signs for all the duration of the experiment (data not shown).

The ELISA assays performed on rabbit sera using wild-SG and lab-SG crude protein extracts were used to evaluate the immunocompetence of the hosts used for the experimental infestation. Moreover, these assays were set up to investigate the potential involvement of *M. mitochondrii* antigens in the antibody response of the two groups of rabbits, as the wild-SG contains *M. mitochondrii* antigens.

Statistical analysis showed that the difference in O.D. values between rabbit groups W and L, and their trends over time both varied depending on the ELISA substrate ($F_{2,\ 8} = 36.3$, $p < 0.0001$ and $F_{2,\ 148} = 12.4$, $p < 0.0001$, respectively). Detailed results of the final model are reported in Supplementary Table 1.

The elicited IgG antibody response against wild-SG protein extract (Fig. 1) increased significantly with time post-infection (parameter estimate ± SE: 0.10 ± 0.01; $t_{148} = 7.13$; $p < 0.0001$). A positive reaction (cut-off value: 0.32) was observed in rabbits of group W starting from week 1 up to the end of the experiment (week 16) and in rabbits of group L starting from week 2 up to the end of the experiment. Rabbits of group L showed significantly lower O.D. values than rabbits of group W in their antibody response against wild-SG crude protein extract (parameter estimate ± SE: -1.63 ± 0.15; $T_8 = -10.94$; $p < 0.0001$). This higher and earlier immune response observed in rabbits of group W compared to group L is consistent with our expectation of *M. mitochondrii* playing an antigenic function in the immune response of vertebrate hosts against ticks. Once a plateau was reached, no decrease in the immune response was observed until the end of the experiment.

The elicited IgG antibody response against lab-SG substrate (cut-off value: 0.22; Fig. 2) significantly increased with time as well (parameter estimate ± SE: 0.05 ± 0.01; $t_{148} = 3.75$; $p = 0.0002$), showing values above the cut-off starting from week 1 for W rabbits and from week 2 for L rabbits, and up to week 16. In this case there was no significant difference in the immune response between the two groups ($p > 0.05$). However, the observed O.D. values of group W and L to the lab-SG substrate were similar to those obtained for rabbits of group L challenged with wild-SG crude protein extract ($p > 0.05$).

This lack of difference suggests that the antigens in lab-SG crude protein extract mostly consist of *I. ricinus* proteins, which are naturally present in both wild and lab ticks. However, the higher response of group W to the wild-SG substrate, also suggest that *M. mitochondrii* antigens might play a role in enhancing the antibody response of infested rabbits. Since no common TBPs were detected in the DNA extracted from the wild-SG, we may exclude that the observed difference in the immune response between groups is due to these microorganisms. However, we cannot exclude that such difference was induced by a different microbiome composition of wild and lab ticks used in the infestation. Environmental factors, as well as feeding/nutritional status of the tick, or genetic differences between the two tick populations can enhance differences in the tick saliva proteome, which can in turn lead to a difference in the protein-based content of the SG (Narasimhan and Fikrig, 2015).

We also tested the IgG antibody response against *M. mitochondrii* rFliD protein (cut-off value: 0.16; Fig. 3), which did not differ significantly between rabbits of group W and group L ($p > 0.05$). Both groups of rabbits exhibited O.D. values hovering around the cut-off from T_1 to T_8. However, the O.D. values of the two groups of rabbits showed a significantly different trend between T_0 e T_1 ($t_4 = 6.01$; $p = 0.0039$). In fact, rabbits of group W exhibited a significant increase in O.D. values (parameter estimate ± SE: 0.04 ± 0.005; $t_4 = 7.59$; $p = 0.0016$) and rabbits of group L showed no significant variation between the two time points ($p > 0.05$). The increase of the antibody response against rFliD in rabbits of group W is consistent with the increase in O.D. values obtained in the ELISA assay using wild-SG as substrate, suggesting that *M. mitochondrii* antigens are inoculated during the blood meal.

The lack of difference in the response against rFliD substrate between rabbits parasitized with wild and lab ticks is surprising for at least two reasons. On the one hand, we did not expect any response from rabbits of group L, as they were infested with *I. ricinus* which tested negative for the presence of *M. mitochondrii*. On the other hand, we expected a stronger response from rabbits of group W because this antigen has been shown to elicit a specific response in humans (Mariconti et al., 2012a), dogs (Bazzocchi et al., 2013) and roe deer (Serra et al., 2018). Indeed, in natural conditions, serological positivity to the symbiont could be attributed to a long-lasting presence of IgG antibodies against *M. mitochondrii* (depending on the analyzed species), to a continuous exposure to *I. ricinus* (as hypothesized by Serra et al., 2018), or to differences in the interactions among *I. ricinus*, *M. mitochondrii* and vertebrate hosts compared to laboratory conditions.

