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Emerging pathogens in vertebrates: biology, genomics and infectivity of bacteria ascribed to the *Midichloriaceae* family

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List of abbreviations

2DE, 2-DE: two dimensional electrophoresis

cal: calreticuline

ELISA: enzyme-linked immunosorbent assay

EM: electronic microscopy

FISH: fluorescence *in situ* hybridization

FliD: the flagellar cap protein (100 kDa)

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

gyrB: gyrase B

igf1: insulin growth factor 1

MLO, MALO: *Midichloria*-like organism

PCR: polymerase chain reaction

qPCR: quantitative real time PCR

rFliD: recombinant form of the flagellar cap protein (38 kDa)

RLO: *Rickettsia*-like organism

RMS: red mark syndrome

SD: strawberry disease

SG: salivary gland

OT: ovaric tissue

Abstract

This PhD thesis is the outcome of a wider project focused on studying the biology, genomics and infectivity of bacteria ascribed to the *Midichloriaceae* family. *Midichloriaceae* are a group of bacteria extremely diversified in terms of the ecological and taxonomic diversity of their hosts. Members of the family *Midichloriaceae* can be considered as a wide bacterial group, still to be investigated under several aspects. In particular, the association of members of the family *Midichloriaceae* with parasites (such as ticks, fleas and parasitic amoebae) suggests that these bacteria could be potentially infectious to vertebrates (including humans) and even responsible for medical and veterinarian diseases. The researchers conducted in this PhD project have been focused on three main topics: i) possible involvement of a midichloriacea in a disease (red mark syndrome; RMS) affecting rainbow trout; ii) in-depth analysis of different aspects concerning the hard tick *Ixodes ricinus* and its intramitochondrial symbiont *Midichloria mitochondrii* (i.e. antibiotic treatment of the arthropod, salivary glands *vs*. ovaric tissue comparative proteomics, transmission of the symbiont to the vertebrate host); iii) presence of *M. mitochondrii*-related bacteria in different tick species.

A brief description of each topic of the work done is here reported:

i) RMS is a chronic skin disease of unknown etiology affecting farmed rainbow trout *Oncorhynchus mykiss* in Europe. It consists of single or multiple skin lesions usually localized on the trunk of fish approaching market size. Many authors suppose that a bacterial infection could be the cause of the disease. Some studies have reported a possible correlation between the presence of a *Rickettsia*-like organism (subsequently ascribed to the family *Midichloriaceae*) and RMS skin lesions. Our work concerning the possible involvement of a midichloriacea in RMS in rainbow trout resulted in the detection of bacteria of this family, besides in skin lesions, in various internal organs and in the bloodstream. No bacteria were found in unaffected fish and in healthy skin samples from affected individuals. Bacteria were detected also in fish ponds water were RMS-affected fish are farmed. These results give support to the hypothesis of the involvement of these bacteria in developing RMS. It is possible that some free-living eukaryotes could host or be the vector of this midichloriacea.

ii) *M. mitochondrii* is the most prevalent symbiont of the hard tick *I. ricinus*, present in 100% of eggs and adult females of wild *I. ricinus* populations. This bacterium is intracellular and is the only known symbiont able to invade the mitochondria of the host cells. However, the role that *M. mitochondrii* plays in the host metabolism has yet to be elucidated. In addition,

multiple lines of evidence indicate the possibility of the transmission of this bacterium to the vertebrate host during the tick blood meal.

In order to investigate the role of *M. mitochondrii* in the biology of the tick host, we performed an antibiotic treatment on *I. ricinus* individuals, with the aim of reducing/eliminating the symbiont, and to potentially observe the dynamic of bacterial infection in the tick host. We microinjected engorged adult females of *I. ricinus* with tetracycline, and we allowed the resulting larvae to feed on gerbils treated with the same antibiotic. The amount of *M. mitochondrii* was evaluated at different stages of the experiment using molecular techniques. In addition we evaluated the presence/absence of the symbiont DNA in the blood of gerbils used for the larval feeding. The performed treatments did not allow to eliminate the symbiont population from the host tick, however it allowed to reduce the multiplication that occurs after the larval blood meal. These results open the way for future experiments, using different antibiotic molecules, different administration methods and antibiotic administration on subsequent tick stages, to fulfill the goal of eliminating *M. mitochondrii* from the host *I. ricinus*, a major step in our understanding of the impact of this bacterium on ticks.

In order to investigate, from a proteomic point of view, the tick *I. ricinus* and its symbiont, the protein profile of the ovary tissue (OT) and of salivary glands (SG) of adult females of this tick species were generated. To compare the OT and SG profiles, 2-DE profiling followed by LC-MS/MS protein identification were performed. We detected 21 spots showing significant differences in the relative abundance between the OT and SG, ten of which showed 4- to 18-fold increase/decrease in density. This work allowed to establish a method to characterize the proteome of *I. ricinus*, and to detect multiple proteins that exhibit a differential expression profile in OT and SG. Additionally, it was possible to use an immunoproteomic approach to detect a protein from the symbiont. Finally, the method developed will pave the way for future studies on the proteomics of *I. ricinus*, with the goals of better understanding the biology of this vector and of its symbiont *M. mitochondrii*.

The transmission of *M. mitochondrii* after *I. ricinus* bite has been evidenced through direct and indirect evidences in human and other vertebrates by different authors. However, the time of seroconversion against the antigens of this symbiont is still unknown, as well as the kinetic of the antibody response against *M. mitochondrii*. In this work we performed an experimental infestation of rabbits with wild *I. ricinus* ticks (harboring *M. mitochondrii*) and *I. ricinus* from a lab colony (free of the symbiont). We were able to show the first seroconversion of a vertebrate model against *M. mitochondrii*. The seroconversion occurs approximately around

the first and the second week after tick infestation, with duration of at least one month after infestation. It has been observed that *M. mitochondrii* represent an actual pack of antigens that can persist in the vertebrate host for a prolonged time. *M. mitochondrii* DNA was also observed circulating in blood of infested rabbits. The flagellar protein FliD of *M. mitochondrii* can be considered an interesting marker for *I. ricinus* bite, at least concerning adult females bite. However, the antibody response against this protein suggests that a possible screening for this marker should be considered within a limited time after tick infestation. Additional markers both from *M. mitochondrii* and *I. ricinus* should be considered in view of developing a possible marker for *I. ricinus* bite detection.

iii) The multiple screening of 17 tick species allowed the detection and quantification of bacteria of the family *Midichloriaceae* in seven of them, including the first report of a representative of this family in a soft tick species (Argasidae), *Ornithodoros maritimus*. Based on sequence identity and phylogenetic analysis we propose that all these bacterial symbionts of ticks could be members of the genus *Midichloria*. The performed screening highlights different prevalence levels and variable bacterial loads in different tick species including one, *Ixodes aulacodi*, where the bacterium is present in all examined individuals, like in *I. ricinus*. This result prompts us to hypothesize different roles of *Midichloria* bacteria in different tick species.

Riassunto

Questo lavoro di tesi è parte integrante di un più ampio progetto inerente allo studio della biologia, genomica ed infettività di batteri ascritti alla famiglia *Midichloriaceae*. Le *Midichloriaceae* costituiscono una famiglia batterica estremamente diversificata in termini di variabilità ecologica e tassonomica dei suoi ospiti. I membri della famiglia *Midichloriaceae* costituiscono un grande taxon batterico il cui studio deve ancora essere approfondito sotto vari aspetti. Un aspetto peculiare della famiglia *Midichloriaceae* è rappresentato dall'associazione di alcuni membri di questa famiglia con organismi parassiti (quali zecche, pulci e amebe patogene), che suggerisce come almeno alcuni componenti possano essere considerati potenzialmente trasmissibili ai vertebrati (uomo incluso) ed essere responsabili dello sviluppo di malattie che interessano l'uomo e altri animali. Le ricerche condotte durante questo progetto di dottorato si sono focalizzate su tre macro-argomenti principali: i) il possibile coinvolgimento di una midichloriacea nello sviluppo di una malattia (red mark syndrome; RMS) nella trota iridea; ii) approfondimento di differenti aspetti riguardanti la relazione tra la zecca dura *Ixodes ricinus* e il suo simbionte intramitocondriale *Midichloria mitochondrii* (i.e. trattamento antibiotico dell'artropode, proteomica comparativa di ghiandole salivari *vs.* tessuto ovarico, trasmissione del simbionte all'ospite vertebrato); iii) presenza di batteri correlati a *M. mitochondrii* in varie specie di zecca.

Una breve descrizione di ciascun argomento e di seguito brevemente riportata:

i) La RMS è una malattia cronica che colpisce la cute delle trote iridee di allevamento (*Oncorhynchus mykiss*) in Europa e la cui causa eziologica risulta essere ignota. La malattia si manifesta con lesioni cutanee singole o multiple sui fianchi di pesci generalmente pronti per l'immissione sul mercato. Diversi autori ipotizzano che un'infezione di tipo batterico potrebbe essere implicata nello sviluppo della malattia. Alcuni studi hanno riportato una possibile correlazione tra la presenza di un organismo *Rickettsia*-like (successivamente ascritto alla famiglia *Midichloriaceae*) e la presenza di lesioni cutanee da RMS. Il lavoro svolto in questo progetto di tesi ha riguardato lo studio del possibile coinvolgimento di una midichloriacea nello sviluppo della RMS, con l'obiettivo di rilevare e quantificare di DNA di batteri appartenenti a questa famiglia, oltre che nelle lesioni cutanee, anche in diversi organi interni e nel torrente circolatorio. La presenza di questi batteri non è invece stata riscontrata nei pesci sani né nel tessuto cutaneo sano di pesci affetti da RMS. Questi risultati supportano l'ipotesi inerente il possibile coinvolgimento di questi batteri nello sviluppo della RMS. È

altresì possibile che alcuni eucarioti a vita libera possano fungere da ospiti intermedi o rappresentare il vettore di questa midichloriacea.

ii) *M. mitochondrii* è il più rilevante simbionte della zecca dura *I. ricinus*, presente nel 100% delle uova e delle femmine adulte delle popolazioni naturali di questa specie di zecca. *M. mitochondrii* è un batterio intracellulare ed è l'unico simbionte tuttora noto in grado di invadere i mitocondri della cellula ospite. Il ruolo giocato da *M. mitochondrii* nel metabolismo dell'ospite è tuttavia ancora poco chiaro. In aggiunta a ciò, vi sono diverse evidenze che indicano come sia possibile che questo batterio venga trasmesso all'ospite vertebrato in seguito al pasto di sangue della zecca.

Al fine di investigare il ruolo di *M. mitochondrii* nella zecca ospite, è stato effettuato un trattamento antibiotico su femmine di *I. ricinus*, con l'intenzione di ridurre/eliminare il simbionte e potenzialmente osservare la dinamica dell'infezione batterica nella progenie della zecca trattata. Femmine adulte replete di *I. ricinus* sono state micro-iniettate con tetraciclina e la progenie derivante, allo stadio larvale, è stata fatta nutrire su gerbilli trattati con lo stesso antibiotico. La quantità di *M. mitochondrii* è stata valutata a diversi stadi dell'esperimento per mezzo di tecniche di biologia molecolare. In aggiunta, la presenza/assenza del simbionte è stata valutata anche nel sangue dei gerbilli usati per l'infestazione larvale. I trattamenti antibiotici effettuati non hanno permesso l'eliminazione del simbionte dall'ospite zecca, tuttavia hanno permesso di ridurre la moltiplicazione di *M. mitochondrii* che normalmente avviene nell'artropode a seguito del pasto di sangue. Questi risultati aprono la strada a futuri esperimenti implicanti differenti molecole antibiotiche, differenti metodi di somministrazione anche per quanto riguarda stadi vitali successivi alla larva, al fine di raggiungere la completa eliminazione del simbionte da *I. ricinus* e compiere un passo importante nella comprensione dell'impatto di questi batteri nella biologia della zecca.

Per investigare, dal punto di vista proteomico, la relazione tra *I. ricinus* e il suo simbionte, è stato generato il profilo proteico del tessuto ovarico (OT) e delle ghiandole salivari (SG) di femmine adulte di *I. ricinus*. A questo fine, è stato effettuato un profilo in 2-DE e successiva identificazione proteica in LC-MS/MS sui due tipi di tessuto. Sono stati identificati 21 spot proteici con abbondanza relativa differenziale tra OT e SG, dieci dei quali hanno mostrato una densità maggiore/inferiore tra le 4 e le 18 volte. Questo lavoro ha permesso di definire un protocollo funzionale per caratterizzare il proteoma di *I. ricinus* e per rilevare diverse proteine che mostrano un profilo di espressione differenziale tra OT e SG. In aggiunta, è stato possibile applicare un approccio di immunoproteomica per identificare una proteina specifica del simbionte. Infine, lo sviluppo di questo metodo apre la strada a futuri studi inerenti alla

proteomica di *I. ricinus*, con lo scopo di comprendere meglio la biologia di questo vettore e del suo simbionte *M. mitochondrii*.

La trasmissione di *M. mitochondrii* in seguito al morso di *I. ricinus* è stata illustrata attraverso evidenze dirette ed indirette nell'uomo e in altri vertebrati da parte di diversi autori. Tuttavia, il tempo di sieroconversione nei confronti di antigeni appartenenti a questo simbionte non è ancora noto, così come la cinetica della risposta anticorpale nei suoi confronti. In questo lavoro è stata effettuata un'infestazione sperimentale di conigli con zecche *I. ricinus* di campo (naturalmente ospitanti *M. mitochondrii*) e *I. ricinus* provenienti da una colonia mantenuta in laboratorio (in cui *M. mitochondrii* è assente). Il lavoro ha permesso di mostrare per la prima volta la sieroconversione in un organismo modello nei confronti di antigeni appartenenti a *M. mitochondrii*. La sieroconversione avviene approssimativamente tra la prima e la seconda settimana successiva all'inizio dell'infestazione sperimentale, con una durata che va da un minimo di un mese per protrarsi fino a 4 mesi dopo l'infestazione. È stato possibile osservare come *M. mitochondrii* costituisce un vero e proprio "pacchetto antigenico" che può persistere nell'ospite vertebrato per un tempo prolungato. Il DNA di *M. mitochondrii* è stato rilevato nel sangue dei conigli infestati anche a distanza di diverse settimane post-infestazione. La proteina flagellare FliD di *M. mitochondrii* può essere considerata un marker interessante per quanto riguarda il morso da *I. ricinus*, perlomeno nel caso di morso di zecche adulte. Dei marker aggiuntivi, appartenenti sia a *M. mitochondrii* che a *I. ricinus*, dovrebbero tuttavia essere considerati come marker per la rilevazione di morso da zecca.

iii) Uno screening multiplo di 17 specie di zecca ha permesso di rilevare e quantificare batteri della famiglia *Midichloriaceae* in sette di esse, compresa una specie appartenete al gruppo delle zecche molli (famiglia Argasidae), *Ornithodoros maritimus*. Basandosi sull'identità delle sequenze e su analisi filogenetiche, con questo lavoro proponiamo che tutte queste midichloriaceae rilevate nelle zecche possano essere incluse nel genere *Midichloria*. Lo screening effettuato evidenzia l'esistenza di livelli di prevalenza differenti e carichi batterici diversi nelle varie specie di zecca. In una specie, *Ixodes aulacodi*, il batterio è risultato essere presente in tutti gli individui analizzati. Questi risultati fanno supporre come i batteri appartenenti al genere *Midichloria* possano giocare ruoli diversi nelle varie specie di zecca.

1 General introduction

1.1 The bacterial family *Midichloriaceae*

The class Alphaproteobacteria encompasses a wide group of Gram-negative bacteria characterized by different lifestyles and physiologies (Ferla et al., 2013). Bacteria belonging to this taxon are found in diverse ecological niches from water to soil, showing association with eukaryotes, including unicellular organisms, bur also metazoans including nematodes, arthropods and mammals (Batut et al., 2004). Many of these bacteria frequently show intracellular lifestyle as plant mutualists or plant and animal pathogens (Williams et al., 2007). Bacteria of the order *Rickettsiales* belong to the class Alphaproteobacteria that comprises small rod-shaped or coccoid microorganisms, with obligate intracellular behavior in eukaryotic cells, i.e. they cannot proliferate in host-cell free media (Dumler and Walker, 2005). Most *Rickettsiales* have established close relationships with their hosts, as evidenced by manipulation of cellular process such as host reproduction (Renvoisé et al., 2011) or possibly mutualistic associations as observed, for example, in members of the genus *Wolbachia* (Werren et al., 2008; Taylor and Hoerauf, 1999). In some cases, strong symbiosis can lead to the integration of bacterial genome fragments into the host cell, as observed for *Wolbachia* bacteria. Horizontal Gene Transfer (HGT) has been found and confirmed into the genome of several arthropod (for example *Drosophila ananassae*) and nematode species (for example in the worms *Brugia malayi* and *Dirofilaria immitis*), and sometimes the transferred sequences represented a significant portion (up to 30%) of the genome (Lacroix and Citovsky, 2016; Dunning Hotopp et al., 2007). The most significant symbiosis occurs when the majority of genes have been transferred to the hosting cell and the gene products needed for the bacterial functions are indeed encoded by the hosting cell. This is what occurred for part of *Rickettsiales* genome when was integrated into the nucleus of its eukaryotic host and another genome fragment was merged into the mitochondria (McCutcheon, 2016). Many evidences support the conclusion that mitochondrial genome originated from the eubacterial domain of life, in particular from the Alphaproteobacteria branch (Gray et al., 2001). Indeed, *Rickettsiales* are most closely related to mitochondria, with a divergence time of around 850–1500 million years ago (Renvoisé et al., 2011). Furthermore, in many studies *Rickettsiales* is recurrently retrieved as a distinct group, separate from all other Alphaproteobacteria (Carvalho et al., 2015).

The order *Rickettsiales* is composed of three families of bacteria (Figure 1): *Anaplasmataceae*, *Rickettsiaceae* and *Candidatus* Midichloriaceae (hereafter *Midichloriaceae*) (Montagna et al., 2013; Ferla et al., 2013). In the latest few years evolutionary relationships within *Rickettsiales* significantly changed and its taxonomy was reorganized. Although the *Anaplasmataceae* and *Rickettsiaceae* were already two established bacterial families encompassed in the order *Rickettsiales* (Brenner et al., 1993), their taxonomy was modified by Dumler and colleagues in 2001 (Dumler et al., 2001), so that these two "classical" families thereafter included respectively the genera *Orientia*, *Rickettsia*, and *Anaplasma*, *Ehrlichia*, *Neorickettsia, Wolbachia.*

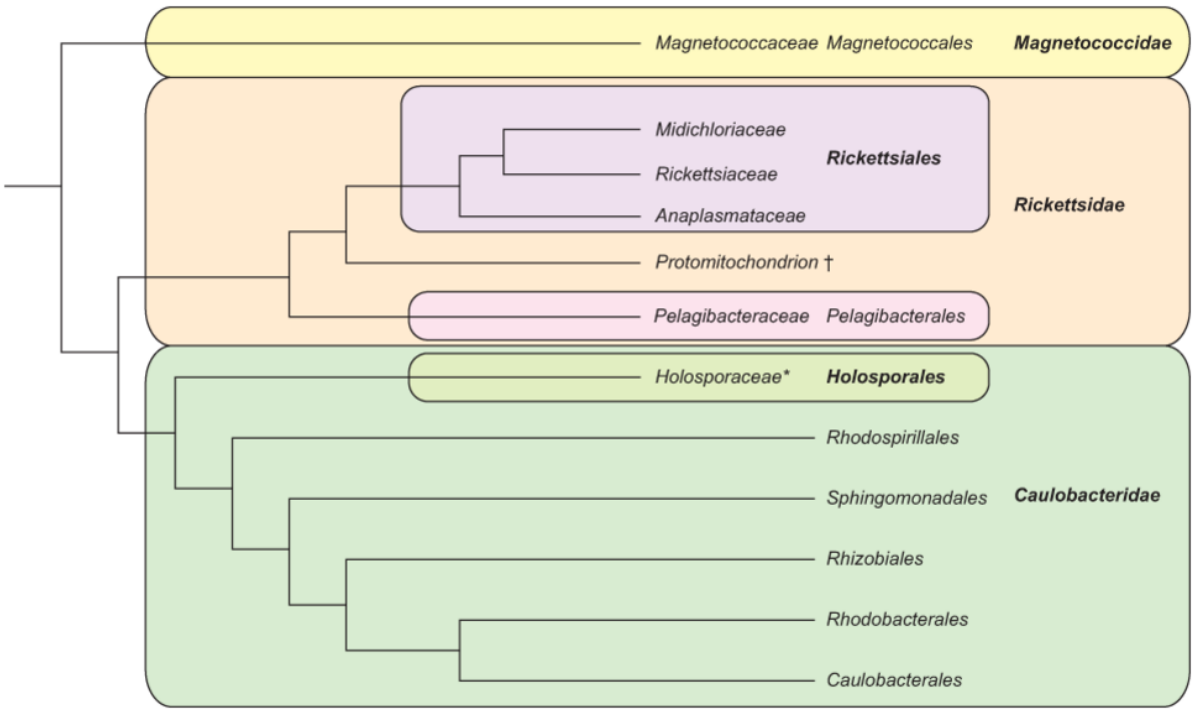

Figure 1 Subclasses of the Alphaproteobacteria proposed by Ferla et al. (2013). The three proposed subdivisions are the Magnetococcidae, the Rickettsidae and the Caulobacteridae. Under this scheme the *Rickettsiales* are comprised of the *Rickettsiaceae*, *Anaplasmataceae* and *Midichloriaceae* (Ferla et al., 2013).

On the contrary, the family *Midichloriaceae* was described by Montagna and colleagues only in 2013 (Montagna et al., 2013). Before *Midichloriaceae* were classified as a separate family within the order *Rickettsiales*, the members of this clade were previously identified as part of the wide and heterogeneous group of "*Rickettsia*-like organisms" (RLO) or "organisms ascribed to the order *Rickettsiales*", without a precise phylogenetic position (Lloyd et al., 2008; Fritsche et al., 1999; Lewis, 1979). During the years, indeed, it become evident that representatives of *Rickettsiales* genera displayed a broader host range than previously

recognized, as observed for *Rickettsia* and *Wolbachia*, that were retrieved respectively, in several non-hematophagous arthropods (Weinert et al. 2009; Perlman et al. 2006), and in filarial nematodes (Werren et al., 2008; Taylor et al., 2005). The type-representing species of the *Midichloriaceae* family, previously described as a RLO, is *Candidatus* Midichloria mitochondrii (hereafter *Midichloria mitochondrii*), intramitochondrial symbiont of the hard tick *Ixodes ricinus* (Sassera et al., 2006; Beninati et al., 2004). Afterwards, with the advent of studies based on detecting and cataloguing the bacterial diversity in environmental and biological samples (Boscaro et al., 2013a; Matsuura et al., 2012; Sunagawa et al., 2010; Vannini et al., 2010; Erickson et al., 2009; Epis et al., 2008; Lloyd et al., 2008; Fraune et al., 2007; Mediannikov et al., 2004; Fritsche et al., 1999), the *16SrRNA* gene sequences retrieved from various samples, showing strong similarity with *M. mitochondrii*, were indicated as MALOs (*Midichloria* and like organisms; Montagna et al., 2013). These previously "unclassified" MALOs were subsequently grouped into the well defined cluster of the family *Midichloriaceae* (Montagna et al., 2013).