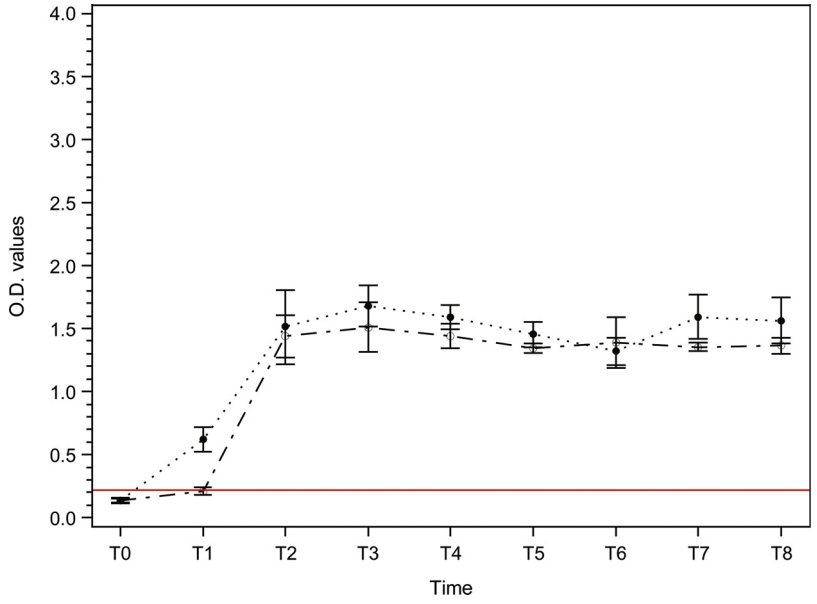

Fig. 2. Mean (± SE) antibody response against lab-SG crude protein extract in rabbits infested with wild (filled circles) and lab (blank circles) *I. ricinus* females. Line indicates the cut-off value (0.22).

The limited immune response observed in the present study in rabbits infested with wild ticks could be attributed to the genetic variability among these vertebrate hosts. Laboratory rabbits are indeed typically more outbred than other laboratory animal models, leading to inter-individual differences in their antibody responses against particular antigens (Sanders et al., 1998). Other causes for the low observed response of group W could be: i) inter-individual differences in *M. mitochondrii* loads among ticks (as observed in Sassera et al., 2008) and which is also suggested by the high variability in *M. mitochondrii* loads observed in wild-SG pools, ii) differences in saliva doses conveyed into the vertebrate host; or iii) interactions among bacteria harbored by ticks. Microbial interactions should be indeed taken into consideration, as possible interferences in the transmission of *M. mitochondrii* could occur when multiple microorganisms are present in the same tick individual. To date, no information is available on the effects of other microorganisms on *M. mitochondrii* transmission in *I. ricinus*. Despite the variability observed among rabbits, these results show that the antibody response against *M. mitochondrii* rFliD, at least in NZW rabbits under experimental conditions, is detectable within one month after *I. ricinus* exposure, suggesting a moderate antigenic activity of the FliD protein under laboratory conditions.

Concerning the positive O.D. values observed for two out of three rabbits of group L, they could suggest the occurrence of cross-reactions. However, *M. mitochondrii* flagellar protein FliD was initially chosen as antigen due to its characteristics of being a surface protein and its high specificity, leading to the lack of cross-reactivity with other bacteria proteins, especially TBPs proteins (for example *Borrelia burgdorferi;* Mariconti et al., 2012a).

After the exclusion of cross-reaction issues, we questioned whether the lab ticks were actually negative to *M. mitochondrii* or were harboring a bacterial load below the detection limit of the qPCR method (10 copies/µl). Moreover, as *M. mitochondrii* is known to exhibit a