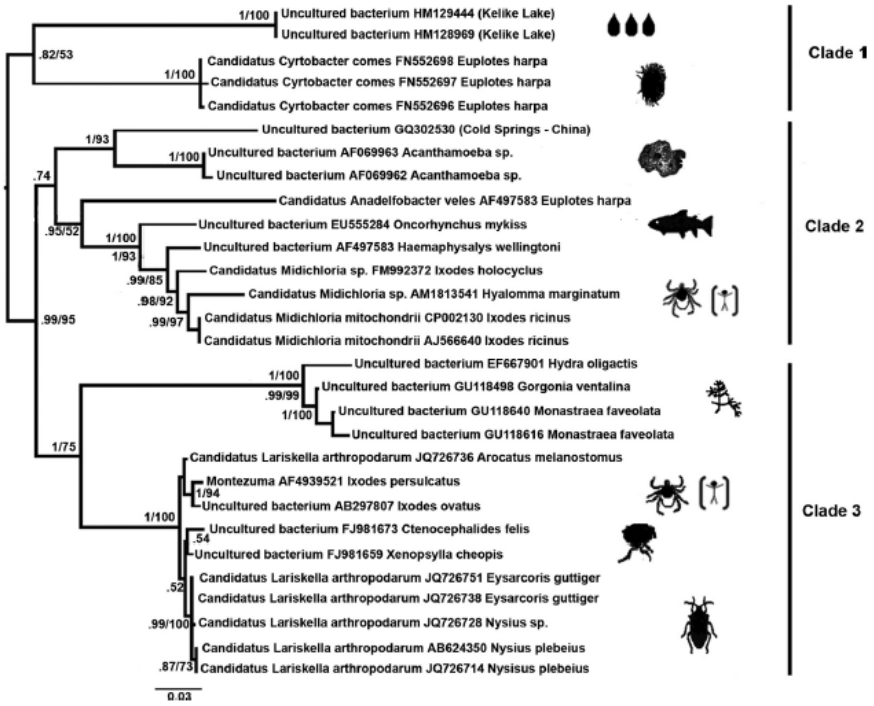

Figure 2 Bayesian phylogram of the *Midichloriaceae* family obtained from the *16SrRNA* gene sequence data set. Where *16SrRNA* gene sequences were obtained from environmental samples, the source is indicated in parentheses. Visual representations of hosts (or sources) of the bacteria are mapped beside the terminal tips. The scale bar indicates the distance in substitutions per site (Montagna et al., 2013).

Any other *Rickettsia*-like organism for which the assignment was based only on morphological features and lacking in molecular characterization (such as a partial *16SrRNA* gene sequence) was not included in this family. *Midichloriaceae*, whose diversity and host ranges are comparable to those of *Rickettsiaceae* and *Anaplasmataceae*, are associated to phylogenetically diverse hosts from protists to Cnidaria and Porifera, from parasitic arthropods to mammals (Figure 2). This widespread distribution, together with the peculiar intramitochondrial behavior (at least in *I. ricinus*) and the association with parasitic hosts (such as parasitic arthropods and parasitic protists, i.e. *Acanthamoeba* strains) let us suppose that these bacteria could be infectious to vertebrates (included humans). This hypothesis is supported by direct and indirect evidences of the transmission of *M. mitochondrii* to vertebrate hosts after *I. ricinus* bite (Bazzocchi et al., 2013; Mariconti et al., 2012b) (see Paragraph 1.2.4) and by a host spectrum at least comparable to the two families *Rickettsiaceae* and *Anaplasmataceae*, which are responsible for several pathological alterations in vertebrates (Dumler and Walker, 2005). As *Midichloriaceae* are, until now, uncultivable microorganisms, their role as potentially infectious agents is far more difficult to be defined as it is impossible to fulfill Koch's Postulates.

In the light of these considerations the research carried out during my PhD course has been focused on two main aspects concerning the family *Midichloriaceae* that will be introduced in the next chapters: 1) expanding the knowledge about the presence and the relationship of *Midichloriaceae* in ticks and studying the infectivity of *M. mitochondrii* in vertebrates parasitized by the hard tick *I. ricinus* (Chapter 1.2); 2) investigating the involvement of a midichloriacea in skin and organs lesions of *Oncorhynchus mykiss* (rainbow trout) individuals affected by red mark syndrome (Chapter 1.3).

1.2 *Midichloriaceae* in ticks and transmission to vertebrate hosts

1.2.1 Ticks and transmission of bacteria: a brief general description

Ticks are highly specialized obligate, hematophagous, nonpermanent, ectoparasitic arthropods of the order Ixodida (subclass Acarina) that can feed on all superior vertebrates, from reptiles to mammals, and amphibians (Mediannikov and Fenollar, 2014; Dantas-Torres et al., 2008) all over the world. Molting and reproduction are regulated by temperature, day-length, and ingestion of blood. Ticks present four life stages: egg, larva, nymph and adult (male and

female); each life stage if followed by molting after the blood meal, which is also fundamental for egg production. Life stages in ticks can be very different, as some species pass through multiple nymphal stages (Argasidae), while others present a single nymphal stage (Ixodidae; Figure 3).

Figure 3 Typical Ixodidae life stages (left to right: adult female, adult male, nymph, larva). www.science-art.com

Generally, the body of ticks is divided into anterior gnathostoma (capitulum) and posterior idiosoma. The mouthparts include the dorsally paired chelicerae and the segmented palps, that do not enter the wound (they are pressed laterally and horizontally against the skin during the blood meal) and the ventral, backward-pointing, denticulate hypostome (used as a holdfast organ and food canal), all mounted on the basis capituli (Anderson and Magnarelli, 2008). The body, posterior to the capitulum, presents the legs and genital pore in the anterior part, while the spiracles (allowing gas exchange) and anus are present in the posterior part. The genital aperture is lacking in larvae and nymphs while is open in adults. In some cases a hardened shield, the scutum, covers the dorsal anterior portion of the body of female and juvenile ticks; the scutum covers the entire body in males. During feeding, the cuticle, with the exception of the scutum, expands to accommodate the ingested blood meal (Anderson and Magnarelli, 2008). Legs are characterized by six segments used for locomotion; larvae have three pairs of legs, whereas nymphs and adults present four pairs of legs (Anderson and Magnarelli, 2008). In order to find hosts and mates, ticks use the Haller's organ, full of a large set of different types of chemoreceptive sensilla, located on the tarsus of the first leg (Klompen and Oliver, 1993), allowing them to detect temperature, air currents, odors and chemicals.

Tick are divided into three families: 1) *Ixodidae* (commonly known as "hard ticks" due to the presence of a sclerotized dorsal plate and a protruding capitulum) with an estimated number

of species higher than 700; 2) *Argasidae* ("soft ticks", characterized by the absence of dorsal plate and the capitulum, except in larvae, positioned sub-terminally) which includes around 200 species (Mediannikov and Fenollar, 2014; Anderson and Magnarelli, 2008); 3) *Nuttalliellidae* with a single species distributed from southern Africa (Namibia and South Africa) to Tanzania and has been considered to be the "missing link" between the hard and soft tick families as well as being a living fossil (Mans et al., 2015).

The family Ixodidae is the most involved taxon in human and animal tick parasitosis, this is the reason why my research has been focused on ticks included in this group. The species belonging to this family are arranged into two major groups (Prostriata and Metastriata; Oliver, 1989). All life stages are able to feed on animals, except for Prostriata (*Ixodes*) adult males, resulting in readiness to mate almost immediately; a blood meal taken soon after ecdysis to the adult stage may accelerate the process. Metastriata males must generally feed as adults in order to undergo meiosis and spermatid production (Oliver, 1989). Ixodid females usually do not become fully engorged unless mated and, if the feeding virgin ixodid female is not mated soon after she has reached the receptive period, she may remain attached to the host for long periods of time or may detach partially fed. Tick bites can be directly harmful for the host by causing mechanical irritation and inflammation of the skin, and causing, in some cases, paralysis, toxicoses and allergic reactions (Parola and Raoult, 2001). However, tick bite can be of even more importance when causing indirect harm to the host, being vehicle of many severe diseases. The lifestyle of ticks, which includes uptake of blood from hosts, secretion of saliva into the host tissues and shifting from one to another host individual, makes them suitable to host and transmit other organisms (Baneth, 2014). For this reason, ticks are considered as the second worldwide vector of human diseases, soon after mosquitoes (de la Fuente et al., 2008). The vectoring ability of hard ticks is enhanced by their relatively long periods (several days) of feeding without a noticeable or painful bite (as salivary secretions contain anti-inflammatory, antihemostatic and immunosuppressive substances; Parola and Raoult, 2001) and strong attachment to the host (Parola and Raoult, 2001). The feeding period comprises three phases: a preparatory phase (about 1 day), a slow feeding period (~7 days) is followed by a rapid engorgement (~1–3 days) (Flynn and Kaufman, 2015). Precisely, larval and nymphal feeding period on warm-blooded animals usually require 3-7 and 4-8 days, respectively. Adult females parasitizing birds and mammals usually require seven to twelve days, and those parasitizing reptiles take longer (Oliver, 1989). While feeding is occurring, there are alternating periods of sucking blood and salivation, with regurgitation occurring frequently, particularly at the end of the rapid engorgement phase (Parola and

Raoult, 2001). As explained, because of this behavior, ixodid ticks can transmit a broad spectrum of pathogens to both humans and animals and they are indeed considered the group of arthropods that can transmit the wider variety of agents (Jongejan and Uilenberg, 2004) such as: viruses, bacteria, protozoa and helminthes, most of which have a life cycle that requires passage through the vertebrate host (Bonneau et al., 2015; Mediannikov and Fenollar, 2014). Among the broad range of organisms that can be transmitted during the tick bite, a large number is represented by prokaryotes. Some of these are involved in symbiosis such as mutualism (where both the host and symbiont reciprocally benefit from the relationship) or commensalism (if the symbiont utilizes the host without benefiting or harming it), without inducing any kind of diseases, while others are able to cross into vertebrate hosts and become pathogenic, such as the Lyme disease agent *Borrelia burgdorferi* (Baneth et al., 2014; Leung and Poulin, 2008).

Ticks get infected with bacteria by feeding on bacteremic hosts, by transstadial and/or transovarial transmission and by co-feeding (ticks feeding in proximity on the host can transmit pathogens from one another; Raoult and Roux, 1997; Gern and Rais, 1996; Labuda et al., 1993). For some bacteria, all these forms of transmission may occur, as seen, for example, in the spotted fever group rickettsiae (e.g. the Rocky Mountain Spotted fever agent *Rickettsia rickettsii*; Karbowiak et al., 2016). Bacteria such as rickettsiae multiply in almost all organs and fluids of ticks, in particular the salivary glands and ovaries, enabling transmission of organisms during feeding and transovarially. Other bacteria may be transmitted transovarially but do not infect the salivary glands of their tick hosts and cannot then be transmitted to susceptible vertebrate hosts where they might cause disease acquired by a tick during feeding, e.g. *Rickettsia peacockii* (Felsheim et al., 2001). Contrary to the family Argasidae, each stage of ixodid tick feeds only once, and bacteria acquired by a tick during feeding can then be transmitted to another host only when the tick has molted to its next developmental stage and performs its next blood meal (Parola and Raoult, 2001). Not all tick species within a genus are able of transmitting bacteria transstadially; for example, not all *Ixodes* species that acquire *B. burgdorferi*, pass the agent transstadially (Parola and Raoult, 2001). When bacteria can be transmitted both transstadially and transovarilly within a tick species, the arthropod host will be considered as reservoir for the bacteria, resulting in the same distribution of the disease caused by the microbial agent identical to that of its tick host (Parola and Raoult, 2001).

Tick-borne diseases (TBDs) have a worldwide distribution, but in the recent years an increase has been observed as never before. Climatic, land-use, and ecological factors underlie the increasing densities and expansion in geographical distribution of ticks, as it has been

observed for *Ixodes ricinus* (the most common tick found parasitizing humans in European countries and vector of Lyme disease agent *B. burgdorferi*) in Europe. Some climate models, indeed, suggest that the range of *I. ricinus* in Europe might double in the future (Medlock and Leach, 2015). These changing scenarios are likely to become an issue of public health relevance, especially considering their effects on the epidemiology of some infectious diseases. Parasites affecting both domestic and wild animals may circulate between and among sympatric populations of animals, thus facilitating potential spread of the infections to humans (Otranto et al., 2015).

In the recent years, an increasing number of novel *Rickettsiales* bacteria has been described since the advent of molecular detection and characterization (Parola et al, 2005). For some of these bacteria, only molecular description has been possible without cultivation of isolates. In some cases DNA sequences were retrieved only in the tick host, while in other cases molecular detection has been performed in blood of human and/or other vertebrate hosts, as seen for *Midichloria mitochondrii* and other midichloriaceae transmitted by ticks (Bazzocchi et al., 2013; Mediannikov et al., 2004). This aspect will be better explained in the next paragraphs.

1.2.2 *Midichloria mitochondrii* in *Ixodes ricinus*

Interactions between arthropods and bacteria can play multiple and important roles in the biology of both. Such relationships range from obligatory mutualistic endosymbiosis, where the removal of the symbiont population results in the death of the host, to full parasitism, with the bacteria having a clear pathogenic effect on the host (Zug and Hammerstein, 2015; Duron et al., 2008; Goebel and Gross, 2001). As already explained, ticks have been reported to harbor complex and highly variable microbial communities (Rynkiewicz et al., 2015) that play important roles in the biology of these arthropods. Among the members of these communities are important pathogens of humans and animals that can be transmitted through the blood meal, including protozoa such as *Babesia* spp. and *Anaplasma* spp. (Stańczak et al., 2004) and a wide range of viruses. However, the diversity of bacterial tick-borne pathogens is even greater, including, for example, *B. burgdorferi* and related species (Chomel et al., 2015). Less is known about the other members of the bacterial community associated with Ixodida, and particularly those that do not cause overt diseases. However, studies focused on reporting and comparing the presence of such bacteria are increasing, with the goal of understanding

their role on host physiology. For example, in *Dermacentor andersoni*, the bacterial symbiont *R. peacockii* is known to prevent the transovarial transmission of the Rocky Mountain spotted fever agent *R. rickettsii* (Felsheim et al., 2009).

One of the most intriguing endosymbionts of ticks is *M. mitochondrii* in the hard tick *I. ricinus*. The European sheep tick *I. ricinus*, known as the "sheep tick" or "castor bean tick", is the most widespread tick species in Europe and is one of the most common tick species infesting humans. The importance of *I. ricinus* is due to its wide area of distribution (i.e. from Scandinavia down to Northern Africa and across to Russia and Turkey; Estrada-Peña et al., 1998), to its low host specificity and capacity to parasitize humans and to its central role in the transmission of multiple infectious agents (Socolovschi et al., 2009).
M. mitochondrii appears as a Gram-negative, non spore form, intracellular, rod-shaped bacterium of ~0.45 µm in diameter and ~1.2 µm long (Beninati et al., 2004). It was observed for the first time during an electron microscopy (EM) study of *I. ricinus*, when intracellular bacteria were discovered in the ovaric tissue (Lewis, 1979). The bacteria were found inside mitochondria as well as in the cytoplasm. It was suggested that the bacteria replicated in the mitochondria, which then burst and released bacteria into the cytoplasm (Lo et al., 2006). Later, in a further experimental study, female *I. ricinus* were found to harbor bacteria with the same characteristics; bacteria were not seen in male specimens (Zhu et al., 1992). Subsequently, EM and fluorescent *in situ* hybridization (ISH) studies on engorged females of *I. ricinus*, showed bacteria within various cells of the reproductive tissue, free in the cytoplasm or included in a host-derived membrane (Beninati et al., 2004; Sacchi et al., 2004). Bacteria were observed inside the mitochondria between the inner and outer membranes and consuming the mitochondrial matrix (Sacchi et al., 2004). Molecular analyses showed that the bacteria seen in EM represented a single species within the Alphaproteobacteria and this species was given the temporary designation IricES1 (*I. ricinus* EndoSymbiont 1), pending further taxonomic studies (Lo et al., 2006). Like many Alphaproteobacteria, IricES1 was found in the cytoplasm enclosed in a host membrane. However, it showed to have the peculiar characteristic of colonizing mitochondria (Beninati et al., 2004), a phenomenon that has previously been observed only in the ciliates *Halteria geleiana* (Yamataka and Hayashi, 1970) and *Urotricha ovata* (de Puytorac and Grain, 1972); however, the phylogenetic status of these bacteria has not been determined yet. The symbiont was then proposed to be renamed "*Midichloria mitochondrii*" (Sassera et al., 2006) with a name able to recall its unique intra-mitochondrial lifestyle. The bacterium has been observed to be vertical transmitted to the eggs

as observed through EM, FISH and molecular analyses and is present in all life stages (Epis et al., 2013; Sassera et al., 2008; Beninati et al., 2004). *M. mitochondrii* has been found ubiquitous in females of *I. ricinus* across its distribution (with a prevalence of 100%), while a 44% prevalence is observed in males (Sassera et al., 2006). The presence in 100% females is typical of a mutualist or of a manipulator of the host reproduction. However, this prevalence is observed throughout the entire geographical distribution of *I. ricinus* and no alterations of the reproduction has been observed in this tick species so far (Sassera et al., 2008). It has been supposed that a lower number of bacteria (or a lacking of them) can be observed in those nymphs that will molt to adult males (Sassera et al., 2008). Unfortunately, currently no molecular method is available to determine the sex of nymphs. Moreover, the loss of the symbiont in tick colonies maintained in the laboratory indicates that the symbiosis is not obligate, suggesting a facultative mutualism (Sassera et al., 2008; Lo et al., 2006).

Little is known about the role of *M. mitochondrii* in its arthropod host. A previous quantitative real time PCR (qPCR) study has shown that bacterial growth is linked to the blood meal (Sassera et al., 2008). It is not clear if this coincides with the production of metabolites by *M. mitochondrii* or if it may reflect competition among symbionts for transmission to the next stage of the tick, although the increase appears to occur in both female and male larvae (Sassera et al., 2008). Other hypotheses about some possible roles include providing help during the host molting, heme detoxification (Sassera et al., 2011) and conferring protection toward different types of pathogens/parasites (Pistone et al., 2011).

1.2.3 *Midichloriaceae* in other tick species

Following the discovery and description of *M. mitochondrii* in *I. ricinus* (Sassera et al., 2006), multiple studies detected closely related bacteria. Screenings of multiple Ixodida species were performed, either searching directly for bacteria closely related to *M. mitochondrii* (Beninati et al., 2009; Epis et al., 2008) or in the context of studies assessing the microbial diversity using universal primers (Loftis et al., 2006; Dergousoff et al., 2011). In addition, other previously described bacteria retrieved from ticks, e.g. *Rickettsiales* bacteria found in *Ixodes persulcatus* ("Montezuma"; Mediannikov et al., 2004) and in *Haemaphysalis wellingtonii* (Parola et al., 2003), have thus been included in the same phylogenetic cluster of *M. mitochondrii* (Epis et al., 2008) and ascribed to the family *Midichloriaceae* (Montagna et al., 2013). A list of midichloriaceae found in ticks is reported in Table 1.

Host		Author
Genus	**Species**	
Amblyomma	*americanum*	Williams-Newkirk et al., 2012
Amblyomma	*tuberculatum*	Epis et al., 2008
Dermacentor	*andersonii*	Dergousoff et al., 2011
Haemaphysalis	*punctata*	Epis et al., 2008
Haemaphysalis	*wellingtoni*	Parola et al., 2003
Hyalomma	*excavatum*	Loftis et al., 2006
Hyalomma	spp. (nymphs)	Loftis et al., 2006
Hyalomma	*marginatum*	Epis et al., 2008
Hyalomma	*truncatum*	Epis et al., 2008
Ixodes	*brunneus*	Goddard et al., 2003
Ixodes	*frontalis*	Palomar et al., 2015
Ixodes	*holocyclus*	Beninati et al., 2009
Ixodes	*ovatus*	Fujita et al., 2007
Ixodes	*persulcatus*	Qiu et al., 2014 Mediannikov et al., 2004
Ixodes	*ricinus*	Beninati et al., 2004
Ixodes	*uriae*	Epis et al., 2008
Rhipicephalus	*bursa*	Epis et al., 2008
Rhipicephalus	*decoloratus*	Najm et al., 2012
Rhipicephalus	*turanicus*	Epis et al., 2008

Table 1 Summary of the *Midichloria* sequences obtained during previous screenings, indicating the tick species analyzed (published in Cafiso et al., 2016)

The overall result is that positive specimens have been detected in species belonging to each of the six most species rich genera (gathering 98% of the species described) of hard ticks (i.e. the genera *Ixodes*, *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Hyalomma* and

Rhipicephalus), while to our knowledge there are no reports indicating the presence of *Midichloria* bacteria in soft ticks (Argasidae).

While the limited number of individuals screened for most species does not allow for a precise prevalence estimate, among species analyzed to date, only the Australian species *I. holocyclus* presents 100% prevalence as in *I. ricinus* (Beninati et al., 2009). An additional study on the microbial community of *I. holocyclus* indicates that the bacterium is not only prevalent, but also extremely abundant in this tick species (Gofton et al., 2015), similarly to what was observed in *I. ricinus*. Nevertheless, the presence of *Midichloria* bacteria was not observed inside mitochondria in *I. holocyclus* (Beninati et al., 2009). Interestingly, a phylogenetic analysis of the *Midichloria* bacteria of multiple tick species did not show signs of co-cladogenesis between the ticks and the symbionts (Epis et al., 2008), suggesting that horizontal transfer could be the main source of diffusion of *Midichloria* among ticks. This is also supported by evidences of the horizontal transmission of *M. mitochondrii* to humans and other animals after *I. ricinus* bite (Bazzocchi et al., 2013; Mariconti et al., 2012b) as illustrated in Paragraph 1.2.4.

1.2.4 Transmission of *Midichloriaceae* from ticks to vertebrate hosts

The presence of *M. mitochondrii* has been detected, in addition, in salivary glands and rostrum of adult females of *I. ricinus* (Mariconti et al., 2012a). This result, together with the association of *M. mitochondrii* with a parasite arthropod (as well as the association of other midichloriaceae with additional parasitic hosts such as multiple tick species, fleas, bed bugs and tabanids; Montagna et al., 2013; Epis et al., 2008) has raised the hypothesis of the possible horizontal transmission and infectivity of *Midichloria* bacteria to vertebrates. Various studies have then demonstrated that *Midichloria* can be directly and indirectly detected in mammalian host. Molecular evidences for the presence of circulating *M. mitochondrii* DNA into the bloodstream have been observed in various vertebrates parasitized by *I. ricinus* (in blood samples from horses, cattle, sheeps and dogs exposed to the risk of tick bite; Bazzocchi et al., 2013) and in roe deer specimens, which are known to be highly parasitized by this tick species (Skarphédinsson et al., 2005). The *16SrRNA* of a *Rickettsiales* bacterium (subsequently added to the *Midichloriaceae* clade) called “Montezuma” was detected in blood from humans parasitized by *I. persulcatus* from Far East of Russia (Mediannikov et al., 2004) that were reported to show acute febrile disease after the tick bite. This bacterium was later

found to be evolutionarily closely related to endosymbionts of other arthropods, such as stinkbugs and fleas, which were indeed collectively included in the new species "*Candidatus* Lariskella arthropodarum" (hereafter *Lariskella arthropodarum*) (Matsuura et al., 2012). The symptoms reported from tick-parasitized patients in this study resembled those of rickettsiosis and anaplasmosis, namely respiratory disorders, left shift of leukocyte formula, increased erythrocyte sedimentation rates, and increased level of serum transaminases (Mediannikov et al., 2004). Despite the patients showed typical ehrlichiosis/tick-borne rickettsiosis symptoms, the performed PCR analyses did not reveal the presence of DNA of the expected tick-borne transmissible agents, including the etiological agents of Lyme disease and babesiosis. The fact that *L. arthropodarum* DNA was found only in 4 out of the 22 patients examined could be due to assay detection limits (for example in the same patient it was found in a biopsy of the area of tick bite but not in the blood). In any case, it was not possible to establish a direct causal link of the organism to the disease. The available data are in part comparable to those on *M. mitochondrii*, as evidence of transmission of molecules to humans from *Midichloriaceae* bacteria after tick bites. The case of *L. arthropodarum* is even more relevant, because disease symptoms were also found. However, the interaction with the vertebrate was less evident, since no specific test for host immune response against *L. arthropodarum*.

Indirect detection of *Midichloria* transmission to vertebrates has been observed by detecting antibodies produced against symbiont antigens. A previous work regarding the genome sequencing of *M. mitochondrii* opened the way for the study of potential antigenic proteins produced by this bacterium (Sassera et al., 2011). *M. mitochondrii* genome sequencing showed, indeed, the presence of 26 genes involved in flagellar synthesis, making it to be the first member of the *Rickettsiales* order presenting a complete set of genes coding for a putative flagellar apparatus (Mariconti et al., 2012a; Sassera et al., 2011). A fragment of the flagellar protein "FliD" (i.e. protein coding for the external cap) was therefore produced in recombinant form and used in a preliminary study as the first marker of the bite of *I. ricinus*, with encouraging preliminary results (positivity of 58.5% in humans exposed to tick bite, 1.18% in the healthy blood donors) (Mariconti et al., 2012b). Another study has reported indirect signs of the presence of *Midichloria* bacteria in sera of dogs exposed to the risk of *I. ricinus* bite through an ELISA test that evaluated the presence of antibodies against the rFliD protein (Bazzocchi et al., 2013). *Midichloria* bacteria can thus be regarded not only as a symbiont of *I. ricinus* and/or other tick species, but also as a potential infectious agent or, at least, as a package of antigens that ticks likely inject into the vertebrate hosts during the blood meal (Bazzocchi et al., 2013). The fate of *Midichloriaceae* in the vertebrate after the tick bite

is indeed still unexplored, but the evidences explained above show that *Midichloriaceae* might play a primary or secondary role in pathologies deriving from the tick bite. Some authors agree in considering the tick bite (and thus the infection by pathogens transmitted by the bite) as a risk factor for the development of various chronic degenerative diseases. Probably the most discussed correlation is the one between tick bite and Amyotrophic Lateral Sclerosis (ALS), for which there are several lines of evidence (Harvey and Martz, 2007; Hänsel et al., 1995; Halperin et al., 1990). Other debated associations are those between tick-borne infections and rheumatologic (Hsieh et al., 2007) and cardiovascular diseases (e.g. forms of dilatative cardiomyopathy; Lelovas et al., 2008). Moreover, as already explained, *L. arthropodarum* is supposed to be the causative agent of a tick-borne disease observed in humans and transmitted through bites of *Ixodes* ticks in Russia (Mediannikov et al., 2004). These hypotheses could be taking into account considering the ability of *M. mitochondrii* to invade mitochondria.

1.3 Involvement of a bacterium of the family *Midichloriaceae* in rainbow trout *Oncorhynchus mykiss* affected by red mark syndrome

Farmed fish is susceptible to multiple diseases whose etiological agents are often unknown. Infectious diseases and parasites could represent a limiting factor for intensive aquaculture, often resulting in big costs for fish farmers (Ghittino et al., 2003). Notably, farmed rainbow trout *Oncorhynchus mykiss* (Walbaum) is affected by different skin pathologies caused by unknown etiologies (for example European warm water strawberry disease, US strawberry disease, red mark syndrome) (Oidtmann et al., 2013). Red mark syndrome (RMS) is a chronic skin disease with unknown etiology. The condition was first detected in Idaho, USA, during the 1950s, and by the 1970s was endemic in some hatcheries in the western and Pacific North-Western regions (Olson et al., 1985; Erickson, 1969). In the USA, the disease is referred to as strawberry disease (SD), which reflects the bright red coloration of the lesions resulting from dilation and congestion of the microvasculature (Olson et al., 1985).

In Europe, lesions resembling those of SD (Figure 4) were detected for the first time in Scotland in 2003 (Verner-Jeffreys et al., 2008), and this disease was referred to as RMS. Since then, it has been spreading in various continental European countries from France to Italy (Verner-Jeffreys et al., 2008; Schmidt-Posthaus et al., 2009; Oidtmann et al., 2013) and also in

Turkey (Kubilay et al., 2014) and Iran (Sasani et al., 2016). The first European RMS outbreak has been thought to start from an imported batch of eggs from the USA (Metselaar, 2012) and it thus plausible to think that SD has been imported to Europe and some possible differences in the pathology could be due to genetic variations in the host response (Metselaar, 2012). Because of their morphological and histopathological similarity, the two conditions are considered substantially the same disease (Ferguson et al., 2006).

Figure 4 Representative red mark syndrome lesion near the pelvic fin.

RMS consists of multiple bright red skin lesions on the fish body, mostly observed on the flanks (Oidtmann et al., 2013), of farmed rainbow trout approaching the market size (above 100 g weight), with no effects on weight or behavior (Lloyd et al., 2008). The disease emerges at water temperatures below 16 °C, but skin lesions tend to resolve spontaneously in 6-8 weeks when left untreated and healing improvement is seen when fish is transferred to warmer water (Ferguson et al., 2006). Some authors reported, in the past, rare cases of skin lesions resembling those of RMS/SD in few individuals of wild brown trout, cutthroat trout and whitefish (Oidtmann et al., 2013), but, thus far, no other cases than those of *O. mykiss* have been reported in wild conditions. In fish farms, RMS has been reported to affect only rainbow trout, even when other species are kept in the same farm (i.e. brown or brook trout; Schmidt-Posthaus et al., 2009).

Histological observations show severe thickness dermatitis, with dermis and hypodermis presenting large infiltration of lymphocytes, heterophils and macrophages; scale resorption is reported with neutrophilic infiltration in scale pockets (Oidtmann et al., 2013; Schmidt-Posthaus et al., 2009; Noguera et al., 2008; Verner-Jeffreys et al. 2008). In comparison to the dermis, the epidermis is largely unaffected or only mildly affected, showing mild epidermal hyperplasia and exocytosis, possibly with some lymphocytic infiltration (Oidtmann et al., 2013). Compared to SD skin lesions, heterophils can be seen within established RMS lesions

in connective tissues, between the dermis and epidermis, and dermis and subcutis (Metselaar et al., 2012). Besides skin, various organs of RMS-affected individuals can be sometimes involved, resulting in acute inflammation of intestinal muscle, exophthalmia, necrotizing myocarditis, splenic congestion, peritonitis and perivascular lymphocyte infiltration (Oidtmann et al., 2013). Grade of lesions can be classified with a scale from 1 to 3: 1) presence of small spots with increased mucus production; 2) lesions appear red in the central area; 3) fully developed and extensive lesions, with the affected area appearing raised and highly inflamed (Metselaar, 2012).

Although mortality level is low, morbidity can reach up to 60% of the fish breeding causing downgrading of the product and subsequently significant economical losses for fish farmers (Metselaar et al., 2010). The value of RMS-affected fish is compromised for both table market and restocking (Oidtmann et al., 2013).

The causative agent for RMS and SD is still unknown, but in many authors' opinion the possible cause could be found in a bacterial agent. Indeed, healing improvement of the pathology has been observed when affected fish are treated with antibiotics such as Oxolinic acid or some molecules belonging to tetracycline family, for example Oxytetracyclyne (Metselaar et al., 2010; personal comment from a DVM working in a rainbow trout farm in Italy) and, in addition, some studies have demonstrated how the pathology can be transmitted from affected to naïve fish (Verner-Jeffreys et al., 2008). Oman reported experimental infection through inoculation with SD lesion homogenate (Oman, 1990). Ferguson suggested a possible association with *Flavobacterium psychrophilum*, but no data confirmed this hypothesis (Ferguson et al., 2006). However, no specific bacterial agents have been unequivocally found as the causative agent of this skin pathology. There is evidence for a correlation between the presence of RMS/SD skin lesions and the detection of the *16SrRNA* gene sequence of a *Rickettsia*-like organism (RLO) (Lloyd et al., 2008). This RLO was then included in a phylogenetic study that showed it to belong to the recently described family *Midichloriaceae*, within the order *Rickettsiales* (Montagna et al., 2013). The bacterium associated to RMS/SD has thus been defined as *Midichloria*-like organism (MLO). Considering the ability of the *I. ricinus* tick symbiont *M. mitochondrii* to invade the mitochondria of its host (Beninati et al., 2004), it is possible that this ability does not exclusively belong to *M. mitochondrii* from *I. ricinus*, and this MLO could be found as the actual cause (or one of the factors) of RMS.

In the absence of definitive information about the etiological agent, disease triggers and routes of transmission, the utilization of good biosecurity procedures is recommended to reduce the

introduction and spread of disease through a site (Adam, 2009). It appears that some sites have been able to eradicate the condition through systematic clearance of livestock, and disinfection or liming of ponds (Rodger, 2008; Verner-Jeffreys et al. 2008). However, the continual emergence of RMS in new countries and the persistent recurrence of the disease on production sites in the UK and in Europe underline the importance of identifying the etiological agent, so that detection and preventive measures can be targeted more effectively.

Although no vector or reservoir of the MLO associated to RMS has been identified until now, it is possible to make some speculations about this topic. In the light of the current phylogenies, two groups of protists seem to be of particular relevance as hosts for *Midichloriaceae*: ciliates (Alveolata) and Acanthamoeba species (Amoebozoa) (Serra et al., 2016). This let us hypothesize the possible involvement of an aquatic eukaryotic single-celled vector/reservoir for the midichloriacea associated to RMS. There are various examples of protists hosting *Midichloriaceae.* A well defined and rich clade of midichloriaceae associated to ciliates is the one with *Ca*. Bandiella woodruffii (endosymbiont of the ciliate *Euplotes woodruffi*; Senra et al., 2016). The other described endosymbionts of ciliates include the genus *Lyticum* in *Paramecium* sp. (formally two species *Lyticum sinuosum* and *L. flagellatum*, though over 99% similarity for the *16S rRNA*; Boscaro et al., 2013b). Others are *Ca*. Anadelfobacter veles found in *Euplotes harpa* (Vannini et al., 2010), the two species of *Ca*. Cyrtobacter in *Euplotes aediculatus* and *E. harpa* (Boscaro et al., 2013a), *Ca*. Defluviella procrastinata in *Paramecium nephridiatum* (Boscaro et al., 2013a) and a long time known endosymbiont of *Paramecium* sp., which has been only recently affiliated to the family thanks to molecular characterization and now called *Ca*. Fokinia solitaria (Szokoli et al., 2016). For all of these (except *Lyticum*) not much data other than *16SrRNA* gene sequence and intracellular localization in the host by FISH is available. Concerning amoebas, there are two endosymbionts, which are the first representatives of *Midichloriaceae* characterized by *16SrRNA gene* and microscopy (formally belonging to the same species, over 99% similarity). *Acanthamoeba* sp. (strain UWC36) symbiont, which was recently described as *Ca.* Jidaibacter acanthamoeba, is the sister strain to the endosymbiont of *Acanthamoeba* sp. UWC8 and colonizes host-derived vacuoles (Schultz et al., 2015; Fritsche et al., 1999). The two endosymbionts were independently sequenced, though one in a draft form, and represent, together with *M. mitochondrii*, the only three complete (or almost) genome sequences produced from the family *Midichloriaceae*. While the ciliate hosts cited above have not been described as harmful for vertebrates, pathogenic *Acanthamoeba* sp. strains UWC8 and

UWC36 (containing their endosymbionts) were retrieved from infected human corneal tissues (Schultz et al., 2015; Fritsche et al., 1999).

Considering that breeding water in fish farms is derived from stream water, it is possible that the MLO associated to RMS skin lesion could be brought to the fish ponds and harbored by some protist inhabiting the incoming waters. This eukaryotic cell could serve as simple vector for the bacteria or as a reservoir of the disease.

2 Purpose of the PhD project

The phylogenetic analysis of the family *Midichloriaceae* associated with different eukaryotic hosts clearly indicates that they were repeatedly able to move and become adapted from one host to another (Senra et al. 2016; Schulz et al. 2015). Anyway, little is known about the relationship of these bacteria with their eukaryotic hosts. For example, the biology of *M. mitochondrii* in *I. ricinus* has yet to be explored. It is not clear if the presence of this bacterium is beneficial to the tick host, how ticks can cope the invasion of its mitochondria and how the fitness of individuals can be modified after the removal of the symbiont.

Moreover, several lines of evidence convincingly indicate that the family *Midichloriaceae* includes agents transmissible to humans and other vertebrates, and possibly involved in the development of pathological changes. These data obviously need to be better investigated in order to evaluate the effective propagation and diffusion of these bacteria inside vertebrates and the possible pathogenic effects, also considering that a formal proof of their dependency is lacking. For example, human or animal diseases suspected to be linked with tick bites could be re-interpreted and further investigated, in particular those clinical cases compatible with rickettsiosis or ehrlichiosis, but characterized by the absence of antibody titers for known pathogenic *Rickettsiales*, similar to the case of Montezuma/*L. arthropodarum* observed in Russia (Mediannikov et al., 2004). *Midichloria* bacteria are involved in modulating the host immune response after the tick bite, as humans and other animals infested by *I. ricinus* have been found seropositive to FliD protein of *M. mitochondrii* (Bazzocchi et al., 2013; Mariconti et al., 2012b). No information is available about the seroconversion time and antibody response kinetic towards antigens belonging to *M. mitochondrii*, as well as there is no information about the possible replication of *M. mitochondrii* inside the vertebrate host.

Aside from the aspects related to ticks, a representative of the family *Midichloriaceae* has been found strictly associated to RMS skin lesions in *O. mykiss*. This point needs to be deeper investigated in order to define if this MLO represents an opportunistic or commensal microorganism or if it represents the actual etiological agent of the disease. Considering the presence of several *Midichloriaceae* in aquatic hosts, in particular different ciliates (Senra et al., 2016; Szokoli et al., 2016; Boscaro et al., 2013a,b; Vannini et al., 2010), and parasitic *Acanthamoeba* sp. strains UWC8/UWC36 (Schultz et al., 2015; Fritsche et al., 1999), protists could be involved as reservoirs of bacteria infectious for aquatic invertebrates and vertebrates, especially in aquaculture.

The principal aims of my PhD project are:

1) to investigate the relationship between the MLO and RMS. At this purpose, a novel qPCR (quantitative PCR) based approach is used for absolute quantification of the MLO associated with RMS in *O. mykiss*, possibly detecting the presence of this MLO in organs of affected fish. Moreover, this qPCR approach is able to highlight which tissue involved in skin lesion (epidermis/derma or underlying muscle) presents the highest bacterial load. In addition the presence of the MLO in entering/exiting farming water will be investigated;
2) to investigate how an antibiotic treatment on *I. ricinus* individuals can act in reducing/eliminating the symbiont *M. mitochondrii* and to potentially observe the resulting dynamic of bacterial infection in the tick host;
3) to expand the knowledge about the distribution of *Midichloria*-related bacteria in ticks;
4) to study the time and the kinetic of seroconversion against antigens of *M. mitochondrii* (rFliD protein and salivary glands crude protein extract) in a model organism (rabbit).

3 Performed researches

3.1 Molecular evidence for a bacterium of the family *Midichloriaceae* (order *Rickettsiales*) in skin and organs of the rainbow trout *Oncorhynchus mykiss* (Walbaum) affected by red mark syndrome

Authors: Cafiso, A., Sassera, D., Serra, V., Bandi, C., McCarthy, U., and Bazzocchi, C.

Paper published in: Journal of Fish Diseases (2016) Apr, 39(4): 497-501.

3.1.1 AIM OF THE STUDY

The *16S rRNA* gene sequence of a RLO (and now ascribed to the family *Midichloriaceae*) has been found in association with skin lesions of RMS-/SD-affected specimens (Metselaar et al., 2012; Lloyd et al., 2011) and is thought to be, at the moment, the most plausible etiological agent of this skin disease. A novel specific method for absolute quantification of the MLO associated with RMS in *O. mykiss,* based on a quantitative Sybr-green real time PCR approach (qPCR), is here presented. Furthermore, the second aim of the work is to detect the presence of this MLO in organs (heart, liver, spleen, intestine, kidney) of affected fish.

3.1.2 MATERIALS AND METHODS

In order to investigate the presence of the MLO in *O. mykiss*, we collected a set of samples from three farm sites (A, B and C) located in Scotland. The sites are operated independently and are well separated geographically, without any transfer of eggs, fish or equipment. A and B are pond sites while C is a loch site. Samples were: i) a fragment of skin lesion from one individual from site A strongly affected by RMS and used as positive control (F1); ii) sections of tissues from two RMS positive individuals (F2 and F3) from site A with different severity in lesions; iii) skin and organ fragments from one *O. mykiss* individual from a RMS free site (named D), used as negative control (F4); iv) skin (healthy and with lesion, when present) and organ samples from six individuals from site B (F5-F10), four of which were visibly affected by RMS (F7-F10); v) skin (healthy and with lesion, when present) and organ samples from four individuals from farm C (F11-F14), three of which were visibly affected by RMS (F12-F14). See Table 1 for a description of all skin and organ samples. Skin and organ samples

from fish F2 and F3 were fixed in formalin for 24 h. Following routine processing for histology, 3 µm sections were placed on Polysine slides and dried overnight at 45°C before use. The severity of lesions and the health of the skin were evaluated after hematoxylin and eosin staining of histological sections (Fig. 1).

3.1.3 RESULTS AND DISCUSSION

In normal rainbow trout skin, the stratum compactum of the dermis was seen as a dense layer of collagen fibers underlying the stratum spongiosum (Fig. 1b). Early lesions appeared as lymphocytic infiltration in the stratum spongiosum immediately surrounding the scale pockets, becoming more pronounced as the lesion progressed, with infiltration directly below the dermis and spreading into the stratum compactum (Fig. 1c). In more advanced lesions, infiltration was seen throughout the dermis and sometimes extending into the epidermis (Fig. 1f). In some fish sampled during the summer, evidence of infiltration was apparent even in skin which appeared grossly normal (Fig. 1e).

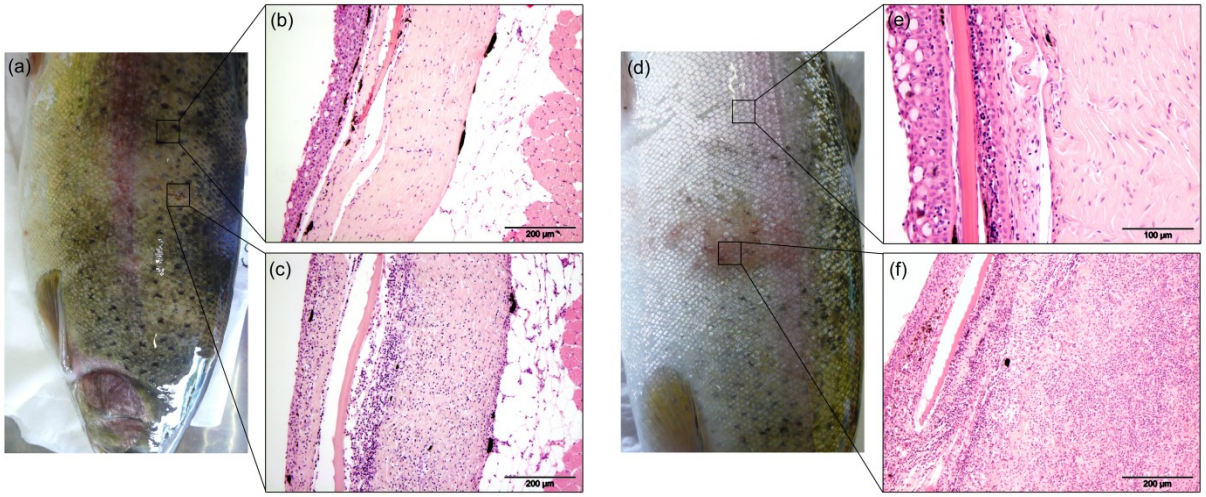

Fig. 1 (a) Fish 2, early stage lesion, gross appearance; (b) Fish 2 section of normal skin (bar=200 µm); (c) F2 section of skin lesion (bar=200 µm); (d) F3 mid stage lesion, gross appearance; (e) F3 section of grossly normal skin (bar=100 µm); (f) F3 section of skin lesion (bar=200 µm). All sections are stained with haematoxylin and eosin.

DNA from fresh samples (F1 and F4-F14) was extracted using the DNeasy Blood and Tissue kit (Qiagen). DNA from glass slides (F2 and F3) was extracted using the QIAmp DNA Investigator kit (Qiagen). A previously obtained alignment of *Midichloriaceae 16SrDNA*

(Montagna *et al.* 2013) was used to design a specific primer set to amplify the *16S rDNA* of the MLO previously detected in lesions of RMS/SD affected fish, targeting a variable region of the gene (16SrDNA-F: GCGGTTATCTGGGCAGTC and 16SrDNA-R: TGCGACACGAAACCTAAG; amplification size: 127 bp). Qualitative PCR was performed (95 °C for 15 s and 60 °C for 30 s for 40 times; final primers concentration: 250nM) on F1 to evaluate the specificity of the amplification. PCR product was purified and cloned into the pGEM®-T Easy Vector (Promega). Ten resulting clones were purified and sequenced confirming the specificity of the amplification. A fragment of the *O. mykiss* insulin growth factor I (*igf1*) gene was amplified as described by Lloyd et al. (2011). This PCR product was cloned into the pGEM®-T Easy Vector also. One clone for each target (*16S rDNA* and *igf1*) was used as standard for setting up qPCR reactions. Plasmids containing the target genes were serially diluted from 10^9 copies μl^{-1} to 1 copy μl^{-1} to evaluate the efficiency (*16S rDNA*: 105%; *igf1*: 101%) and the detection limit of each PCR protocol (10 copies in both cases). PCR conditions for both genes were: 95 °C for 2 m, 40 cycles at 95 °C for 15 s and at 60 °C for 30 s, melt curve from 55 °C to 95 °C with increments of 0.5 °C per cycle; final primers concentration: 250nM. qPCRs were performed on each DNA sample in triplicate. Number of MLO *16S rDNA* and host *igf1* gene copies were obtained through a comparison of the qPCR results of each sample with those of serial dilutions of purified plasmid (containing known copy numbers). Melting curves showed the presence of specific amplified fragments belonging to the target sequences confirming the specificity of the method. Results were expressed as the ratio of *16S rDNA*/*igf1* x 1000.

This method was set up on F1 and validated on fixed tissue sections from early stage lesion (F2) and advanced stage lesion (F3). F2 and F3 skin lesions were positive for the presence of MLO bacteria as shown in Fig. 2. No detectable presence of MLO was observed in F2 and F3 healthy skin and in organ samples from F4 (negative control). Moreover, the amount of bacteria detected in the F3 skin lesion was higher than in F2 in accordance with histological results that showed a different severity of lesions in the two fish. All analysed organs from both F2 and F3 were positive, with an amount of bacteria higher in spleen and liver with respect to kidney, heart and intestine (Fig 2).

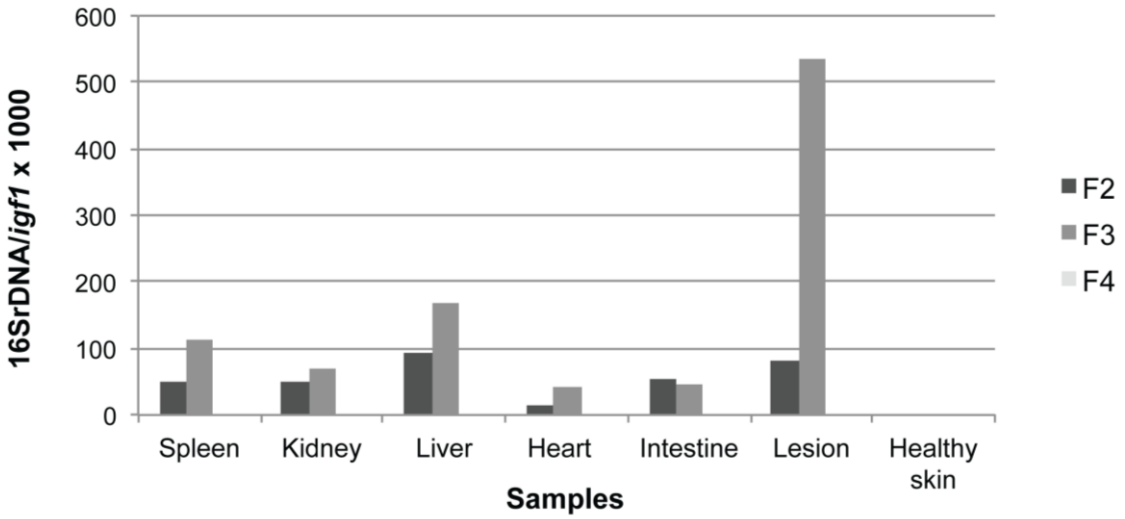

Fig. 2 MLO quantity expressed as *16S rDNA*/*igf1* x 1000 in organs (spleen, kidney, liver, heart, intestine), healthy skin and lesion from F2 (with an early skin lesion) and F3 (with a mid-stage skin lesion) and in organs and skin from a RMS-free control (F4).