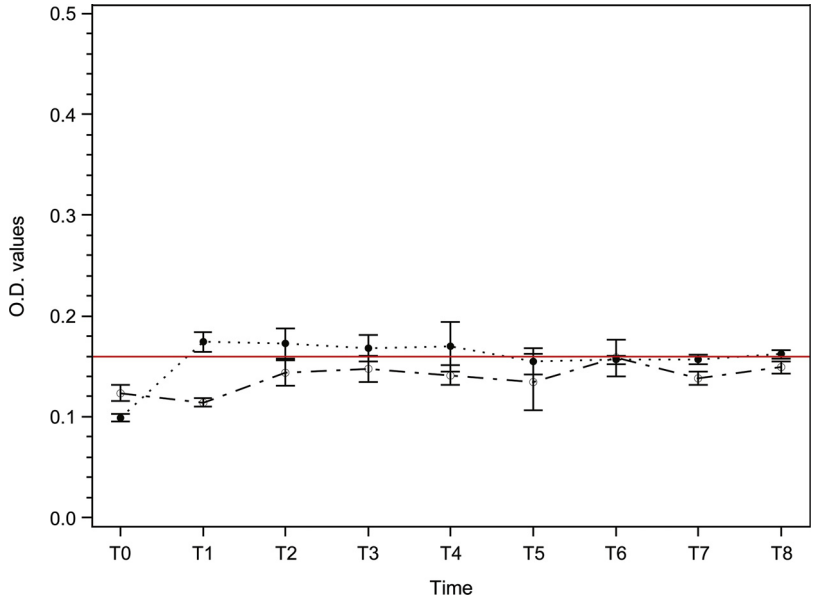

Fig. 3. Mean (± SE) antibody response against *M. mitochondrii* rFliD in rabbits infested with wild (filled circles) and lab (blank circles) *I. ricinus* females. Line indicates the cut-off value (0.16).

bloom following the blood meal (Sassera et al., 2008), the lab strain ticks could harbor extremely reduced numbers (hardly detectable in unengorged ticks), but the endosymbiont could reach higher load, sufficient for its transmission to the host - during blood meal - as observed for *Borrelia* spp. (Piesman et al., 2001).

3.3. Nested PCR analyses on I. ricinus ticks and salivary glands pools

The sensitivity of a recently developed nested PCR analysis was tested to evaluate whether such protocol presents a higher detection sensitivity than the qPCR. The nested PCR assay could indeed show a positive amplification starting from as few as 3 *gyrB* gene copies/µl of the plasmid DNA (data not shown). The 30 laboratory colony representative specimens were thus subjected to a second PCR screening, using the nested PCR approach. Eighteen out of 30 (60%) specimens resulted positive to *M. mitochondrii*, pointing out that some lab individuals were still harboring a highly reduced *M. mitochondrii* bacterial load. However, it was not possible to determine how many lab *I. ricinus* used in the experimental infestation were harboring the symbiont.

DNA samples extracted from lab-SG were also analyzed with the nested PCR protocol and one out of five samples was found positive to *M. mitochondrii*, showing that the symbiont is also present in the SGs of at least some lab *I. ricinus* specimens. However, the lab-SG pool used as substrate for the ELISA assay was negative to both qPCR and nested PCR protocols.

3.4. PCR analyses on blood samples

DNA extracted from blood samples were all negative to *M. mitochondrii* using the qPCR approach. However, consistently with the reduced bacterial load found in some lab specimens, *M. mitochondrii* circulating DNA was detected using the more sensitive nested PCR approach (Table 1). *M. mitochondrii* DNA was detectable at most time points for rabbits infested with wild ticks (from week 1 to week 16 for R1, from week 1 to week 8 for R2 and only at week 1, week 8 and week 12 for R3). Even though lab ticks were characterized by a reduced amount of *M. mitochondrii* load, blood samples of rabbits infested with them resulted positive to *M. mitochondrii* circulating DNA at several time points, starting from week 3 post-infestation for R4 and R6 and from week 4 for R5. Notably, *M. mitochondrii* DNA in R4 was detected up to the end of the experiment, week 16 post infestation.

Amplicons obtained from the nested PCR on the *gyrB* gene of *M. mitochondrii* were sequenced and submitted to BLAST analysis, resulting in 100% identity with the *M. mitochondrii gyrB* gene sequence present in GenBank (accession number LT575859.1).

Nested PCR approaches are common diagnostic tools used for the detection of various TBPs in whole blood samples because of their high sensitivity (*e.g. Rickettsia* spp., *Coxiella*-like endosymbionts, *Anaplasma* spp., *Ehrlichia* spp.; Mediannikov et al., 2004; Çetinkaya et al., 2016; Paris and Dumler, 2016; Seo et al., 2016). *Rickettsiales* bacteria (such as *Orientia tsutsugamushi*, most *Rickettsia* and some *Ehrlichia* species) have endothelial cells as their main targets, which can result in a very low concentration in the blood, sometimes even undetectable by molecular analysis (Souza et al., 2009; Valbuena and Walker, 2009). As a member of the *Rickettsiales*, *M. mitochondrii* could share with these pathogens a similar behavioral pattern. This would explain why *M. mitochondrii* DNA was not always detected at consecutive time points in the analyzed blood samples, even with a nested PCR approach.

Table 1
Results of molecular (nested PCR for the amplification of a fragment of *gyrB* gene) performed on blood samples obtained from rabbits infested with wild (R1-R3) and lab (R4-R6) *I. ricinus* to evaluate the presence of circulating *M. mitochondrii* DNA. Results are expressed as: + positive sample; - negative sample.