The qPCR method was then tested on healthy skin, on lesions when present, and on organ samples from ten fish coming from two different farms. Table 1 shows the qPCR results expressed as *16S rDNA*/*igf1* x 1000. qPCR on samples from fish F5, F6 and F11, considered healthy at initial inspection, did not detect presence of MLO. In 57% of pathological samples from sites B and C (4/7), spleen was positive for the presence of MLO and in 43% of affected individuals (3/7) liver was positive. Kidney did not show any positivity for the presence of MLO in any analysed fish. Skin lesions were positive in all the affected fish while 28% skin samples (2/7) with no visible lesions (healthy skin) of pathological fish were positive. See Table 1 for all qPCR results.

			16SrDNA/igf1 x 1000						
Farm	**Sample**	**RMS signs**	**Healthy skin**	**Lesion**	**Liver**	**Spleen**	**Kidney**	**Intestine**	**Heart**
Site A	F1	++	0	7.85×10^{-1}	NA	NA	NA	NA	NA
	F2	+	0	8.46×10^{1}	9.40×10^{1}	5.28×10^{1}	5.15×10^{1}	5.53×10^{1}	1.59×10^{1}
	F3	++	0	5.73×10^{2}	1.71×10^{2}	1.16×10^{2}	7.19×10^{1}	4.80×10^{1}	4.25×10^{1}
Site D	F4	-	0	healthy	0	0	0	0	0
Site B	F5	-	0	healthy	0	0	0	NA	NA
	F6	-	0	healthy	0	0	0	NA	NA
	F7	+	0	1.29×10^{-1}	0	3.92×10^{-3}	0	NA	NA
	F8	+	0	5.72×10^{-2}	0	0	0	NA	NA
	F9	+	0	1.54×10^{-1}	0	4.55×10^{-2}	0	NA	NA
	F10	+	3.93×10^{-2}	3.40×10^{-1}	1.85×10^{-2}	1.31×10^{-2}	0	NA	NA
Site C	F11	-	0	healthy	0	0	NA	NA	NA
	F12	+	0	1.82	7.46×10^{-3}	2.63×10^{-2}	NA	NA	NA
	F13	+	0	7.10×10^{-3}	0	0	NA	NA	NA
	F14	+	1.50×10^{-1}	1.63×10^{-1}	2.72×10^{-3}	0	NA	NA	NA

Table 1 Analyzed fish samples and presence/absence of MLO DNA based on 16SrDNA/*igf1*x1000 ratio. NA - not available; NLD - no lesion detected; * samples from microscope slides.

We were not able to find a correlation in single fish, or among the samples, between the quantity of bacteria present in the lesions and the quantity detected in positive organs. This result may indicate that distribution of the MLO may not be homogeneous in the fish, or that the dynamics of the infection in the various organs are not simultaneous. The negativity to MLO of kidneys obtained from fresh samples could thus be attributed either to the above explanation or to the sensitivity of the qPCR method (10 copies μl^{-1} of *16SrDNA* gene). Additional studies, focused on monitoring the different stages of the pathology, could give more clues on these issues. Positive results in apparently healthy skin, from diseased fish, could suggest a possible spread of the pathology and could represent the beginning of the infection in the analysed skin section.

Our work shows that the MLO is not only localized in skin lesions of RMS affected fish, but can invade various organs, and can be detected in skin sections that do not present pathological alterations. As spleen and liver in both samples showed the highest *16S rDNA*/*igf1* ratio values, this can lead to the hypothesis that melano-macrophages in spleen and liver could be primarily involved in the elimination of the MLO. Even though our data show a molecular evidence to support an association of this bacterium with RMS, further studies are needed to prove if this bacterium is actually involved in the pathology.

3.2 Antibiotic treatment of the hard tick *Ixodes ricinus*: influence on *Midichloria mitochondrii* following blood meal

Authors: Ninio, C., Plantard, O., Serra, V., Pollera, C., Ferrari, N., Cafiso, A., Sassera, D., and Bazzocchi, C.

Paper published in: Ticks and Tick-borne Diseases (2015) Jul, 6(5): 653-7.

3.2.1 AIM OF THE STUDY

M. mitochondrii can be transmitted to the vertebrate host during the tick bite; this has been proven by both direct and indirect lines of evidence: detection of bacterial DNA in the vertebrate blood (Bazzocchi et al., 2013) and detection of antibodies against *M. mitochondrii* antigens in the vertebrate host (Bazzocchi et al., 2013; Mariconti et al., 2012b). These results are coherent with the evidence for the presence of *M. mitochondrii* in the salivary glands of adult females of *I. ricinus* (Mariconti et al., 2012a). The finding of high loads of *M. mitochondrii* in tick females (Sassera et al., 2008), their location in the ovaries and the phylogenetic relatedness of *M. mitochondrii* to *Wolbachia*, a well-known manipulator of arthropods reproduction (Werren, 1997), lead to the question of whether the physiology and the reproduction of the arthropod is influenced by the presence of these bacteria. Administration of antibiotics has been a useful approach for investigating the role of microbial symbionts associated with invertebrate hosts (Oliver et al., 2010). An example is the use of tetracycline, which is effective against *Rickettsiales*, to elucidate the mutualistic role of *Wolbachia* endosymbionts toward its host *Dirofilaria immitis* (Bandi et al., 1999). Bacterial decrease/removal after antibiotic treatment can have different effects on the invertebrate hosts. In order to test whether antibiotic treatment could influence the numbers of *M. mitochondrii* bacteria in the tick progeny and their dynamic within the invertebrate host, we administered tetracycline both to adult ticks by microinjection and to gerbils used for larvae blood meal by adding the antibiotic to the drink water. We then monitored the amount of *M. mitochondrii* bacteria in adult ticks and their progeny by qPCR.

3.2.2 MATERIALS AND METHODS

Tick rearing and antibiotic treatment

Five fully engorged *I. ricinus* females were collected on roe-deer in Gardouch Research Station (south-west of France; 43°22'15.11"N, 1°40'25.89"E) the 13th of October 2011 (n=3) and in the Chizé Forest (western France; 46° 7'18.89"N, 0°25'3.72"O) the 1st of March 2012 (n=2). Two of the five ticks were microinjected with tetracycline, while the other three females were used as controls. To conduct the antibiotic injection, a Nanojet II™ Injector (Drummond) was used under a dissecting microscope. A hole in the tick tegument was first produced between the coxae of the first and the second pair of legs using a minutien pin (0,10 mm in diameter; Entomoravia Austerlitz Insect Pins®, Czech Republic) mounted on a mandrel. Subsequently a stretched glass capillary (nine cm in length) was inserted in this hole of the tegument and a single dose of tetracycline (15ng/mg of ticks) diluted in PBS 1x was injected. The five ticks were then kept at 20°C, 80% humidity and 6h/18h photo-period conditions to allow oviposition and subsequent egg hatching.

Fifteen unengorged larvae from each of the five mothers were collected immediately after egg hatching and conserved at -80°C for subsequent molecular analysis. The remaining larvae from each of the five ticks were divided into two groups, for a total of ten larvae groups. Larvae groups were positioned each on one gerbil (ten gerbils in total) to allow blood feeding. One group of larvae from each of the five starting adult ticks was allowed to feed on a control gerbil, while the other group of larvae from each tick was allowed to feed on a gerbil treated with the antibiotic tetracycline. Gerbils treatment was performed in two steps. Firstly, 10mg/kg of antibiotic was administered to each of the five gerbils by force feeding each day during the two days preceding the deposit of larvae. Secondly, after the deposit of larvae, tetracycline 1.2% (w/v) was administered to the five gerbils through drinking water for 6 days. This second administration method was chosen after deposit of larvae to prevent the manipulation of gerbils and its possible negative effect on larval engorgement (squashing of engorged ticks during manipulation). After completion of the blood meal, larvae were collected in the water located below the metallic mesh harboring the gerbils. Ten engorged larvae from each group were stored at -80°C for subsequent molecular analysis. The remaining larvae were allowed to moult to nymphs at the following conditions: 20°C, 80% humidity and 6h/18h photoperiod. Ten unengorged nymphs from each group were collected

and conserved at -80°C for subsequent molecular analysis. Details on this experiments are summarized in Table 1.

Adult *I. ricinus*	Number of larvae	Gerbil treatment	No. of nymphs
Tick 1 (C)	553	C	427
	456	T	344
Tick 2 (C)	353	C	190
	270	T	269
Tick 3 (C)	372	C	294
	423	T	309
Tick 4 (T)	432	C	389
	298	T	210
Tick 5 (T)	159	C	113
	233	T	213

Table 1 Adult *Ixodes ricinus* females, number of obtained larvae fed on gerbils and amount of obtained nymphs. C: Control (not treated); T: treated with tetracycline

Blood sampling

Fifty microliters of blood from control and treated gerbils were collected from the tip of the gerbil's tail before and during the engorgement of larvae of *I. ricinus* and stored at -80°C until analysis. In detail, blood samples were collected at the following time points: before the attachment, at one, two, four, and six days after the attachment.

Molecular studies

DNA was extracted from larvae, nymphs and blood samples using the DNeasy Blood and Tissue Kit (Qiagen) following manufacturer instructions. DNAs were eluted in 50 microliters of sterile water and stored at -20°C until molecular analysis.

Seventy five unengorged larvae (15 larvae from each of the five adult females), 100 engorged larvae (50 larvae after the blood meal on control gerbils, 50 larvae after the blood meal on gerbils treated with tetracycline) and 100 unengorged nymphs (50 nymphs derived from larvae that performed blood meal on control gerbils, 50 nymphs derived from larvae that performed blood meal on treated gerbils) were analyzed for quantification of *M. mitochondrii* using a previously described Sybr green real-time PCR approach (Sassera et al., 2008). Briefly, a fragment of the *M. mitochondrii gyrB* gene (coding for the protein gyrase B) and a fragment of the *I. ricinus* nuclear gene *cal* (coding for the protein calreticulin) were cloned

into a pGEM-T easy plasmid vector (Promega corporation) to produce standards for absolute quantification. PCR efficiency was assessed by serial dilutions of purified plasmid DNA starting from 10^9 copies/reaction to 10 copies/reaction. Number of symbiont and nuclear gene copies were obtained through a comparison of the real-time PCR results of each sample with those of serial dilutions of purified plasmid (containing known copy numbers).

DNA extracted from blood samples (n=50) were analyzed for the presence of circulating *M. mitochondrii* DNA, using a previously described PCR protocol, with primers designed on the gene coding for the 16S ribosomal RNA (*16SrDNA*) (Epis et al., 2008). All DNA samples were also examined using universal mammalian primers, targeted on the 12S rRNA gene (Wang et al., 2000), for DNA quality control.

Statistical analysis

The amount of *M. mitochondrii* in larvae and nymphs was estimated through the ratio between *gyrB* and *cal* copy numbers.

To test whether the variability of *M. mitochondrii* infection of the whole sample of larvae was different in respect to the variability between larvae derived from single tick mothers, the *gyrB*/*cal* ratio was analyzed with General Linear Model (GLM) considering *gyrB*/*cal* as response variable and tick mother code as explanatory variable.

Through Generalised Linear Mixed Model (GLMM) we analyzed factors affecting *M. mitochondrii* amount in larvae and nymphs considering as explanatory variables treatment in adult females, treatment of feeding gerbil and blood meal (i.e. engorged versus unengorged). The influence of treatment in adult females was assessed by comparing larvae derived from microinjected versus control ticks. The influence of antibiotic treatment of gerbils was evaluated by comparing ticks fed on gerbils treated with tetracycline versus controls gerbils, while the effect of blood meal was assessed comparing unengorged versus engorged groups of larvae. To account for non independence of the observation on ticks derived from the same mother and fed on the same gerbil, the identification codes of mother tick and feeding gerbil were considered as random factors (Pinhero and Bates, 2009).

To address the following issues these models have been considered: 1) the influence of microinjection of adult females in unengorged larvae was assessed considering female treatment as explanatory variable; 2) the influence of microinjection of adult females and antibiotic treatment of gerbil in engorged larvae was determined considering female treatment

and gerbil treatment as explanatory variables; 3) the influence of microinjection of adult females and blood meal in engorged larvae was determined considering female treatment and blood meal as explanatory variables; 4) the influence of microinjection of adult females and antibiotic treatment of gerbil in unengorged nymphs was determined considering female treatment and gerbil treatment as explanatory variables.

All models were fitted considering the second order interaction between explanatory variables. As the ratio *gyrB*/*cal* showed an aggregate distribution, in all models log *(gyrB/ca*l+0.001) was used as response variable and models were fitted using gaussian error distribution with graphical residual analysis in order to check the fit of the model.

All analyses were performed using the software GenStat 12th Edition (VSN International 2000-14) considering as significance threshold $p<0.05$.

3.2.3 RESULTS

Absence of *M. mitochondrii* in blood samples

A total of 50 gerbil blood samples were analyzed using a PCR approach. The quality of the extracted DNAs was assessed using universal mammalian primers targeted on the mitochondrial *12S rRNA* gene that led to positive amplification from all samples. The presence of circulating *M. mitochondrii* DNA was tested using a previously described PCR protocol targeted on the *16S rRNA* gene *of M. mitochondrii* (Epis et al., 2008). This non-quantitative PCR protocol was chosen due to its very high robustness and capacity to amplify symbiont DNA from blood samples. The real-time PCR protocol used to quantify symbiont load in tick samples (see materials and methods) does not possess these characteristics, and it is therefore not appropriate for DNA extracted from vertebrate blood. All gerbil blood samples were negative for the presence of circulating *M. mitochondrii* DNA at each analyzed time points (i.e. blood samples collected both before and after larvae feeding).

Quantity of *M. mitochondrii* in unengorged larvae

In order to obtain absolute quantification of *M. mitochondrii* using a real-time PCR approach, purified plasmids containing the *gyrB* and *cal* gene fragments were serially diluted to evaluate the efficiency (104% for *gyrB* and 100,2% for *cal*) and detection limit (10 copies in each case) of each PCR protocol. Threshold cycle values were manually set at 200 for both protocols.

The amount of bacteria was normalized on the amount of tick DNA and data were expressed as ratio of *gyrB/cal* copy number x 1000. PCRs were then performed in triplicate on the DNA samples of 15 larvae from each of the five starting adult females (i.e. a total of 75 unengorged larvae analyzed individually).

Results showed that there was a considerable variability in the load of *M. mitochondrii* bacteria in the 75 analyzed larvae with *gyrB/cal* ratios spanning from 0 to 0.611. Moreover *gyrB/cal* ratios distributions were strongly skewed with 84% of samples showing values below the mean *gyrB/cal,* corresponding to 0.022 (Figure 1).

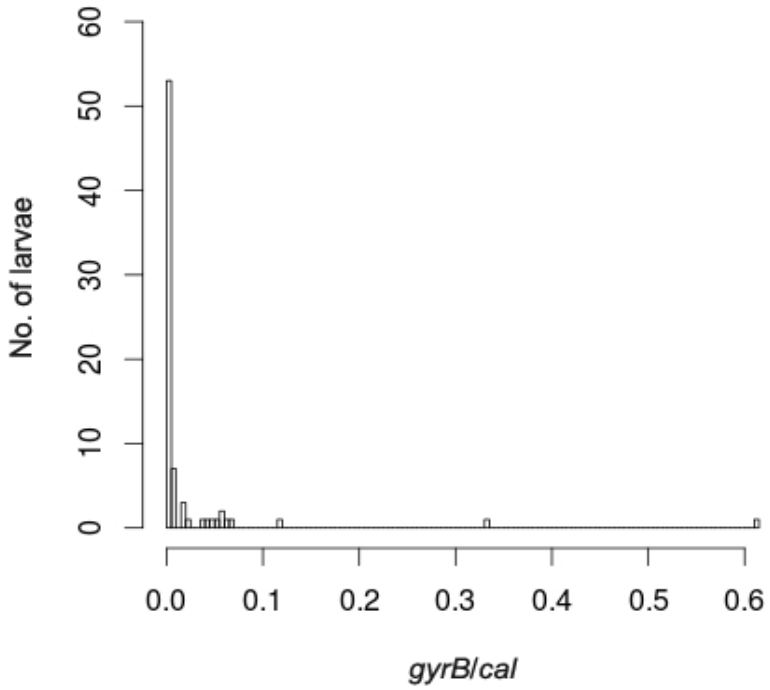

Figure 1 Frequency distribution of the *gyrB*/*cal* ratio in the analyzed unengorged larvae.

Tetracycline microinjection of adult female ticks did not have a statistically significant effect on the amount of bacteria present in their offspring. In fact, based on the results obtained, no difference was found (Wald = 0.04, df = 1, p = 0.847) between the amount of *M. mitochondrii* present in larvae derived from ticks microinjected with tetracycline and those derived from the three females used as control. This variability among the larvae born from different

mothers was higher than that observed between the 15 larvae derived from each single mother tick (deviance = 180.6; gl: = 1, p value<0.004).

Quantity of *M. mitochondrii* in engorged larvae after blood meal on gerbils

The load of *M. mitochondrii* was analyzed also in 100 engorged larvae using the real-time PCR protocol described above. No difference in the amount of *M. mitochondrii* was detected between larvae that were fed on control gerbils and gerbils treated with tetracycline nor between larvae derived from microinjected females compared with larvae from control females (Table 2).

Factor	Wald	g.l.	P
Female treatment	0.79	1	0.374
Gerbil treatment	0.40	1	0.528
Female treatment : gerbil treatment	0.63	1	0.427

Table 2 Model results of the effect of treatment on females (microjection vs. control) and treatment of gerbil on *M. mitochondrii* abundance in engorged larvae.

Starting from the above result, larvae after the engorgement on control and treated gerbils were analyzed as a single group for subsequent analysis. A significant increase in the amount of *M. mitochondrii* in larvae after the blood meal was detected (Table 3; Figure 2), confirming data published previously (Sassera et al., 2008). Interestingly, the statistical analysis showed that the increase in the amount of *M. mitochondrii* from unengorged to engorged larvae derived from untreated ticks was higher than the increase observed for larvae derived from microinjected ticks (P<0.001, Table 3 and Figure 2).

Factor	Wald	g.l.	P
Female treatment	0.60	1	0.438
Blood meal status	1156.076	1	<0.001
Blood meal status: female treatment	19.30	1	<0.001

Table 3 Model results of the effect of blood meal on the larvae deriving from microinjected vs. control tick on their *M. mitochondrii* abundance.

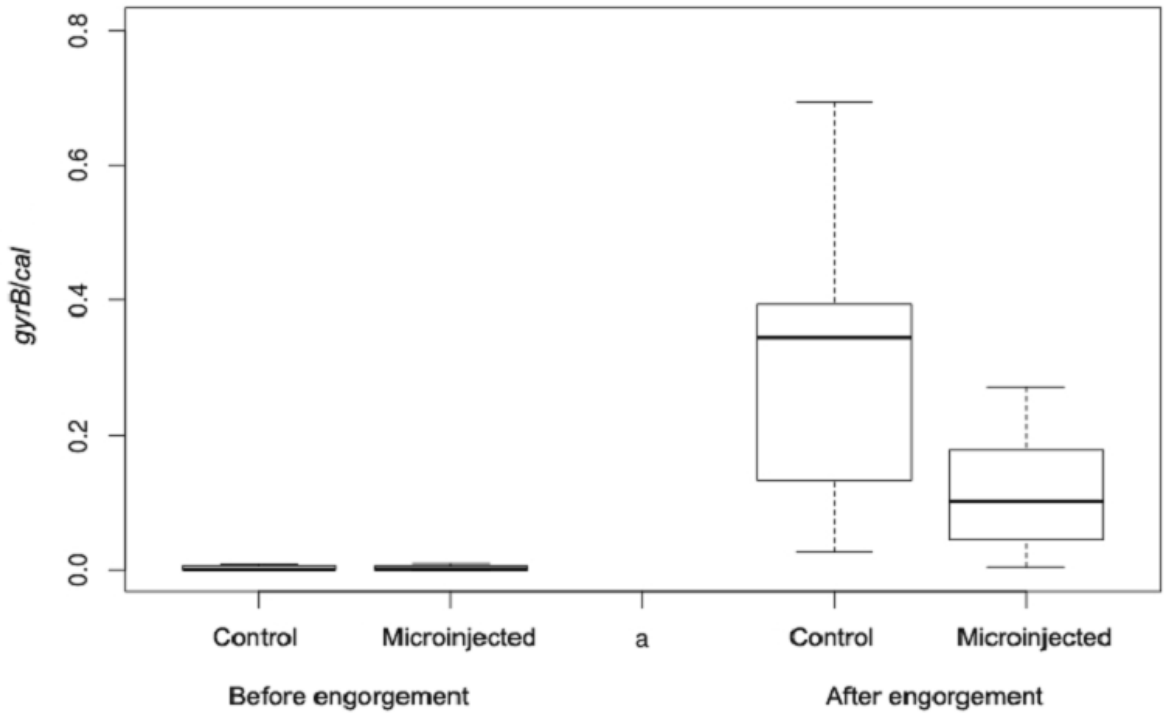

Figure 2 Effect of blood meal on *gyrB*/*cal* ratio in larvae derived from control and microinjected ticks.

Quantity of *M. mitochondrii* in unengorged nymphs from larvae fed on gerbils

Results of qPCR on nymphs derived from control and microinjected ticks that were fed on control or treated gerbils showed no statistically significant variability in the amount of *M. mitochondrii* among the nymphs derived from the two ticks groups (Table 4). Additionally, no variability was detected in the amount of bacteria in nymphs derived from larvae that were fed on treated and untreated gerbils (Table 4).

Factor	Wald	g.l.	P
Female treatment	0.54	1	0.464
Gerbil treatment	0.21	1	0.649
Female treatment : gerbil treatment	1.38	1	0.240

Table 4 Model results of the effect of treatment on mother tick (microinjected vs. control) and gerbil treatment on *M. mitochondrii* abundance in unengorged nymphs.

3.2.4 DISCUSSION

In this work we present the results of tetracycline treatment on adults and larvae of the hard tick *I. ricinus*. Adult engorged females were microinjected with the antibiotic, while larvae were allowed to feed on treated gerbils. The effects of the treatments on ticks and on the endosymbiont *M. mitochondrii* were investigated.

PCR analysis of gerbil blood showed no presence of *M. mitochondrii* DNA. These results seem in contrast with a previously presented report (Bazzocchi et al., 2013), in which the presence of *M. mitochondrii* DNA in the blood of dogs, sheep and horses exposed at the risk of tick bite was documented. However the present experiment analyzes the results of infestation from *I. ricinus* larvae, while the animals from the previous study were mostly infested by adults, as is the case for larger mammals in the field (Manilla, 1998). We could thus hypothesize that larvae, unlike adults of *I. ricinus,* are not able to transmit *M. mitochondrii* through the blood meal. It must be noted in fact that a previous report shows the presence of this symbiont in the salivary glands of adult *I. ricinus* (Mariconti et al., 2012a); however, the available data regarding the localization of the *M. mitochondrii* population in the organs of immature stages of *I. ricinus* suggests the presence only in the primordia of the genital apparatus (Epis et al., 2013). The amount of *M. mitochondrii* found in larvae is known to be lower than the load of nymphs or adult females (Sassera et al., 2008). An alternative hypothesis could be that larvae can transmit *M. mitochondrii* but in quantity that are insufficient to be detected by our method. However, the PCR protocol used in this study has proven to be sensitive enough to detect positivity in the blood of large mammals parasitized by small numbers of ticks (Bazzocchi et al., 2013). Thus, considering the limited amount of blood in a gerbil (approximately 70 ml) and the attachment of fifteen larvae on each gerbil, the first hypothesis seems more likely.

No statistically significant effect on the load of *M. mitochondrii* was achieved with tetracycline microinjection of adults. Different hypotheses could explain this result: 1) the antibiotic tetracycline, regardless of its proven effect on closely related bacteria (e.g. *Wolbachia*, also belonging to the family Anaplasmataceae; Bandi et al., 1999), may not produce any effect on *M. mitochondrii*; 2) the dose of microinjected tetracycline was not sufficient for the removal of bacteria; 3) microinjection of ticks at the end of the blood meal (when the embryogenesis has already begun) could be too tardive, and thus not able to affect the symbiont population and in turn to influence the amount of bacteria transferred to the

eggs. Additional experiments using different antibiotics, different concentrations and different treatment time-lines are now necessary to better elucidate this issue.

The *M. mitochondrii* load in the analyzed larvae was highly variable both within and among the progenies of different ticks. This result could be explained considering that adult females present highly variable loads of *M. mitochondrii* (Sassera et al., 2008) and consequently we could hypothesize that they transmit different amounts of symbionts to their offspring. Moreover, the variability observed within the progeny of each tick indicates a non-uniform distribution of transmission of bacteria from the mother to the eggs. Such a distribution is coherent with the results of previous reports of variable levels of transovarial transmission of another tick-borne bacterium, *B. burgdorferi* (Rollend et al., 2013).
It is notable that the amount of bacteria in unengorged nymphs shows a decrease compared to engorged larvae. Such a decrease between engorged larvae and non-engorged nymphs was already observed (Sassera et al., 2008) suggesting that the amount of *M. mitochondrii* increases after the blood meal but is reduced following the molt of larvae. In our experiment, this reduction erased the difference observed between engorged larvae having received antibiotics versus control.

Furthermore 57% of nymphs, equally distributed among treated and untreated, were found devoid of *M. mitochondrii* bacteria. As adult males have been previously shown to exhibit low prevalence and reduced symbiont load (Sassera et al., 2008), it is reasonable to hypothesize that at least some individual nymphs devoid of *M. mitochondrii* are male nymphs. Unfortunately, currently no molecular method is available to determine the sex of nymphs. This uncertainty about the origin of *M. mitochondrii*-free individuals (i e. resulting from antibiotic treatment *versus* male nymphs) will reduce our ability to draw conclusions concerning the effect of antibiotic on *Midichloria*.

The tetracycline treatments that we conducted did not remove the symbiont population, neither in larvae derived from microinjected females, nor in engorged larvae fed on treated gerbils. However, we detected a significant effect on the multiplication of *M. mitochondrii* symbiont population in the progeny of microinjected females. However, this effect is not immediate, but is actually delayed to the larval stage of the following generation, where a more limited increase was observed after the blood meal. This effect can be explained if we consider that the mechanism of action of tetracycline (i.e. inhibition of the protein synthesis)

is bacteriostatic and not bactericidal, and that the population of *M. mitochondrii* symbionts seems not to multiply constantly but in bursts of growth associated with the tick blood meal. This finding will allow to better design future experiments of antibiotic treatment, which will have to be performed just before the blood meal in order to obtain maximum efficiency.

3.3 Molecular screening for *Midichloria* in hard and soft ticks reveals variable prevalence levels and bacterial loads in different tick species

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3.3.1 AIM OF THE STUDY

One of the hypotheses concerning the spreading of *Midichloria* among tick asserts that horizontal transfer could be the main source of diffusion, as argued in a previous study for the screening of *Midichloria* bacteria in multiple tick species (Epis et al., 2008); this study did not show signs of co-cladogenesis between the ticks and the symbiont. Moreover, to date, no soft tick species have been found to harbor *Midichloria* bacteria. In order to expand our knowledge of the distribution of *Midichloria* in ticks, samples from multiple species were collected and qualitative and quantitative molecular screenings followed by phylogenetic analysis performed.

3.3.2 MATERIALS AND METHODS

Tick sampling

Ninety-two tick specimens belonging to 16 species of the Ixodidae 112 family and to one species of the Argasidae family were collected in three continents, sampled free in the environment or directly from the hosts, then conserved in ethanol at 4°C or frozen alive at -80 °C. All specimens were identified using standard taxonomic keys (including Hillyard, 1997 and Pereiz-Eid, 2007 for European species; Arthur, 1965 for the African species). Ticks genus, species, number of collected individuals, life stage, geographical origin, vertebrate host and conservation protocol are summarized in Table 1.

Genus	Species	No. of specimens	Stage/ Sex	Geographical origin	Host (if present)	Positive samples for *16S rRNA* PCR	Positive samples for *gyrB* PCR
Ixodes	*arboricola*	1*	♀	Belgium	Laboratory strain	ND	ND
Ixodes	*aulacodi*	14*	♀	Akure (Nigeria)	*Thryonomis* spp.	14	14
		2*	♂	Akure (Nigeria)	*Thryonomis* spp.	2	2
		1*	♀	Owerri (Nigeria)	*Thryonomis* spp.	1	1
		2*	♀	Owerri (Nigeria)	*Cricetomys gambianus*	2	0
		7*	♀	Ondo (Nigeria	*Thryonomis* spp.	7	7
Ixodes	*acuminatus*	2•	♀	Pleine-Fougere (France)	Laboratory strain	ND	ND
		4•	♀	Pleine-Fougere (France)	Micromam mal		
		4•	♀	Pleine-Fougere (France)	*Mustela nivalis*		
Ixodes	*canisuga*	2*	♀	Bernay (France)	close to *Vulpes vulpes*	ND	ND
		2*	nymph	Bernay (France)	close to *Vulpes vulpes*		
Ixodes	*colasbelcouri*	2*	♀	Anjozorobe (Madagascar)	*Rattus rattus*	1	2
Ixodes	*frontalis*	8*	♀	Sautron (France)	*Streptopelia decaocto*	4	3
		1*	♀	Nantes (France)	*Gallinula chloroplus*	1	1
		1*	♀	Nantes (France)	*Melopsittacus undulatus*	1	1
Ixodes	*lividus*	3•	nymph	Anetz (France)	*Riparia riparia*	ND	ND
		3*	nymph	Anetz (France)	*Riparia riparia*		
Ixodes	*loricatus*	1*	♀	Morteros (Argentina)	*Didelphis albiventris*	ND	ND

Ixodes	*rubicundus*	1*	♀	Tüssen-Die-Riviere (South Africa)	*Tragelaphus oryx*	ND	ND
Ixodes	*simplex*	1•	♀	Rancognes (France)	close to *Miniopterus schreibersi*	ND	ND
		1•	♂	Rancognes (France)	close to *Miniopterus schreibersi*		
Ixodes	*trianguliceps*	4*	nymph	Combraille (France)	Micromam-mal	ND	ND
Ixodes	*ventalloi*	4*	♀	Saint Pierre de Quiberon (France)	*Oryctolagus cuniculus*	0	1
		6*	nymph	Portugal	–	0	3
Ixodes	*vespertilionis*	1•	♀	Bernay (France)	close to *Rhinolophus ferrumequi-um*	0	1
		1•	♂	Bernay (France)	close to *Rhinolophus ferrumequi-um*	0	0
Amblyomma	*variegatum*	1*	♀	Guadeloupe (France)	Laboratory strain	1	1
Hyalomma	*anatolicum*	3*	♀	Turkey	–	0	3
Hyalomma	*excavatum*	4*	♀	Turkey	–	2	4
Ornithodoros	*maritimus*	5*	♀	Valuec Island (France)	*Phalacroco-rax aristotelis*	2	4

Table 1 List of the analyzed samples, indicating tick species, life stage, sex, geographical origin, host (when present), method of preservation (asterisk indicates Ethanol preservation, dot indicates samples were frozen at -80) and overall positive samples obtained from both qualitative PCR (*16S rRNA* gene) and qPCR (*gyrB* gene).

DNA extraction

Before proceeding with DNA extraction, ethanol preserved specimens were rehydrated and washed twice in PBS 1X for 20 minutes and then left to dry for additional 20 minutes, in order to remove all the ethanol residues. Frozen ticks were boiled for 5 minutes before processing them. After these steps, DNA was extracted from all ticks using DNeasy Blood & Tissue Kit (Qiagen) with the following changes to the manufacturer instructions: proteinase K incubation was carried on overnight at 56 °C and DNA was eluted in two steps with 25 µl

each of sterile water pre-heated at 72 °C (as explained in Epis et al., 2008), quantified and stored at -80 °C until use.

Qualitative PCRs

In order to evaluate the quality of the extracted DNA, a fragment of the mitochondrial *12S rRNA* of the tick was amplified using a previously published protocol (Epis et al., 2008). Qualitative PCR to detect *Midichloria* bacteria was performed using a modified version of the protocol described by Epis et al. (2008) with two sets of primers targeting the *16S rRNA* bacterial gene. The first set of primers (Midi-F: GTACATGGGAATCTACCTTGC; Midi-R: CAGGTCGCCCTATTGCTTCTTT; primers final concentration: 1 µM; amplification size: 1100 bp) was used for a first round of amplification. The second set of primers (Midi-F2: CAAAAGTGAAAGCCTTGGGC; Midi-R2: TGAGACTTAAAYCCCAACATC) was used to perform two semi-nested PCRs (Midi-F/Midi-R2, primers final concentration: 1 µM, amplification size: 691 bp; Midi-F2/Midi-R, primers final concentration: 1 µM, amplification size: 675 bp). PCRs were carried out using the same thermal profile (30 s at 95 °C, 30 s at 58 °C and 45 s at 72 °C for 40 times).

Real Time PCR

DNA samples were subsequently subjected to a Real Time PCR (qPCR) based on a previously published protocol designed for amplification of a fragment of the *gyrB* gene from *M. mitochondrii* (Sassera et al., 2008). For bacteria quantification, a purified plasmid containing the *gyrB* fragment was serially diluted starting from 10^9 copies/ µl to 1 copy/µl to evaluate the efficiency and detection limit of the PCR protocol. The amplification of *gyrB* gene (a single copy gene per genome; Sassera et al., 2008) was obtained starting from 25 ng of DNA for each tick sample and the quantification was performed comparing the qPCR results with those of serial dilutions of cloned fragments (containing known copy numbers).

DNA sequencing and phylogenetic analyses

16S rRNA and *gyrB* genes PCR products were loaded on agarose gel, excised and purified with Wizard® SV Gel and PCR Clean-Up System (Promega) and then subjected to Sanger sequencing. After manual correction of the electropherograms, the obtained *16S rRNA*

sequences were added to a dataset of published *16S rRNA* sequences of *Midichloriaceae* bacteria. Sequences were aligned using the software Muscle (Edgar, 2004) and the alignment was used for phylogeny reconstruction using the software RaxML (Stamatakis et al., 2008) with the GTRCAT model and 1000 bootstrap replicates. A heatmap representing the variable levels of identity percentages was obtained from the *16S rRNA* alignment using an in-house python script. *gyrB* gene amplicons were subjected to BLAST analyses in order to verify the specificity of the amplification.

3.3.3 RESULTS

A total of 92 tick individuals, belonging to 16 Ixodidae and one Argasidae species, were collected and DNA was successfully extracted, as confirmed by the *12S rRNA* gene amplification (data not shown).

A qualitative semi-nested PCR to amplify a fragment of the *16S rRNA* of *Midichloria* bacteria, showed a total of 38 out of 92 specimens positive. These individuals belong to six out of 17 analyzed species (five Ixodidae and one Argasidae species), with different prevalence levels. All DNAs were subjected to a qPCR approach based on the amplification of the *gyrB* gene of *Midichloria*. This quantitative method allows the detection of as low as 10 copies of *gyrB* gene per PCR reaction. The qPCR showed a total of 48 out of 92 specimens positive, belonging to a total of nine out of 17 species (eight Ixodidae and one Argasidae species). In several samples that resulted positive in qPCR, the estimated quantification was below the sensitivity threshold of 10 copies/µl. In all cases the obtained amplicons were sequenced, confirming the specific amplification of a fragment of *gyrB*. We defined as "$< 10^1$ copies/µl" all the values from tick samples for which the quantification was lower than the sensitivity threshold (see Table S1).

Positive specimens are indicated in Table 1 and bacterial loads are shown in Table S1. DNA sequences were obtained for all *16S rRNA* and *gyrB* genes amplicons and submitted to the EMBL database (*16S rRNA* accession numbers LT575860-LT575865, *gyrB* accession numbers LT575850-LT575859).

Twenty-six individuals of the species *I. aulacodi* were examined (24 females and 2 males), and all were positive for the presence of *Midichloria* bacteria using the qualitative PCR approach, while qPCR showed positivity in 24 out of 26 ticks (92%). The two *I. aulacodi*

negative in qPCR were the two females sampled from the Gambian Pouched Rat *Cricetomys gambianus* (*I. aulacodi* 23 and 24) while the 24 others were sampled from Grasscutter *Tryonomys* spp. All the *16S rRNA* amplicons showed a 99% similarity with *M. mitochondrii* IricVA strain *16S rRNA*. *gyrB* sequences showed instead 97% similarity with *M. mitochondrii* partial *gyrB* gene. Bacterial load varied substantially between ticks, ranging from about 1.18 x 10^5 bacteria per tick to 2.49 x 10^8 bacteria per tick, with no evident difference among female and male samples.

One *I. colasbelcouri* was positive for the presence of *Midichloria* bacteria in qualitative PCR and the obtained *16S rRNA* fragment sequence showed 99% similarity with the *16S rRNA* gene of *M. mitochondrii*. Moreover, both the *I. colasbelcouri* individuals were positive in qPCR and the *gyrB* gene sequence of both specimens showed a 97% similarity with the *gyrB* gene fragment of *M. mitochondrii* in *I. ricinus*. The two individuals of *I. colasbelcouri* harbored different amounts of *Midichloria* bacteria: more than 3.71 x 10^6 bacteria were found in the individual that was also positive in qualitative PCR and around 4.53 x 10^5 in the other specimen.

Six specimens of *I. frontalis* were positive (60%) for *Midichloria* using the qualitative approach. Five out of six of these samples were also positive with the qPCR investigation. The *16S rRNA* sequences were identical and related to *M. mitochondrii* (99% identity). Moreover, all the *gyrB* amplicons from positive samples were identical and related to *M. mitochondrii* (97% identity). Copies of *gyrB* varied from a maximum of 8.23 x 10^8 per tick sample to a minimum lower than 5 x 10^2 copies.

In *I. ventalloi*, four samples (one adult female and three nymphs) were positive only using a qPCR approach, with bacterial load between a minimum lower than 5 x 10^2 and a maximum of 1.08 x 10^3 copies per tick. *gyrB* gene sequence was identical among the four samples and showed 99% similarity with *M. mitochondrii gyrB* gene. In *I. vespertilionis* the one female sample examined was found to be positive for *Midichloria* bacteria, with a *gyrB* gene sequence identical to the one of *M. mitochondrii*.
The only available *A. variegatum* sample was positive both in qualitative (99% identity with *M. mitochondrii*) and in quantitative PCR (97% similarity with *M. mitochondrii*) with *gyrB* gene copies < 5 x 10^2 per total extracted DNA.

All the three individuals belonging to *H. anatolicum* species harbored *Midichloria*, which was detectable only with qPCR approach (bacterial loads were < 5 x 10^2 *gyrB* copies for total extracted DNA) showing a 97% similarity with *gyrB* of *M. mitochondrii*.

Of the four analyzed specimens of *H. excavatum,* two showed positivity in qualitative PCR (99% identity with *M. mitochondrii* and 99% with a *Rickettsiales* bacterium found in *H. excavatum* by Loftis et al. in 2006) while qPCR detected the presence of *Midichloria* in all the four specimens (*gyrB* similarity with *M. mitochondrii* was 95%). Quantification of *gyrB* ranged from < 5 x 10^2 to 7.98 x 10^4 copies.

Two specimens out of five of the soft tick *O. maritimus* were positive with the qualitative PCR (99% identity with *M. mitochondrii*). The qPCR amplification showed positivity for *Midichloria* in four out of five samples. In this case three out of four qPCR positive samples showed a similarity of 97% with *gyrB* of *M. mitochondrii*, while one specimen showed 100% sequence identity with *M. mitochondrii*).
The sample presenting the same *gyrB* sequence of *M. mitochondrii* (*O. maritimus* 3) was also the one showing the highest amount of bacteria compared to the other individuals of the same species (over 9.91 x 10^5 bacteria compared to an average < 5 x 10^2 for the other three individuals). No samples of the other 10 species were positive to either of the PCR protocols applied.

All the obtained *16S rRNA* sequences were added to an alignment of sequences of *Midichloriaceae* from ticks and other organisms retrieved from the databases and three *Rickettsiales* sequences used as outgroups. The manually curated alignment was subjected to Maximum Likelihood phylogenetic reconstruction. The resulting tree showed that all the novel sequences belong to a highly supported monophyletic clade (in grey in Fig. 1) including sequences of *Midichloria* previously obtained from tick species, as well as sequences detected in the blood of mammals parasitized by ticks. As previously reported (Boscaro et al., 2013a), *16S rRNA* single gene alignments cannot solve all nodes of *Midichloriaceae* phylogeny, and indeed our tree presents multiple polytomies. However the support for the monophyly of the clade encompassing sequences of ticks symbionts is strong, validating our claim of clustering them within the *Midichloria* genus.

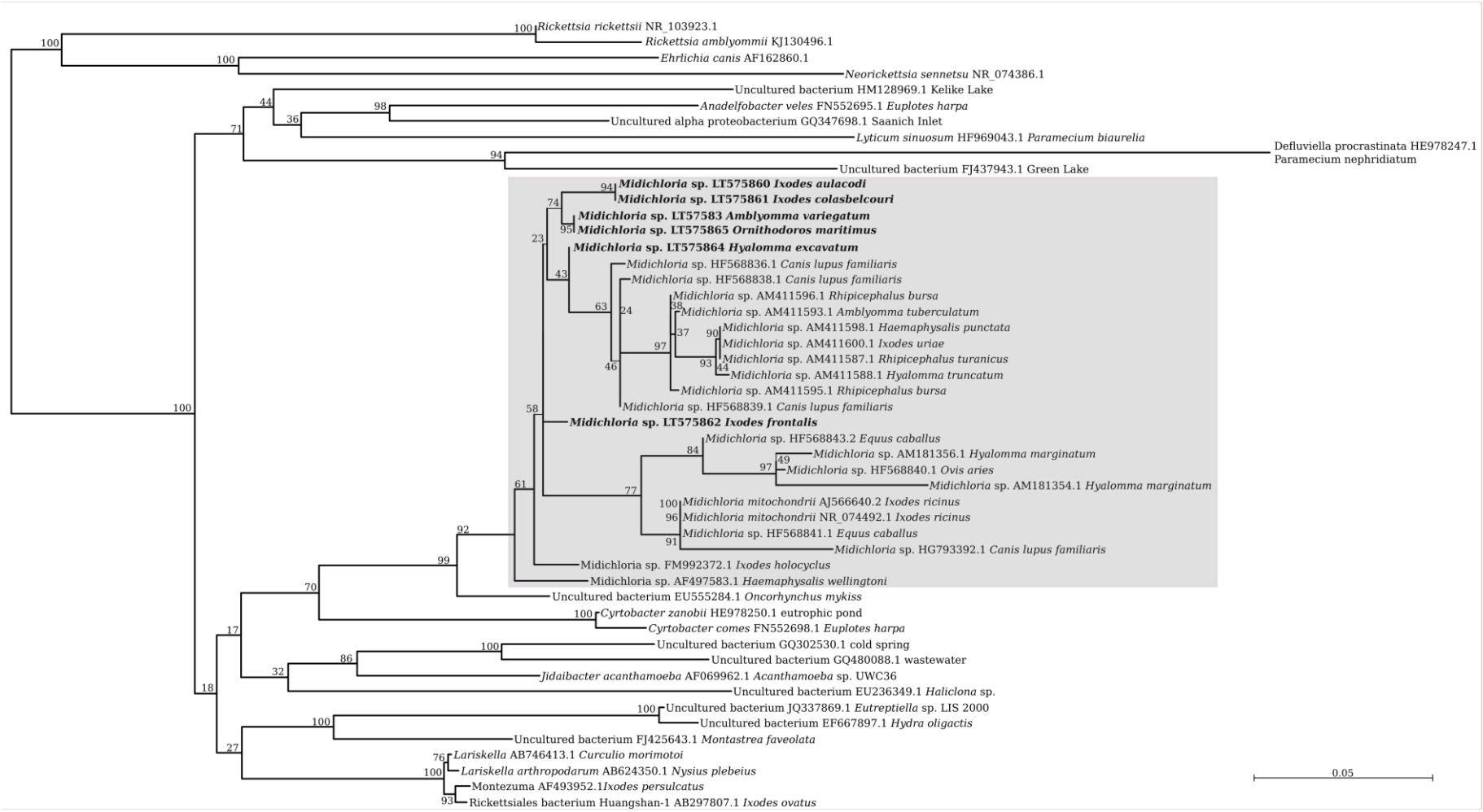

Fig. 1 Phylogenetic tree of the family *Midichloriaceae*, obtained after Maximum likelyhood analysis of a *16S rRNA* gene alignment. For each analyzed sequence, the name of the bacterium, the accession number, and the host/collection site are indicated. Sequences obtained in this study are shown in bold, the clade proposed to represent the genus *Midichloria* is shaded in grey, bootstrap values are shown above each node. Branches with support values below 50 were collapsed.

3.3.4 DISCUSSION

This work presents a molecular screening for the presence of bacteria related to *M. mitochondrii* in various species of ticks. Two PCR protocols were used to achieve this aim, a dual semi-nested qualitative PCR for the *16S rRNA* gene and a qPCR for the *gyrB* gene. The qPCR sensitivity was assessed to 10 copies per µl using a plasmid standard. It is interesting to note that the used methods did not give fully congruent results (Table 1), with 38/92 samples positive to one or both the *16S rRNA* semi-nested reamplifications and 48/92 samples positive to the qPCR (35 out of 92 are positive using both qPCR and at least one semi-nested). It appears that the three protocols show different sensitivities in different tick species (Table S1). These results could be due to the presence of mismatches in the primers regions among different species, resulting in cases of suboptimal primer annealing. Indeed, there was a clear pattern of specific protocols being more sensitive in single species. For example, *I. aulacodi*, showing high bacterial load, was positive with all protocols (including the *16S rRNA gene* PCR performed before the two semi-nested). *I. frontalis* samples were positive to *gyrB* qPCR and to one of the semi-nested protocols (Midi-F/Midi-R2), while *I. colasbelcouri* was positive to the qPCR and to the second semi-nested protocol (Midi-F2/Midi-R) (Table 1).

All the PCR amplicons were sequenced, in order to verify the specificity of amplification and to determine the genetic variation within the *Midichloria* bacteria detected in all tick species. The novel *16S rRNA* sequences were used for a phylogenetic approach by adding them to the tree of *Midichloriaceae* (Fig. 1). All the novel sequences belong to the monophyletic group containing exclusively sequences of *Midichloriaceae* associated to ticks (see the group shaded in grey in Fig. 1). Within this group, *16S rRNA* identity is always above 93% (Fig. 2) and the same group formed by all the *Midichloriaceae* associated to ticks is observed in the clustering analysis based on identity percentages among sequences. In this analysis however, the group also includes the sequence associated to the rainbow trout *Oncorhynchus mykiss.* This sequence is the sister group of all "*Midichloriaceae* associated to ticks" in the phylogenetic analysis (Fig. 1), thus illustrating the proximity of this sequence to the ones associated to ticks. The unexpected location of this sequence in the clustering analysis may be due to known skews/bias of clustering – i. *e.* phenetic – methods relatively to phylogenetic reconstruction). Based on the strong phylogenetic support and a previously performed generalized mixed Yule coalescent analysis (Montagna et al., 2013), we propose that all the bacteria of this clade, the MALO cluster 2 (Montagna et al., 2013), should be classified within the genus *Midichloria*.