Rabbit	Time sampling								
	T_0	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8
R1	−	+	+	+	+	+	+	+	+
R2	−	+	+	+	+	+	+	−	−
R3	−	+	−	−	−	−	+	+	−
R4	−	−	−	+	−	−	+	+	+
R5	−	−	−	−	+	+	−	−	−
R6	−	−	−	+	−	+	−	−	−

The molecular analyses on blood samples showed that the positivity to *M. mitochondrii* DNA is consistent with the amount of *M. mitochondrii* harbored by wild and lab *I. ricinus* individuals used for the infestation. Rabbits of group W were positive to *M. mitochondrii* DNA starting from week 1 post-infestation, while positivity in rabbits of group L was highlighted only from around week 3 post-infestation. These results are in accordance with serology, which shows a positive antibody response against rFliD starting from week 1 post-infestation in rabbits of group W and a delayed positivization observed in at least some of the rabbits of group L. This result indicates that *M. mitochondrii* transmission can thus occur even when *I. ricinus* ticks harbor highly reduced bacterial loads. The delayed positivization of blood samples and sera of group L rabbits to *M. mitochondrii* could be attributed to the lower loads of bacteria inoculated by lab *I. ricinus* requiring longer to replicate enough to be detected through PCR and serological analyses. The replication hypothesis is also supported by the detection of *M. mitochondrii* DNA in blood samples obtained up to 16 weeks post-infestation in both groups of rabbits. The symbiont is thus probably able to survive into its vertebrate host after inoculation and to replicate, even though no clinical signs were observed in any of the rabbits. In the light of low DNA concentrations in systemic circulation (which can result in insufficient material for PCR amplification), additional tissues, such as bone marrow, brain tissue, synovial and cerebrospinal fluids (as performed, for example, for the detection of *B. burgdorferi*; Schwaiger et al., 2001; Wilske, 2003), should be analyzed to investigate their role in harboring *M. mitochondrii* bacteria.

4. Conclusions

The seroconversion against *M. mitochondrii* rFliD in rabbits infested with wild *I. ricinus* was observed to occur one week post-infestation. Furthermore, the symbiont seems to replicate inside the vertebrate host, as revealed by molecular and serological analyses showing positivity up to 16 weeks after experimental infestation. As a support to the replication hypothesis in the vertebrate host, future studies focused on *M. mitochondrii* RNA recovered from blood and organs of parasitized vertebrates could be envisioned to provide information about the viability of this symbiont after inoculation. Additionally, the positivity of blood and sera to *M. mitochondrii* in rabbits infested with lab ticks suggests that transmission of the *I. ricinus* symbiont can be effective even when symbiont loads are extremely reduced.

If the loss of *M. mitochondrii* is not a rare event in natural tick populations (as suggested by their decreasing prevalence following successive generation in tick lab strains), a frequent re-acquisition *via* horizontal transmission would explain why the prevalence of the symbiont in wild females is 100%. Our results, by experimentally demonstrating the transmission of *M. mitochondrii* to vertebrate hosts following tick bite, provide additional support for this hypothesis. Moreover, our findings support the hypothesis that co-feeding on a shared host could be an important determinant for the dispersal of *M. mitochondrii*, and more in general of tick symbionts, with vertebrate hosts acting as “ecological arenas” for the exchange of symbionts (Duron et al., 2017). If the transmission of endosymbionts by ticks to their hosts is a frequent event, this could accelerate the turn-over of tick symbionts also at the interspecific level. In the case of *M. mitochondrii* (and other bacteria of the genus *Midichloria*), vertebrate hosts can serve as intermediate routes for inter-/intra-specific transmission among

Ixodida, as suggested by similar 16S rDNA gene sequences shared among distantly related tick species (Cafiso et al., 2016).

Further investigations are needed to unveil whether *M. mitochondrii* can colonize host tissues. However, the transmission and replication of *M. mitochondrii* into the vertebrate host do not necessarily imply a pathogenic role of this endosymbiont, especially when no experimental or epidemiological evidence of disease has ever been reported. *M. mitochondrii* potential ability to take part in the development of tick-borne illnesses for which no etiological agent has been yet identified needs to be deeply investigated.

Ethic statements

The experimental infestation of rabbits was carried out in strict accordance with good animal care practices recommended by the European guidelines. The protocol was approved by the Ethic Committee for Animal Experiments of the region Pays de la Loire (CEEA PdL 06) (Permit Number: 2015-29).

I. ricinus ticks were collected from roe deer (*Capreolus capreolus*) in the Chizé forest, in accordance with the recommendations on the French National charter on the ethics of animal experimentation and the DIRECTIVE 2010/63/EU OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 22 September 2010 on the protection of animals used for scientific purposes. The protocol was approved by the "Comité d'Ethique en Expérimentation Animale de l'Université Claude Bernard Lyon 1" (CEEA-55; DR2014-09). The capture of roe deer was performed by competent people without causing avoidable pain, suffering, distress or lasting harm to animals.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: https://doi.org/10.1016/j.ttbdis.2018.08.008.

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