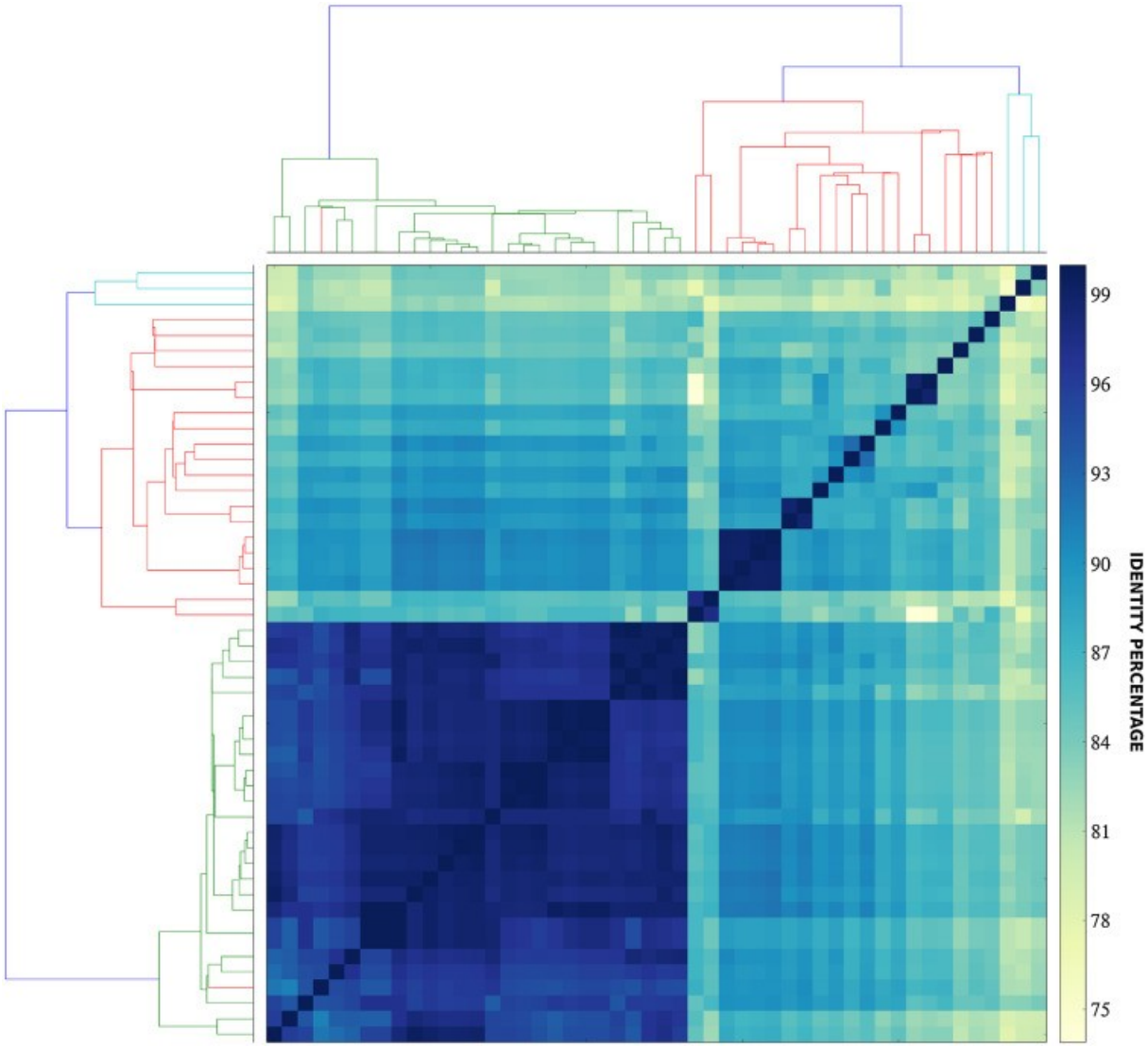

Fig. 2 Phylogenetic tree of the family *Midichloriaceae*, obtained after Maximum likelyhood analysis of a *16S rRNA* gene alignment. For each analyzed sequence, the name of the bacterium, the accession number, and the host/collection site are indicated. Sequences obtained in this study are shown in bold, the clade proposed to represent the genus *Midichloria* is shaded in grey, bootstrap values are shown above each node. Branches with support values below 50 were collapsed.

Even if the *16S rRNA* marker, due to limited genetic variability, is not suitable for fine phylogenetic discrimination (the *gyrB* marker being even worse due to its reduced length), the tree did not show evidence of co-cladogenesis between the bacteria and their tick hosts. For example, within the group of *Midichloria* associated to ticks, *Midichloria* associated to different species of the *Ixodes* genus do not form a monophyletic group, but are scattered in various clusters of the phylogenetic tree, sometimes clustering with sequences of *Midichloria* associated to other tick genera or even other tick families (*id est I. aulacodi* or *I. colasbelcouri* clustering with *A. variegatum* or *O. maritimus* that belong to the Amblyommidae and

Argasidae families respectively; or *I. uriae* clustering with *Haemaphysalis punctata* and *Rhipicephalus turanicus* that belong to the Amblyommidae family). This result, together with the presence of sequences obtained from mammalian blood samples (Bazzocchi et al., 2013), provides additional support to the hypothesis that bacteria of the genus *Midichloria* can transfer horizontally between ticks through vertebrate hosts, probably through the blood meal.

We believe that a multigene phylogenetic approach could be very useful in order to better define the boundaries of the genus *Midichloria*, and to provide a definite answer to the question of the co-cladogenesis. A Multi Locus Sequence Typing (MLST) effort, restricted to the symbionts of ticks, or even wider, including other members of the family, could elucidate this issue.

We examined individuals from 17 species of hard and soft ticks, detecting positive ticks in nine of them. For most species the limited number of available samples did not allow for a precise evaluation of the prevalence levels. The only species which exhibited 100% prevalence with a significant number of specimens (n=26) was *I. aulacodi*. In this tick species, both males (n=2) and females (n=24) appear to host *Midichloria* bacteria. The presence of *Midichloria* in 100% females could indicate a vertical transmission of the symbiont to the offspring, as demonstrated for *M. mitochondrii* of *I. ricinus*. The qPCR negativity of two samples could be due to a lower sensitivity of the method in this species.
Unfortunately it was not possible to fully evaluate the prevalence of *Midichloria* bacteria in the males of this species in order to determine whether or not they were all infected with the bacterium, as only two males were collected (in *I. ricinus* only 44% males host the *M. mitochondrii* symbiont).

The qPCR was efficient in evaluating the *Midichloria* load in all tick samples. The numbers were very variable, ranging from values below 5×10^2 copies to 8.23×10^8 gene copies per tick. While the limited number of samples did not allow for statistical analysis of these data, we observed a high variability of bacterial load in the species *I. frontalis* and *O. maritimus* (Table S1). The presence of a bacterium of the genus *Midichloria* in *I. frontalis* confirms the recent discover by Palomar and colleagues (Palomar et al., 2015). The species with the highest number of examined samples, *I. aulacodi*, has a median *Midichloria* load of 1.08×10^6 bacteria. Such high numbers, coupled with 100% prevalence, strongly suggest an important role for this bacterium in the physiology of the host. Indeed these data could

indicate a mutualistic relationship between *I. aulacodi* and *Midichloria*, as previously suggested for the *I. ricinus – M. mitochondrii* symbiosis. It must be noted that a previous report indicates that the number of *M. mitochondrii* symbionts varies greatly through the *I. ricinus* life cycle (Sassera et al., 2008). Additional *I. aulacodi* individuals, belonging to different life stages, should be examined in order to investigate this issue.

The presence of a *Midichloria* bacterium in *H. excavatum* is in accordance with the detection of a *16S rRNA* gene sequence belonging to a *Rickettsiales* bacterium found by Loftis et al. (2006) in one *H. excavatum* specimen and in one pool of *Hyalomma* spp. nymphs. Three of the four *O. maritimus* individuals that were positive for *Midichloria* had low bacterial load and identical gene sequences, while the remaining positive sample presented very high load and a different *gyrB* sequence. This result could simply indicate a gene polymorphism in *Midichloria* bacteria in *O. maritimus.* However another possible explanation could be that *O. maritimus* can host multiple *M. mitochondrii* strains. Indeed it is interesting to observe that the higher bacterial load was found in the sample with a *gyrB* sequence that is identical to the one belonging to *M. mitochondrii* of *I. ricinus.* Following this reasoning, we could hypothesize that the ancestral *Midichloria* behavior is that of tick-borne bacteria that travel horizontally through ticks and mammalian hosts at low prevalence and low bacterial load, as is often the case for tick-borne rickettsiae (which are close relatives of *Midichloriaceae*). Such behavior could have evolved, once or multiple times, in a stronger relationship, in which the bacterium assumed a mutualistic role within the host, with a strong increase in load per individual, higher prevalence and possibly vertical transmission. More studies are necessary to test whether this hypothesis is correct in *I. ricinus, I. holocyclus* and/or *I. aulacodi*.

Future studies should thus be focused on developing and applying genetic markers on faster evolving characters, in order to investigate the genetic variability of *Midichloria* in ticks using a multi-gene phylogenetic approach and test the above hypotheses. Such investigations, including the selection of a gene with a mutation rate providing suitable polymorphism, are facilitated by the knowledge of the whole genome of *M. mitochondrii* (Sassera et al., 2011). It would also be interesting to analyze tick ovaries by means of transmission electron microscopy, in order to assess if the bacteria are present inside the mitochondria of infected cells (as does *M. mitochondrii* of *I. ricinus*) or just in the cytoplasm (as observed in *I. holocyclus*). This would answer the question of whether the intramitochondrial tropism is a unique character of *M. mitochondrii* or if it is widespread in the genus *Midichloria*.

3.3.5 SUPPORTING INFORMATION

Table S1 Quantifications of the *gyrB* gene through qPCR in tick samples positive for *Midichloria*.

Genus	Species	Sample code	Stage/ Sex	Positivity for *16S rDNA* gene	*gyrB* copies in 25 ng DNA	*gyrB* copies in total DNA eluted in 50 µl
		1	♀	+	1.52×10^3	7.61×10^5
		2	♀	+	1.31×10^3	2.62×10^5
		3	♀	+	6.29×10^3	1.26×10^6
		4	♀	+	1.47×10^5	2.20×10^7
		5	♀	+	1.58×10^4	7.89×10^5
		6	♀	+	9.93×10^3	2.98×10^6
		7	♀	+	4.04×10^4	1.29×10^8
		8	♀	+	3.48×10^4	1.25×10^8
		9	♀	+	6.57×10^3	2.13×10^7
		10	♀	+	2.22×10^4	1.67×10^7
		11	♀	+	1.91×10^3	4.78×10^5
Ixodes	*aulacodi*	12	♀	+	7.20×10^3	7.20×10^5
		13	♀	+	6.34×10^4	6.34×10^6
		14	♀	+	1.15×10^4	2.31×10^6
		15	♀	+	2.77×10^5	2.49×10^8
		16	♀	+	6.03×10^3	6.03×10^5
		17	♀	+	9.05×10^3	9.05×10^5
		18	♀	+	2.35×10^3	1.18×10^5
		19	♀	+	3.41×10^3	5.11×10^5
		20	♀	+	5.69×10^4	8.54×10^6
		21	♀	+	5.30×10^3	5.30×10^5
		22	♀	+	5.49×10^3	5.49×10^5
		23	♀	+	0	0

		24	♀	+	0	0
		25	♂	+	3.74×10^{4}	1.87×10^{6}
		26	♂	+	2.56×10^{3}	1.28×10^{5}
Ixodes	*colasbelcouri*	1	♀	+	3.71×10^{4}	3.71×10^{6}
		2	♀	-	9.05×10^{3}	4.53×10^{5}
Ixodes	*frontalis*	1	♀	+	$< 10^{1}$	$< 5 \times 10^{2}$
		2	♀	+	1.98×10^{5}	8.23×10^{8}
		3	♀	+	1.01×10^{5}	3.37×10^{8}
		4	♀	+	0	0
		5	♀	+	$< 10^{1}$	7.81×10^{3}
		6	♀	+	$< 10^{1}$	$< 5 \times 10^{2}$
Ixodes	*ventalloi*	1	♀	-	$< 10^{1}$	$< 2.8 \times 10^{3}$
		5	nymph	-	1.50×10^{1}	9.01×10^{2}
		7	nymph	-	1.80×10^{1}	1.08×10^{3}
		10	nymph	-	$< 10^{1}$	1.50×10^{1}
Ixodes	*vespertilionis*	1	♀	-	$< 10^{1}$	$< 5 \times 10^{2}$
Amblyomma	*variegatum*	1	♀	+	$< 10^{1}$	$< 5 \times 10^{2}$
Hyalomma	*anatolicum*	1	♀	-	$< 10^{1}$	$< 5 \times 10^{2}$
		2	♀	-	$< 10^{1}$	$< 5 \times 10^{2}$
		3	♀	-	$< 10^{1}$	$< 5 \times 10^{2}$
Hyalomma	*excavatum*	1	♀	+	1.33×10^{2}	7.98×10^{4}
		2	♀	-	$< 10^{1}$	$< 5 \times 10^{2}$
		3	♀	-	$< 10^{1}$	$< 5 \times 10^{2}$
		4	♀	+	1.10×10^{1}	1.17×10^{3}
Ornithodoros	*maritimus*	1	♀	-	$< 10^{1}$	$< 5 \times 10^{2}$
		2	♀	-	$< 10^{1}$	$< 5 \times 10^{2}$
		3	♀	+	1.98×10^{4}	9.91×10^{5}
		4	♀	+	$< 10^{1}$	$< 5 \times 10^{2}$

3.4 *Ixodes ricinus* and its endosymbiont *Midichloria mitochondrii*: a comparative proteomic analysis of salivary glands and ovaries

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3.4.1 AIM OF THE STUDY

With the advent of proteomics, the screening of proteins as potential biomarkers has achieved important progresses. Detection and identification of proteins in different organs/tissues, with the aim of understanding whether they represent an attractive tool for monitoring alterations in these districts, is currently an area of increasing interest. Recently, studies have been focused on the characterization of *I. ricinus* salivary glands and midgut proteomes, in a much-needed effort to better understand the role of these organs, fundamental in the tick bite and metabolism (Schwarz et al., 2015; Vu Hai et al., 2013).

The plan of this work was then to expand the knowledge of *I. ricinus* protein profiles by applying two dimensional electrophoresis (2-DE) as a tool for comparing the protein pattern of the ovary with that of salivary glands (i.e. the sialome). The first goal of this study is to give insight into the process of oogenesis, central to the tick life cycle. Additionally it was planned to provide clues on the symbiotic relationship between *I. ricinus* and its symbiont *M. mitochondrii*, which is highly prevalent in the ovaries. Moreover, to seek the best possible protocol for future studies on *I. ricinus* proteomics, the work was focused on a careful optimization of the proteomic analysis pipeline.

3.4.2 MATERIALS AND METHODS

Ethics Statement

I. ricinus ticks were collected from roe-deer (*Capreolus capreolus*) in the Chizé forest (Northern France) in February 2014, in strict accordance with the recommendations in 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 deers was carried out only by competent persons using methods which do not cause the animals avoidable pain, suffering, distress or lasting harm.

Ticks collection, protein and DNA extraction

One hundred and twenty semi-engorged *I. ricinus* ticks were collected from roe deer (*Capreolus capreolus*) in the Chizé forest (Northern France) in February 2014. The semi-engorged ticks were selected as this stage presents the highest combined development of the two investigated organs, ovaries and salivary glands, and also presents a high concentration of *M. mitochondrii* symbionts (Sassera et al., 2006). Ticks were manually dissected under a stereomicroscope Leica (Wetzlar, Germany), to collect salivary glands and ovaries. Salivary glands and ovaries from twenty ticks were pooled in 100 µL PBS with 1.5 µL of 1x protease inhibitor (Sigma). After mechanical disruption of tissues, 20 µL of lysate were recovered for subsequent DNA extraction. The remaining volume was subjected to sonication with Digital Sonifier 450 (Branson Ultrasonic Corporation, Danbury, CT, USA), with three five-second treatments. Each sample was then centrifuged at maximum speed for 10 min and supernatants were recovered and stored at -80°C until use. DNA from each sample was extracted using the Qiagen DNeasy Blood and Tissue Kit (Hilden, Germany) following manufacturer instructions. DNAs were eluted in 50 µL of sterile water and stored at -20°C until molecular analysis.

PCR

The presence of common tick-borne pathogenic bacteria (Michelet et al., 2014) in the extracted DNA was screened using previously described PCR protocols for *Borrelia burgdorferi* (Pistone et al., 2010), *Anaplasma* spp., *Ehrlichia* spp. and *Rickettsia* spp. (Pesquera et al., 2015). Samples negative for the presence of pathogens were subsequently analyzed for absolute quantification of *M. mitochondrii* content using a previously described Sybr green real-time PCR approach (Sassera et al., 2006) based on the amplifications of a fragment of the *M. mitochondrii gyrB* gene (coding for the protein gyrase B) and a fragment

of the *I. ricinus* nuclear gene *cal* (coding for the protein calreticulin). Results were expressed as ratio of *gyrB*/*cal* copy numbers.

Quantification of proteins

The Bicinchoninic Acid (BCA) assay (Smith et al., 1985) was applied to obtain the exact quantification of each pool of proteins extracted from salivary glands and ovaries. Bovine serum albumin was the standard protein used for the production of calibration curves, in the range of concentrations between 5 and 25 µg/mL.

Two-Dimensional Electrophoresis (2-DE)

About 250 µg of extracted proteins were dissolved in 125 µL of rehydration buffer (8 M urea, 4% CHAPS (w/v), 65 mM DTE, 0.8% carrier ampholytes (v/v), 0.5% bromophenol blue) and loaded onto 7 cm IPG strips, with nonlinear (NL) pH 3–10 or linear pH 4–7 gradient range, Amersham Biosciences (Amersham, UK). Strips were rehydrated without applying voltage for 1 h at 20 °C. The first-dimensional IEF was carried out at 15°C using an Ettan IPGphor system (Amersham Biosciences), programmed with the following voltage gradient: 30 V for 8 h, 120 V for 1 h, 500 V for 0.5 h, 1000 V for 0.5 h and 5000 V until a total of 25-27 kV/h was reached. Reduction/alkylation steps were applied between the first and the second dimension. The focused IPG strips were incubated for 15 min at room temperature in 6 M urea, 2% (w/v) SDS, 50 mM Tris pH 6.8, glycerol 30%, containing 2% (w/v) DTE, followed by a second incubation of 15 min in the same buffer containing 2.5% (w/v) iodoacetamide and 0.5% bromophenol blue. At the end of the IEF step, strips were hold in place with 0.4% low melting temperature agarose and loaded onto 8x6 cm slabs, 12.5% SDS polyacrylamide gels. Electrophoresis was carried out at a constant current of 10 mA per gel in a PROTEAN II xi 2-D Cell equipment Bio-Rad (Berkeley, California), until the buffer front line was 1 mm from the bottom of the gels. The 2-DE gels were stained with ‘‘Blue silver’’ (colloidal Coomassie G-250 staining), according to Candiano et al. (2004). Digital images of stained gels were acquired using VersaDoc Imaging Model 3000 (BioRad) and then subjected to quali/quantitative analysis using the PD Quest (BioRad) version 8.0.1 software. Scanned images were filtered and smoothed to remove background noise, vertical/horizontal streaking and gel artifacts and then normalized to eliminate the variability of each sample. The software then determined the amount of spots present and calculated their intensity by applying the following algorithm: peak value (ODs/image units) $*\sigma_x*\sigma_y$ (standard deviations in x and y).

Reproducibility of the study

To verify the reproducibility of the study, 2-DE maps were obtained in triplicate for each of the analyzed salivary glands (SG) and ovary tissues (OT) pools. Those presented in this report are the best representative gels among all generated that showed spots consistently present. Experimental steps concerning sample preparation, electrophoretic run and gel staining were performed ‘‘in parallel’’ on all samples.

***In situ* enzymatic digestion**

Enzymatic digestion was performed as previously described (Giuliano et al., 2014). Briefly, the selected spots were carefully excised from the gel, placed into Eppendorf tubes and broken into small pieces. This material was then washed twice with aliquots (200 µL) of 100 mM ammonium bicarbonate buffer pH 7.8, 50% acetonitrile (ACN) and kept under stirring overnight, until complete destaining. Gels were dehydrated by addition of ACN (100 µL). After removal of the organic solvent, reduction was performed by addition of 50 µL of 10 mM Dithiothreitol (DTT) solution (40 min at 37°C). DTT was replaced with 50 µL of 55 mM iodoacetamide for 45 min at 56 °C. This solution was removed and the gel pieces were washed twice with 200 µL of 100 mM ammonium bicarbonate for 10 min, while vortexing. The wash solution was removed and gel dehydrated by addition of 200 µL of ACN until the gel pieces became an opaque-white color. ACN was finally removed and gel pieces were dried under vacuum. Gels were rehydrated by addition of 75 µL of 100 mM ammonium bicarbonate buffer pH 7.8, containing 20 ng/µL sequencing grade trypsin (Promega, Madison, WI, USA) and digestion was performed incubating overnight at 37°C. Following enzymatic digestion, the resultant peptides were extracted sequentially from gel matrix by a three-step treatment (each step at 37°C for 15 min) with 50 µL of 50% ACN in water, 5% trifluoroacetic acid (TFA) and finally with 50 µL of 100% ACN. Each extraction involved 10 min of stirring followed by centrifugation and removal of the supernatant. The original supernatant and those obtained from sequential extractions were pooled, dried and stored at -80°C until mass spectrometric analysis. At the moment of use, the peptide mixture was solubilized in 100 µL of 0.1% formic acid (FA) for MS analyses.

LC-MS/MS

All analyses were carried out on an LC-MS (Thermo Finnigan, San Jose, CA, USA) system consisting of a thermostated column oven Surveyor autosampler controlled at 25°C, a quaternary gradient Surveyor MS pump equipped with a diode array detector, and an Linear Trap Quadrupole (LTQ) mass spectrometer with electrospray ionization ion source controlled by Xcalibur software 1.4. Analytes were separated by RP-HPLC on a Jupiter (Phenomenex, Torrance, CA, USA) C_{18} column (150 x 2 mm, 4 μm, 90 Å particle size) using a linear gradient (2–60% solvent B in 60 min) in which solvent A consisted of 0.1% aqueous FA and solvent B consisted of ACN containing 0.1% FA. Flow-rate was 0.2 mL/min. Mass spectra were generated in positive ion mode under constant instrumental conditions: source voltage 5.0 kV, capillary voltage 46 V, sheath gas flow 40 (arbitrary units), auxiliary gas flow 10 (arbitrary units), sweep gas flow 1 (arbitrary units), capillary temperature 200°C, tube lens voltage –105 V. MS/MS spectra, obtained by CID studies in the linear ion trap, were performed with an isolation width of 3 Th *m/z*, the activation amplitude was 35% of ejection RF amplitude that corresponds to 1.58 V.

Data processing was performed using Peaks studio 4.5 software. An ad-hoc database was obtained selecting from the NCBI database all the protein sequences belonging to the following taxonomic groups: *Ixodida* (taxid:6935), *Cervidae* (taxid:9850), *Borrelia* (taxid:138), *Rickettsiales* (taxid:766). The mass lists were searched against the SwissProt and the ad-hoc protein database under continued mode (MS plus MS/MS), with the following parameters: trypsin specificity, five missed cleavages, peptide tolerance at 0.2 Da, MS/MS tolerance at 0.25 Da, peptide charge 1, 2, 3+, and experimental mass values: monoisotopic.

Western Blotting

Western blot analysis was effected starting from 100 micrograms of proteins extracted from the OT and SG pool that exhibited the highest concentrations of *M. mitochondrii* based on *GyrB/cal* gene ratio. Separated proteins were transferred onto nitrocellulose membrane by using a Trans Blot Electrophoresis Transfer Cell (BioRad) and applying a current of 200 mA for 1.20 h in running buffer (25 mM Tris pH 8.3, 192 mM glycine, 20% methanol). To verify the transfer of proteins, the membrane was stained with Ponceau Red and washed with PBS (10 ml) for 10 min. After 1h incubation in 5% milk (10 ml) diluted in PBS and three additional washes with PBST(0,1% Tween (10 ml), the membrane was incubated overnight with th polyclonal antibodies against the *M. mitochondrii* flagellar protein FliD (Mariconti et

al., 2012b) at a dilution 1:5000 in 1% milk. After washing the membrane three times with PBST (10 ml), incubation with the secondary antibody (Dako, Glostrup, Denmark) was carried out for 1 h at room temperature with polyclonal goat anti-rabbit immunoglobulin diluted 1:2000 in 1% milk in PBST. The membrane was finally washed three times with PBS and incubated in ECL Prime solution (GE Healthcare, Uppsala, Sweden). Immunoblots were acquired with the ImageQuant LAS 4000 analyzer (GE Healthcare).

3.4.3 RESULTS

PCR

Concentration of *M. mitochondrii* and presence of tick-borne pathogens was assessed in all the OT and SG pools by performing PCR on the DNA extracted from the samples. Two out of six OT and SG pools were positive to tick-borne pathogens and were thus excluded from subsequent analyses. PCR for the detection of *M. mitochondrii* was performed on the remaining samples and all OT and SG pools resulted, as expected, positive to *M. mitochondrii.* The copy numbers of *gyrB* and *cal* genes and the *gyrB/cal* x1000 ratios are provided in Table S1 of Supporting Information.

Two-dimensional electrophoresis with nonlinear pH 3–10 gradient range

To identify *I. ricinus* proteins differentially expressed between OT and SG, as well as proteins of the *M. mitochondrii* symbiont, parallel 2-DE analyses were performed on the four salivary glands (SG) and ovary tissues (OT) of *I. ricinus* adult ticks that were infected by *M. mitochondrii* that resulted free from other bacterial pathogen presence. Gels were scanned and spots were detected using the spot detection wizard tool, after defining and saving a set of detection parameters. Following spot detection, the original gel scans were filtered and smoothed to clarify spots, remove vertical and horizontal streaks, and remove speckles. Three dimensional Gaussian spots were then created from filtered images. Three images were created from the process: the original raw 2-D scan, the filtered image, and the Gaussian image. A match set for each pool was then created for comparison after the gel images had been aligned and automatically overlaid. If a spot was saturated, irregularly shaped, or otherwise of poor quality, then the Gaussian modeling was unable to accurately determine quantity. In these cases, the spot was defined in the filtered image using the spot boundary

tools. Thus, for each pool, a master gel was produced which included protein spots only if present at least in two out of the three gels. The mean spot number in Coomassie stained gels was 235±29 in SG and 221±21 in OT. A set of spots chosen from the two master gels were excised, destained, digested with trypsin, and peptides were submitted to LC-MS/MS. The MS fragmentation data were searched against the SwissProt and the ad-hoc designed protein databases, and the queries were performed using the Peaks studio 4.5 software. A total of 47 proteins were identified, 20 from SG and 27 from OT. A complete list of the identified proteins is presented in Table S2.

The master gels from both SG and OT pools showed similar patterns of proteins such that they could be matched to each other. This facilitated the correlation of gels and the creation of a virtual image, indicated as high master gel (HMG), comprehensive of all matched spots derived from master gels. The procedure described is summarized in Fig 1.

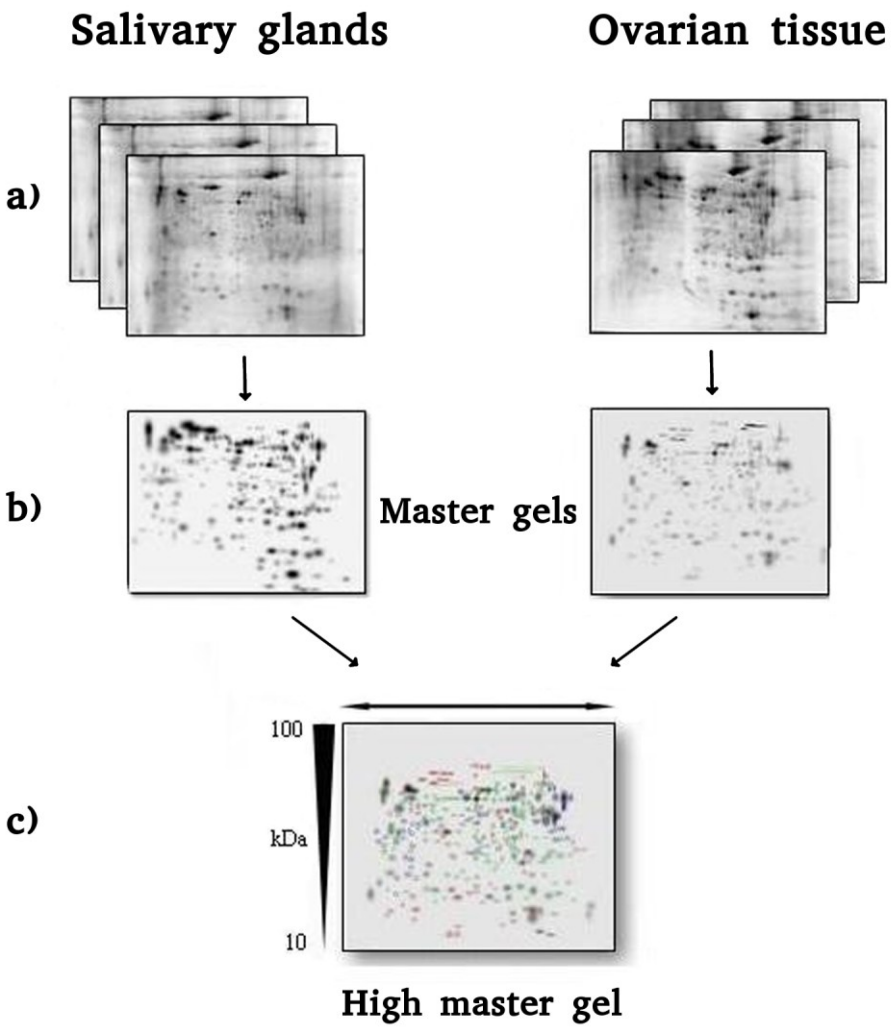

Fig 1. (A) 2-DE maps of three different pools of salivary gland (SG, left) and ovarian tissue (OT, right) of *I. ricinus*, obtained by performing IEF on 7 cm IPG strips with 3–10 NL pH range and SDS-PAGE in the second dimension on 8x6 cm slabs, 12.5% T gels. (B) SG and OT master gels obtained merging the three gels for each sample type. (C) 2-DE High Master Gel created comparing the SG and OT gels.

Differentially expressed proteins

Comparison of 2-DE patterns for SG and OT revealed several qualitative and quantitative differences between the two sets of pools. In terms of presence/absence of spots, qualitative differences are represented in Fig 2. As shown, while the majority of spots were common to both SG and OT (170 ± 25, evidenced in green), some protein spots present in SG profile were absent from the OT one and viceversa. In particular, 81 spots (marked in red) were exclusive of SG and 57 spots (labeled in blue) were detected solely in OT.

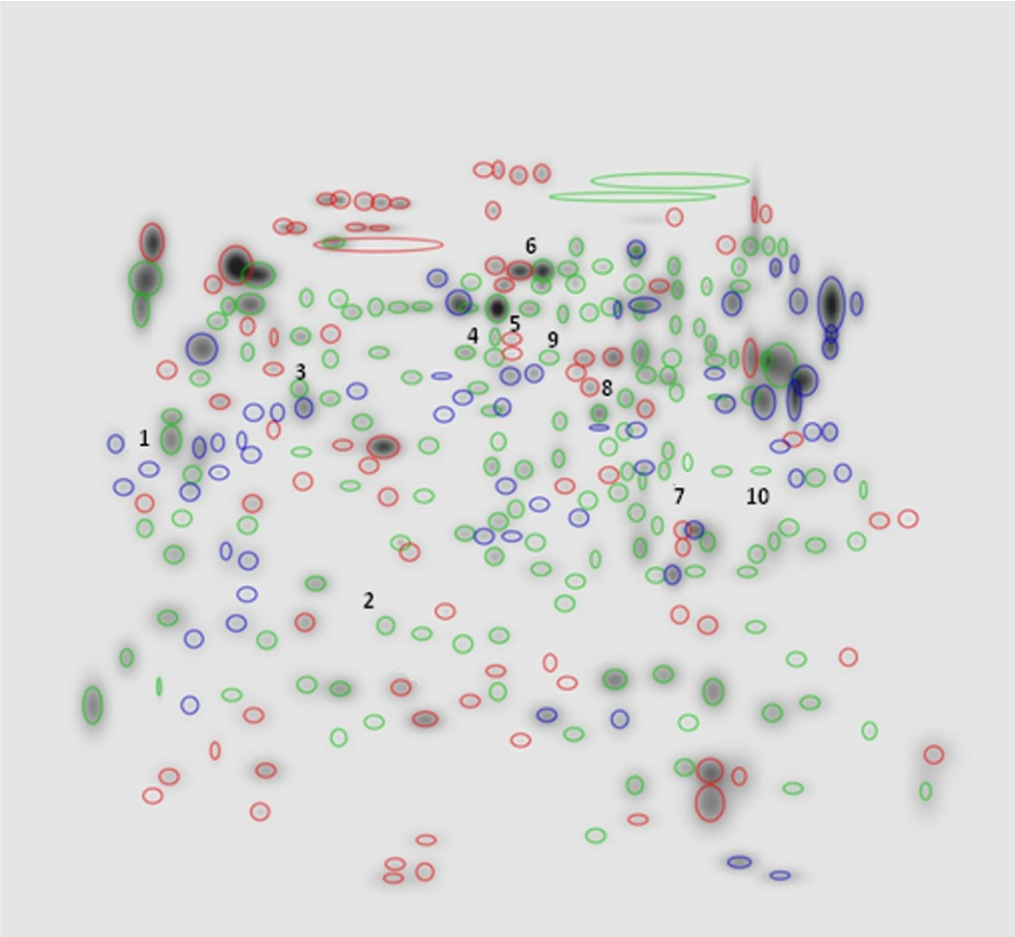

Fig 2. High Master gel, showing qualitative differences between the SG and OT 2-DE master gel patterns (NL, pH 3-10 gradient range). Labeled in green: spots (n = 170 ± 25) common to both SG and OT. Labeled in red: spots (n = 81) exclusive of SG. Labeled in blue: spots (n = 57) detected solely in OT.

Spot quantities of all gels were normalized to remove non expression-related variations in spot intensity, and data were exported as clipboard for further statistical analysis. The raw amount of each protein in a gel was divided by the total quantity of all proteins (spots) that were included in that gel. The results were evaluated in terms of spot optical density (OD). Statistical analysis of PDQuest data allowed to assess differences in protein abundance on a protein-by-protein basis. According to guidelines for differential proteomic research, only spots that showed a change in density at $p < 0.01$ (by Student's t-test) were considered "differentially expressed" in the two pools of samples. This term was used here meaning differential protein abundance determined by several processes, including changes in protein biosynthesis and modification or degradation. Using these criteria, 21 spots differed by the ratio indicated above and were selected by the statistical program as spots having significant differences in the relative abundance between SG and OT. In particular, ten among these spots

(indicated by numbers 1 to 10 in HMG of Fig 2) showed 4- to 18-fold increase/decrease in density. A set of panels, shown in Fig 3, was generated to highlight density variances of these spots between the two sets of pools (i.e. SG and OT).

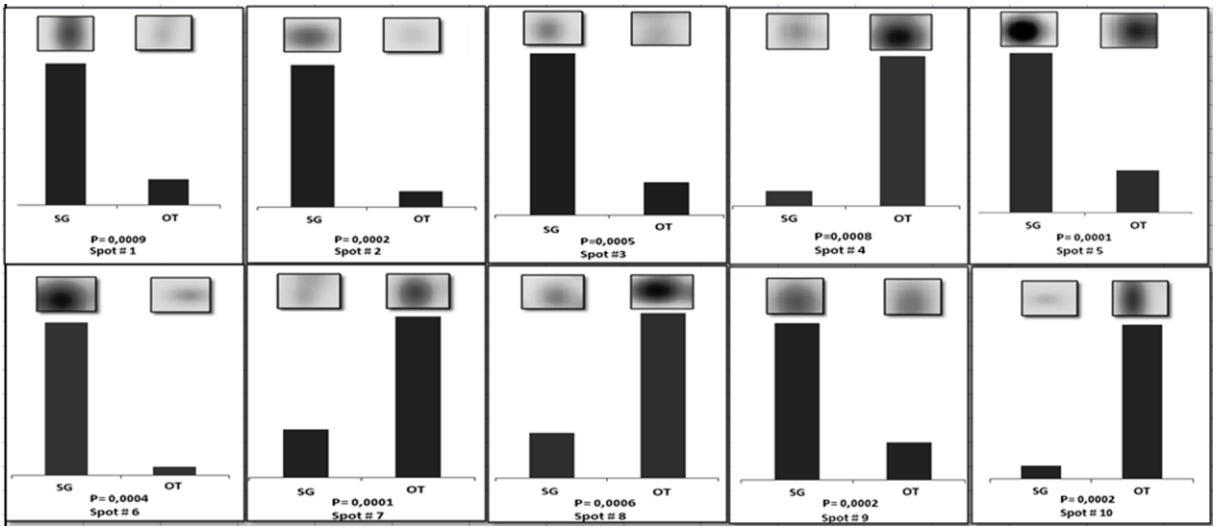

Fig 3. Set of panels showing the density variances between SG and OT pools for spots 1 to 10. In each panel the region of the stained gel containing the spot of interest was magnified (inset) and the up-/downregulation graphically represented. Pvalue indicating statistical significant density variance (T-test) is reported in each panel.

In these panels the region of stained gel containing the spot of interest was zoomed and up-/downregulation graphically represented. Efforts have been devoted to the identification of these proteins, to investigate whether they might have an involvement in the biological processes characteristic of ovaries and salivary glands, or in the interaction between *M. mitochondrii* and *I. ricinus*.

These spots were thus carefully excised from the gel, destained, digested with trypsin, and peptides were submitted to LC-MS/MS following the procedure detailed in the Materials and Methods section. The MS fragmentation data were searched against the SwissProt and the ad-hoc designed protein databases, and the queries were performed using the Peaks studio 4.5 software. All but two (spots 2 and 9) of the queried proteins were identified. The low abundance of proteins corresponding to spots 2 and 9 most likely determined the poor quality of their MS signals and failure in their identification. The fact that unique proteins were identified for all other analyzed spots suggested that, at least for these spots, spot overlap was minimized.

Detailed identification data, including accession number, theoretical p*I*, molecular mass, percent of sequence coverage, number of peptides identified, and MOWSE score of each of the nine proteins identified are reported in Table 1.

Spot	Accession	Description	Mass	Score (%)	Coverage (%)	Query matched
1	gi|442756551|gb|JAA70434.1|	Putative heat shock 70 kda protein 5 [Ixodes ricinus]	72.595	99	8.05%	5
2	/	Not detected	/	/	/	/
3	gi|322422107|gb|ADX01224.1|	Beta actin [Ixodes ricinus]	16.038	90	6.94%	1
4	gi|215497327|gb|EEC06821.1|	Enolase, putative [Ixodes scapularis]	21.493	90	4.52%	1
5	gi|442753241|gb|JAA68780.1|	Putative enolase [Ixodes ricinus]	47.145	99	23.79%	6
6	gi|215491972|gb|EEC01613.1|	Protein disulfide isomerase, putative [Ixodes scapularis]	54.929	98	6.38%	4
7	gi|442748259|gb|JAA66289.1|	Putative 3-hydroxy-3-methylglutaryl-coa reductase [Borrelia spp]	10.741	20	22.43%	2
8	gi|597718071|gb|AHN19768.1|	Serum albumin, partial [Cervus nippon]	66.15	75	6.67%	4
9	/	Not detected	/	/	/	/
10	gi|442754645|gb|JAA69482.1|	Putative heat shock protein [Ixodes ricinus]	36.782	82	4.01%	2

Table 1 Up and downregulated proteins identified by LC-MS/MS.

Additional information concerning the primary sequence of all peptides identified for each protein analyzed was included in Table S3 of Supporting Information.

Western blotting

Given the aim of our study, proteins from SG and OT profiles of the pool that exhibited the highest concentrations of *M. mitochondrii* were transferred onto PVDF membranes and incubated with the polyclonal anti-FliD antibodies, followed by anti-rabbit antibody. Based on: i) its position (pI/Mr) on the PVDF membrane and ii) its recognition by the antibody, the protein spot indicated by an arrow in panel A (OT pool) of Fig 4, was tentatively assigned to FliD. As shown in panel B of Fig 4, despite the appearance of interfering spots, the hypothetical FliD spot was undetectable in the SG profile. This result is expected and the load of *M. mitochondrii* bacteria is much higher in ovaries than in salivary glands (Table S1) (Bazzocchi et al., 2013; Mariconti et al., 2012a).

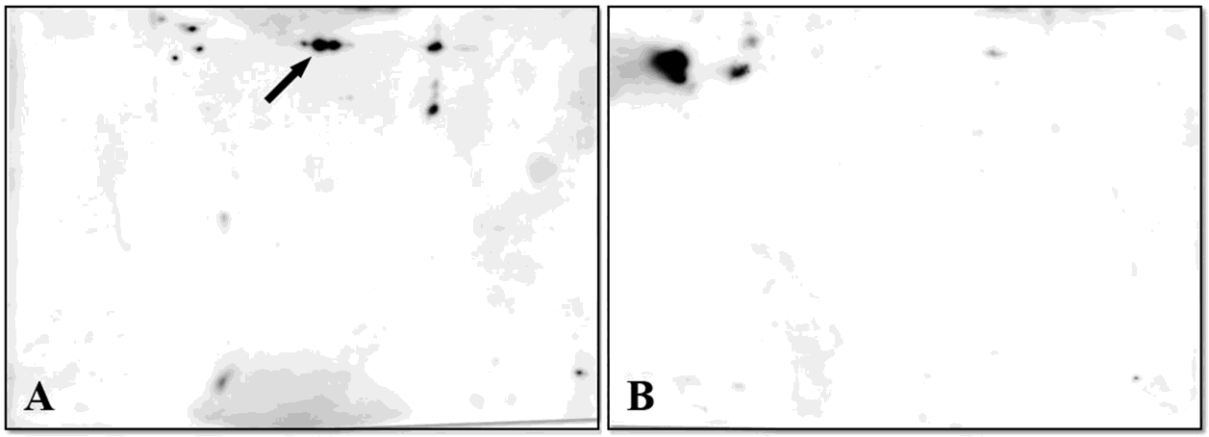

Fig 4. Immunoblotting of proteins from SG and OT profiles generated as indicated in Fig 1. PVDF membranes were incubated with the rabbit polyclonal antibodies anti-FliD of *M. mitochondrii*, followed by anti-rabbit antibody. The protein spot(s) indicated by an arrow in Panel A (OT pool) was tentatively assigned to FliD. Panel B shows the SG profile in which the hypothetical FliD spot is undetectable.

To achieve identification, the immunoreactive protein spot was thus excised from the original OT gel and submitted to the LC-MS/MS procedure indicated above. The results (shown in Table 2) confirmed what appeared evident from the visual inspection of the gel, i.e. the analyzed spot, rather than comprising a single polypeptide chain, was a mixture of at least three components, i.e. Endoplasmic Reticulum Protein 60, putative actin 2, and an unknown

protein from *Borrelia*. Information concerning the primary sequence of all peptides identified for each protein analyzed have been included in Table S4 of Supporting Information.

Accession	Description	Mass	Score (%)	Coverage (%)	Query matched
gi|442747467|gb|JAA65893.1|	Putative erp60 [Ixodes ricinus]	52.115	98	6.45%	3
gi|556065071|gb|JAB75571.1|	putative actin-2 [Ixodes ricinus]	36.27	89	9.79%	2
gi|6841058|gb|AAF28881.1|	unknown [Borrelia hermsii]	29.518	25	3.92%	1

Table 2 List of proteins identified under the immunoreactive spot.

We hypothesized that the fact that the putative flagellar protein FliD was, most likely, less abundant compared to the bulk of other proteins present in the spot, prevented its identification. This hypothesis was strengthened by the poor quality of MS sequence data obtained from third spot (or cluster of spots). This indeed made it difficult to define exactly whether the FliD protein was actually present within the spot(s) considered.

Two-dimensional electrophoresis with pH 4–7 gradient range: identification of FliD

In an effort to overcome the limitations indicated above and to definitively establish (or exclude) the presence of FliD under the spot(s) examined in the OT pool, we worked on the optimization of the electrophoretic conditions. After performing a extensive set of trials with various electrophoretic conditions, the best option was found to be the application of a narrow range pH gradient (linear pH 4-7). This provided a better resolution of proteins, minimizing potential spot overlaps (Fig 5, A). The immunoreactive protein spot (indicated by an arrow in PVDF membrane, B) was evidenced in OT profile obtained under the experimental conditions mentioned above.

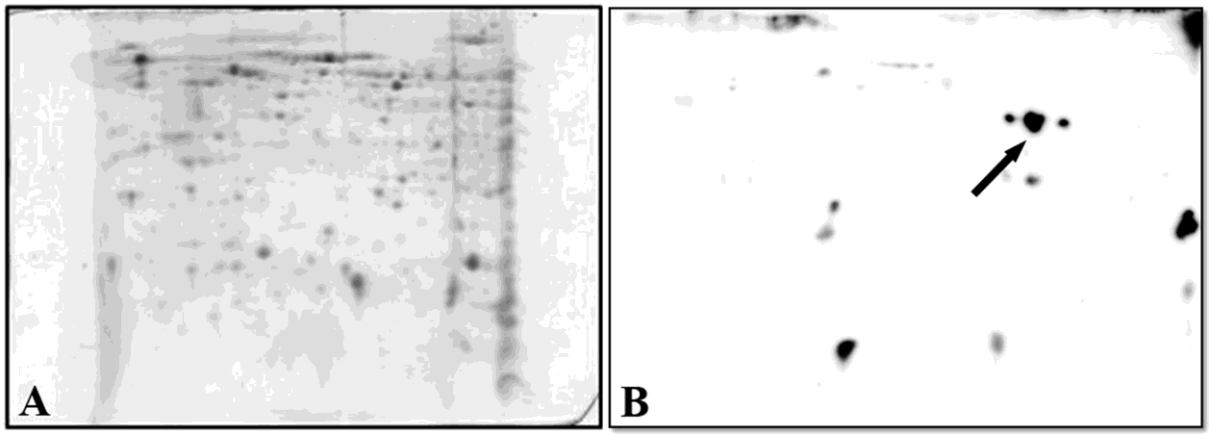

Fig 5. (A) 2-DE map of OT obtained by performing IEF on a 4–7 linear pH range and SDS-PAGE on a constant 12.5 % T in the second dimension, to separate proteins clustered in the single spot shown in Fig 4. (B) Immunoblotting of the gel slab indicated in Panel A. Arrow points to spot originated from separation and identified as FliD.

As a validation of data discussed above, no spot was reactive against the antibody in SG profile (data not shown). After spot excision and tryptic digestion, LC-MS identification confirmed the presence of flagellar protein FliD under this spot (Table 3).

Accession	Description	Mass	Score (%)	Coverage (%)	Query matched
/	Flagellar protein FLID OS=Midichloria mitochondrii	100.58	84	14.64%	13
gi|442747467|gb|JAA65893.1|	Putative erp60 [Ixodes ricinus]	52.115	98	6.45%	3

Table 3 List of proteins that confirmed the presence of FliD.

The primary sequence of peptides found for identification of protein analyzed was included in Table S5 of Supporting Information.

3.4.4 DISCUSSION

Analysis of common proteins differentially expressed

A recent study presented the identification of hundreds of proteins in salivary glands of *I. ricinus* in the presence of the pathogenic tick-borne spirochete *B. burgdorferi,* demonstrating that the expression of proteins modulated by infection differed as a function of the various strains of *B. burgdorferi* (Cotté et al., 2014). In the present study, a total of 47 spots, 27 from OT and 20 from SG were selected for sequencing. Our results were fully congruent with those previously published, validating our approach and confirming the high expression level of a number of proteins, such as Heat shock protein, Protein disulfide isomerase, Enolase, Actin, Hemelipoglycoprotein precursor (putative) etc. This sequencing effort detected only proteins that could be readily identified as belonging to the tick proteome, or that could not be identified unambiguously, but did not reveal the presence of proteins belonging to the bacterial symbiont *M. mitochondrii*. To evaluate whether detection of symbiont proteins was possible, we thus designed a specific immunoproteomic approach, described above and discussed below.

To explore qualitative and quantitative differences between SG and OT of *I. ricinus* infected by *M. mitochondrii,* and to identify tissue-specific proteins, as well as proteins involved in the interaction between the bacterium and the tick, we compared the proteomic profiles of these tissues. The reproducible patterns generated for both tissues evidenced 170 ± 25 spots shared between SG and OT. In addition, 81 protein spots were exclusive of SG profile and 57 spots were detected solely in OT. In particular, we selected and investigated the 10 proteins that exhibited the largest changes in density (4- to 18-fold increase/decrease, see Fig 2, Fig 3 and Table 1). Proteins under spots 1 and 10, both identified as putative heat shock proteins (HSP), were heavily differentially expressed. Putative HSP70 detected under spot 1 was 6-fold more abundant in SG than in OT. By contrast, the putative HSP identified under spot 10 was 11-fold more abundant in OT than in SG. Heat shock proteins are chaperones that, together with other stress response proteins, are well known to protect cells and organisms from environmental stress. HSP70, is involved in many cellular processes, including folding and refolding of nascent and/or misfolded proteins, protein translocation across membranes, and degradation of terminally misfolded or aggregated proteins (Mayer and Bukau, 2005). The role played by HSP proteins in the growth and survival of *I. ricinus* is potentially very important. Being involved in the binding and presentation of antigens to the immune system, they constitute candidate molecules that could be involved in tick immune response to pathogen infection.

Interestingly, a connection may exist between pathogen infection and tick response to stress conditions. In response to heat and other stress (cold, hunger), nearly all ticks undergo diapause. Indeed HSPs involved in the diapause of multiple species of insects were reported (Rinehart et al., 2007), suggesting that they may play key roles in the physiological response to stress of other arthropods, such as ticks. HSP70 is more expressed in salivary glands than in ovarian tissue and midgut and its expression increases with female tick feeding, suggesting a possible role of this protein during blood ingestion and/or digestion (Busby et al., 2012). We speculated that this could be ascribed to the great changes in structure that the salivary glands of hard ticks undergo during blood feeding with an increase in size and the acceleration of protein synthesis (Sonenshine and Hynes, 2008). However, the fact that HSP70 was found to be down regulated in *Anaplasma phagocytophilum* infected whole *Ixodes scapularis* ticks, guts and salivary glands (Busby et al., 2012), may suggest that these proteins have a different function during pathogen infection.

The protein identified under spot 3 was β-actin. The reason of its 5-fold higher expression in SG compared to OT is still a matter of speculation. First, we hypothesized that this was a consequence of the importance of SG in tick feeding. Actin is an important structural protein required for exoskeleton rearrangement during tick engorgement (Narasimhan et al., 2004) and is a common target of many bacterial proteins. It has been shown that the cellular responses induced by a variety of stimuli and pathogens involve changes in cell morphology and the polymerization state of actin (Stevens et al., 2006; Gouin et al., 2005; Cameron et al., 2000). Studies in prokaryotes and eukaryotes demonstrated that nutrition and stress affect the expression of housekeeping genes (Horigane et al., 2007). For example the low expression of actin shown in the unfed first instars nymphs of *I. scapularis* is likely due to low nutrition levels since they have not yet taken the blood meal and the nutrients incorporated into the eggs have been depleted by larval development (Schwan, 1996). The significant differences observed during and immediately after feeding in females are likely related to the dynamic changes that occur in the physiology of ticks preparing for reproduction. It has also been shown that silencing the expression of actin in the soft tick *Ornithodoros moubata* resulted in impairment of tick feeding by a global attenuation of tick activity unrelated to specific function associated with engorgement (Horigane et al., 2007). Finally, further aspects should be taken into consideration. *I. ricinus*, as *I. scapularis*, are vectors of bacterial pathogens including *A. phagocytophilum,* and *B. burgdorferi* (Schwan, 1996; Dumler et al., 2005). To persist in their hosts, obligate intracellular bacteria have evolved a variety of mechanisms including modulating host signaling and the actin cytoskeleton (Bhavsar et al., 2007). If this

hypothesis proves correct, it may be speculated that the high concentration of actin detected in SG of *I. ricinus* could be the result of a sort of survival strategy developed by the symbiont *M. mitochondrii* to persist in its arthropod vector.

The identification of enolase under spots 4 and 5 attracted our interest. Alpha-enolase, one of the most abundantly expressed proteins in human cytosol, is a key glycolytic enzyme that converts 2-phosphoglycerate to phosphoenolpyruvate (Pancholi, 2001). In blood-feeding arthropods this protein is secreted in saliva and inoculated into the host during feeding. The finding of 4-fold higher expression of enolase in SG compared to OT (spot 5) was not surprising. This result may account for one of the pivotal roles of this enzyme. Enolase, in fact, promotes fibrinolysis and maintains blood fluidity during blood ingestion and distribution in the tick midgut. Fibrinolysis is the natural process of fibrin clot solubilization and, in ticks, this process is essential for dissolving any clot that might be formed during feeding, as well as preventing clotting of the ingested blood meal in its midgut (Maritz-Olivier et al., 2007). This said, the higher expression level in OT (10-fold more expressed than in SG) of another putative enolase (spot 4) was a result apparently in contradiction with the previous one. However, the multifunctionality of this protein in both prokaryotes and eukaryotes may probably account for this finding. In fact, it has been shown in *Rhipicephalus microplus* (Moraes et al., 2007) that, to support the energy-intensive processes of embryogenesis, before blastoderm formation, glycogen reserves are preferentially mobilized. As a consequence, protein degradation and gluconeogenesis intensify to supply the embryo with sufficient glucose to allow glycogen re-synthesis. If glycogen is the main energy source during the early stages of *R. microplus* embryogenesis, protein degradation increases during late embryogenesis (Moraes et al., 2007). Thus, the use of amino acids as a substrate for gluconeogenesis and the subsequent glycogen re-synthesis play an important role during the stages of *R. microplus* embryogenesis. Protein metabolism depends strongly on the substantial expression and activity of carbohydrate metabolism enzymes and alpha-enolase is a key glycolitic enzyme (da Silva et al., 2015).

The protein identified under spot 6 was disulfide isomerase (PDI), a 55 KDa multifunctional protein that participates in protein folding, assembly, and post-translational modification in the endoplasmatic reticulum (Liao et al., 2008). The fact that it was 18-fold-more expressed in SG than in OT was not surprising. This protein, together with other saliva enzymes which are putatively associated with antioxidant functions (i.e. glutathion-S-transferase, cytochrome c oxidase, oxidoreductase, NADH dehydrogenase), plays an important role in oxidative stress (Radulović et al., 2014). Tick-feeding, in fact, induces injuries and oxidative stress leading to

production of reactive oxygen and nitrogen species (ROS and RNS) as part of the wound healing mechanism and anti-microbial defenses. Several lines of research have shown that many parasites including ticks are susceptible to ROS and RNS, as revealed by high expression of anti-oxidant enzymes in these parasites or improved survival of these parasites when anti-oxidant system of their hosts are impaired (Radulović et al., 2014). The production of antioxidant enzymes can be considered an evasion mechanism of the immune response used by tick for improving the feeding efficiency, and collaterally enhancing transmission of tick-borne diseases. It is also interesting to note that, given that the tissue destroying effects of oxidative stress products are non-selective, there is a possibility that tick saliva anti-oxidants are protective to host tissue (Radulović et al., 2014).

Identification of FliD

One of the goals of this study was to evaluate whether it is possible to detect proteins from the bacterial symbiont *M. mitochondrii* starting from protein extracts of ovaries and salivary glands of the hard tick *I. ricinus*. Due to the higher symbiont load in OT we expected this to be easier in this tissue, however, none of the 2DE gel spots identified resulted to be of *M. mitochondrii*, neither from the OT, nor from the SG. We thus questioned whether this was a result of the low abundance of symbiont proteins or to some kind of technical issue. To investigate this, we performed an immunoproteomic approach based on the detection of a single *M. mitochondrii* protein (FliD) in a blotting experiment after narrow range pH gradient (linear pH 4-7) 2DE separation of OT and SG (see materials and methods). As shown in Fig 5, a spot potentially corresponding to FliD, based on the chemical properties of the protein, was detected in OT blotting, but not in SG blotting. When analyzing the corresponding region in the original 2DE gel, a cluster of spots was detected. Careful excision of these spots followed by LC/MS-MS allowed the identification of erp60, as well as of the expected *M. mitochondrii* protein FliD. This result indicates that symbiont proteins can indeed be identified using a proteomic approach, but that this is impaired by low protein quantities and by clustering with *I. ricinus* proteins. Specific approaches, such as the use of alternative narrow range pH gradients, immunoproteomic detection are thus needed to investigate the symbiosis between *I. ricinus* and *M. mitochondrii* from a proteomic point of view.

Here we presented a methodological framework that will pave the way for future studies on the proteomics of *I. ricinus*, with the goal of better understanding the biology of this vector and of its symbiont *M. mitochondrii*, but also to be the basis of immunoproteomic approaches

that could prove useful for detecting novel antigenic proteins for innovative diagnostic and vaccination approaches.

3.4.5 SUPPORTING INFORMATION

Table S1 Copy numbers of *gyrB* and *cal* genes and the *gyrB*/*cal* x1000 ratios.

Pool code	*gyrB* copies	*cal* copies	*gyrB/cal* ratio x 1000
OV 1	373899.9594	37.66598829	9926726.373
SG 1	13880.81163	34.89770488	397757.1499
OV 2	1872603.455	1500.759257	1247770.718
SG 2	168.2698052	9.937737539	16932.40585
OV 3	3738999.594	1060.761728	3524825.128
SG 3	2437.79344	487.6015747	4999.560228
OV 4	1265223.304	328.3030099	3853827.916
SG 4	116.9799564	88.44119687	1322.686265

Table S2 Complete list of the identified proteins

Accession	Mass kDa	Score (%)	Description	z	Peptides
gi|215494047|gb|EEC03688.1|	71.165	99	heat shock protein, putative [Ixodes scapularis]	1	ITITNDK
				1	LIGDAAK
				1	NTTIPTR
				1	STLEPVEK
				2	SQVHDIVLVGGSTR
				1	DNNLLGK
				2	VEIIANDQGNR
				2	STAGDTHLGGEDFDNR
				2	WLDTNQLADK
				1	EIAEAYLGK
				2	TTPSYVAFTDTER
				2	NALESYSFNIK
				2	IINEPTAAAIAYGLDK
				2	FEELNADLFR
				2	QKELEQVCNPIITK
gi|442760941|gb|JAA72629.1|	34.898	**99**	Putative carbonic	2	APTDGPILK

			anhydrase, partial [Ixodes ricinus]		
				1	APTDGPILK
				2	QSPIDIVQK
				2	NVQSDPFLAENPLR
				2	EPIEVSQEQFEAFR
gi|442747467|gb|JAA65893.1|	52.115	**99**	Putative erp60 [Ixodes ricinus]	2	TNDPPVPLIK
				2	TLADEDVLVVK
				2	MTNDFSVENLEK
				2	FLEEYLAGNVK
gi|442747295|gb|JAA65807.1|	54.708	**99**	Putative erp60 [Ixodes ricinus]	2	TNDPPVPLIK
				2	MTNDFSVENLEK
				2	TLADEDILVVK
				2	FLEEYLAGNVK
gi|122555|sp|P02073.1|HBB_ALCAA	16.223	**99**	RecName: Full=Hemoglobin subunit beta	1	MLTAEEK
				2	VVTGVANALAHR
				2	LHVDPENFR
				3	LHVDPENFR
				2	VLDAFSEGLK
				1	VLDAFSEGLK
				2	LLVVYPWTQR
gi|122678|sp|P21380.1|HBB_RANTA	16.167	**99**	RecName: Full=Hemoglobin subunit beta	2	VVTGVANALAHR
				2	LHVDPENFR
				3	LHVDPENFR
				2	AAVTGFWGKVK
				1	AAVTGFWGK
				2	LLVVYPWTQR
gi|556054634|gb|JAB70362.1|	161.482	**99**	putative vitellogenin-2 [Ixodes ricinus]	1	GSLLSK
				2	ENAEGKPLGINR
				1	DIALPVYK
				1	YSFTK
				1	SETAYLR
				1	EVYLSAK
				2	KFALDQTQDAK
				2	GVLSIFQLDLVK
				2	FALDQTQDAK
				2	LLNQVVGPQPGSTK
				2	SQVILSSGYDPK

				2	LNNLAVFHEGK
				2	YVTTFDLSTDKDK
				2	EVEDALPITDR
				2	FYYATQNPEWHPR
				2	GLHDYWYESDDR
				3	VHHIAQSFPQSDEAESLDELK
				2	LTDDEAEHFLGK
				3	EVEDALPITDRDYDHVYGR
				1	FESWGLDK
				2	FPEPEWER
				2	YDYGGSSVFAQVR
				2	TNPDLYLLK
				3	TLDLEEVHDANTDTQLPDDLEK
				2	TLDLEEVHDANTDTQLPDDLEK
				1	IAAFVNVLK
				3	FISGLNHLAALEYEDSDIKDVHSK
				3	DYFHGYSFETVSLK
				2	NYFGEVLGYGLNVK
				2	ELQFTLVPAAQDTTTEVEVDLGYK
gi|556054820|gb|JAB70455.1|	175.61	**99**	putative vitellogenin-2 [Ixodes ricinus]	1	GSLLSK
				2	ENAEGKPLGINR
				1	AFIQTTK
				1	DIALPVYK
				1	YSFTK
				1	SETAYLR
				1	EVYLSAK
				2	KFALDQTQDAK
				2	LHVVSQVPPVEK
				2	FALDQTQDAK
				2	LLNQVVGPQPGSTK
				2	SQVILSSGYDPK
				2	LNNLAVFHEGK
				2	YVTTFDLSTDKDK
				2	EVEDALPITDR
				2	FYYATQNPEWHPR
				2	GLHDYWYESDDR
				3	VHHIAQSFPQSDEAESLDELK
				2	LTDDEAEHFLGK
				3	EVEDALPITDRDYDHVYGR
				1	FESWGLDK
				2	FPEPEWER
				2	YDYGGSSVFAQVR
				2	TNPDLYLLK
				3	TLDLEEVHDANTDTQLPDDLEK
				2	TLDLEEVHDANTDTQLPDDLEK
				1	IAAFVNVLK
				3	FISGLNHLAALEYEDSDIKDVHSK
				3	DYFHGYSFETVSLK

				2	NYFGEVLGYGLNVK
				2	ELQFTLVPAAQDTTTEVEVDLGYK
gi|215508461|gb|EEC17915.1|	151.678	**95**	hemelipoglycoprotein precursor, putative [Ixodes scapularis]	3	KKSKNRHR
				1	GSLLSK
				2	LHVVSQVPPVEK
				2	LNNLAVFHEGK
				2	SQLLISSGYDPK
				2	YDYGGATTFGQIR
gi|215504084|gb|EEC13578.1|	177.654	**92**	hemelipoglycoprotein precursor, putative [Ixodes scapularis]	2	HPELVR
				1	GSLLSK
				1	DIALPVYK
				1	YSFTK
				3	EVEDALPVTDREYDHVYGR
				3	TLDLEEVHDANTDTQLPEDLER
gi|215505979|gb|EEC15473.1|	56.912	**99**	protein disulfide isomerase, putative [Ixodes scapularis]	3	VDATIETQLAETYEVR
				1	TFVDASK
				2	GTNEAVEYNGER
				1	TLEGLSK
				2	RPDLVIAK
				2	FDGTANELEHTK
				2	QLVDESSDIK
				2	AAPEEVTEEEEEEEDKEDK
gi|215495481|gb|EEC05122.1|	60.484	**99**	chaperonin subunit, putative [Ixodes scapularis]	2	VGGSSEVEVNEK
				2	VNDALNATR
				1	TGVAIVK
				1	DGVITVK
				1	VGLQVAAVK
gi|215495481|gb|EEC05122.1|	60.484	**99**	chaperonin subunit, putative [Ixodes scapularis]	1	DDTLLLK
				2	DDTLLLK
				1	LASGVALLK

				3	LVQDVANNTNEEAGDGTTTATVLAR
				2	VAQIRDEIDLSNSEYEK
				2	NVILEQSWGSPK
				2	VVEGSDDFGYDALR
				2	GYISPYFINTSK
				2	AAVEEGIVPGGGTALLR
				2	NTYVNMISAGIIDPTK
				2	KISNVQTLIPALELANTQR
				2	TLSDELEVIEGMK
				2	VEFQDALLLFSEK
				3	TALLDASGVASLLTTAEAVVVELPKEEK
gi|442757975|gb|JAA71146.1|	47.874	**99**	Putative protein disulfide-isomerase [Ixodes ricinus]	2	VDATVETQLAETYEVR
				2	LHNLLFVSK
				2	KSPGFEDILK
				1	NFDEVVFDK
				2	SLMEGAVTSESVQSFVK
				1	TFVQDVLDGK
				2	TFVQDVLDGK
				2	QSLLSQDLPEDWDR
				1	ILEFFGLK
gi|254590119|gb|ACT69481.1|	4.326	**23**	hypothetical protein NRI_0501 [Neorickettsia risticii str. Illinois]	2	IKVR
gi|556054818|gb|JAB70454.1|	15.293	**90**	putative ml domain-containing protein [Ixodes ricinus]	2	FEVDFVAER
gi|442746893|gb|JAA65606.1|	17.198	**99**	Putative nucleoside diphosphate kinase [Ixodes ricinus]	2	NIIHGSDSLPSAEK
				2	FMQASEELLQK
				2	EIALWFNEK
gi|215510729|gb|EEC20182.1|	14.859	**99**	fatty acid-binding protein FABP, putative [Ixodes	1	TSTLLK

			scapularis]		
				2	LAQTSKPSVELK
				2	QFGDKEVTIVR
				2	LNDVVAIR
gi|215491972|gb|EEC01613.1|	54.929	**99**	protein disulfide isomerase, putative [Ixodes scapularis]	2	KYGYK
				2	HATDELK
				1	EAGGIVK
				1	SLVTESTK
				1	LAPEYEK
				2	EHDDFIK
				2	SEPVPETNDGPVK
				1	VAVAENFK
gi|442747295|gb|JAA65807.1|	54.708	**99**	Putative erp60 [Ixodes ricinus]	2	GGEFSADYNGPR
				2	DASLHENFLK
				2	TNDPPVPLIK
				2	TLADEDILVVK
gi|442758229|gb|JAA71273.1|	39.174	**99**	Putative fructose-biphosphate aldolase [Ixodes ricinus]	2	ATVTTLQR
				1	ALQASALK
				2	ATAEAIVAPGK
				1	ATAEAIVAPGK
				2	LQGIGVENTEENRR
				2	LQGIGVENTEENR
				2	GILAADESTSTMGK
gi|215504607|gb|EEC14101.1|	39.448	**99**	fructose 1,6-bisphosphate aldolase, putative [Ixodes scapularis]	2	YVAGSIDSLAADR
				2	VTEQVLAAVYK
				1	VTEQVLAAVYK
				1	QYR

gi|215510720|gb|EEC20173.1|	79.111	**90**	elongation factor, putative [Ixodes scapularis]	2	AYLPVNESFGFTADLR

Table S3 Information concerning the primary sequence of all peptides identified for each protein analyzed.

Spot	Accession	Mass	Score (%)	Description	z	Peptides
1	gi|442756551|gb|JAA70434.1|	72.595	99	Putative heat shock 70 kDa protein 5 [Ixodes ricinus]	2	ITINNDQNR
					1	TIEEAVDEK
					3	WLEQHSDADAEELKEQK
					2	QLADTVQPIVAK
					2	NELESYAYSLK
2	/	/	/	Not detectable	/	/
3	gi|322422107|gb|ADX01224.1|	16.038	90	Beta actin [Ixodes ricinus]	2	DSYVGDEAQSK
4	gi|215497327|gb|EEC06821.1|	21.493	90	Enolase, putative [Ixodes scapularis]	2	HIADLAGNSK
5	gi|442753241|gb|JAA68780.1|	47.145	99	Putative enolase [Ixodes ricinus]	2	VNQIGTVTESIR
					2	NPNSNPGDFLER
					2	AAVPSGASTGIHEALELR
					2	IDIGMDVAASEFYK
					2	MPITKIFARQIFDSR
					2	EALELIMSAISAAGYTGK
6	gi|215491972|gb|EEC01613.1|	54.929	98	Protein disulfide isomerase, putative [Ixodes scapularis]	1	SLVTESTK
					2	EHDDFIK
					1	LNFAVSNK

					2	GGEFSADYNGPR
7	gi|442748259|gb|JAA66289.1|	10.741	20	Putative 3-hydroxy-3-methylglutaryl-coa reductase [Borrelia spp]	2	EVRLFHSVLR
					2	MRPTGTVLQTQR
8	gi|597718071|gb|AHN19768.1|	66.150	75	Serum albumin, partial [Cervus nippon]	2	VGEYGFQNALIVR
					1	IVTDLTK
					1	ADFAEVTK
					2	TVMENFVAFVDK
9	/	/	/	Not detectable	/	/
10	gi|442754645|gb|JAA69482.1|	36.782	82	Putative heat shock protein [Ixodes ricinus]	2	LGDYPNAR
					2	IPDTLAK

Table S4 Information concerning the primary sequence of all peptides identified for each protein analyzed

Accession	Mass	Score (%)	Description	z	Peptides
gi|442747467|gb|JAA65893.1|	52.115	98	Putative erp60 [Ixodes ricinus]	2	GGEFSADYNGPR
				2	DASLHENFLK
				2	FLEEYLAGNVK
gi|556065071|gb|JAB75571.1|	36.27	89	putative actin-2 [Ixodes ricinus]	3	VAPEEHPVLLTEAPLNPK
				2	SYELPDGQVITIGNER
gi|6841058|gb|AAF28881.1|	29.518	25	unknown [Borrelia hermsii];	2	NTPVRFYLNDK

Table S5 The primary sequence of peptides found for identification of protein analyzed.

Accession	Mass	Score (%)	Description	z	Peptides
/	100.58	84	Flagellar protein FliD OS=Midichloria mitochondrii	3	IEVNIKRELANIAGIR
				3	IDVEEIKKIEVNIK
				2	SAHIKSLFNR
				3	AKLTEPVTR
				2	IDVEEIKKIEVNIK
				3	KISSAPLDNIPEEVK
				2	KISSAPLDNIPEEVK
				2	ANFKDIGIDFKQEK
				3	IKFDGVAKSFK
				3	DEVNNRHKLALISK
				1	INSAKAGVR
				2	AFGVDTIIGK
				1	SDAFRR
gi|442747467|gb|JAA65893.1|	52.115	58	Putative erp60 [Ixodes ricinus]	2	GGEFSADYNGPR
				2	DASLHENFLK
				2	FLEEYLAGNVK

3.5 Study of the seroconversion against antigens of the endosymbiont *Midichloria mitochondrii* in rabbits parasitized by *Ixodes ricinus*

In preparation for submission.

3.5.1 AIM OF THE STUDY

The transmission of *M. mitochondrii* after *I. ricinus* bite has been evidenced through direct and indirect evidences in human and other vertebrates (Bazzocchi et al., 2013; Mariconti et al., 2012b). However, the time of seroconversion against the antigens of this symbiont is still unknown. In addition, the kinetic of the antibody response against *M. mitochondrii* has not been investigated yet. The fate of *M. mitochondrii* after inoculation into the vertebrate host, has still to be unveiled, it is not clear if bacteria can survive and replicate inside the vertebrate hosts or if they can be eliminated by the host. The aim of the work was to evaluate the time of seroconversion against *M. mitochondrii* antigens and monitoring the kinetic of the antibody response against the bacterial symbiont in rabbits parasitized by *I. ricinus* ticks, over a time span of 4 months. At this purpose, in this work we performed an experimental infestation of a vertebrate model (rabbit) with wild *I. ricinus* ticks (harboring *M. mitochondrii*) and *I. ricinus* from a lab colony (free of the symbiont) with a follow-up of 16 weeks. Because *M. mitochondrii* bacteria have been observed in previous studies to disappear in tick strains maintained in the lab through generation (Lo et al., 2006), those ticks were used as a control to compare the antibody response of rabbits against ticks harboring *Midichloria*. The obtained results will be considered in the light of including *M. mitochondrii* antigens as potential markers for *I. ricinus* bite. We also screened the presence of circulating *M. mitochondrii* DNA in blood of infested rabbits at different time points after infestation, in order to expand the knowledge about the fate of *M. mitochondrii* once inoculated into the vertebrate host.

3.5.2 MATERIALS AND METHODS

Experimental design

This study 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).

Sixty wild unengorged *I. ricinus* females (naturally infected with *M. mitochondrii*) were obtained from the molting of wild fully engorged nymphs collected from roe deer (*Capreolus capreolus*) in the Chizé forest (western France; 46° 7' 18.89" N, 0° 25' 3.72" W) in November 2014. Sixty unengorged *I. ricinus* from a colony, that has been maintaining in ONIRIS BioEpAR laboratory (Nantes, France) since its sampling in a natural population in the year 2000, were used (lab strain ticks).

Twenty additional lab strain *I. ricinus* (from the same colony of the 60 one used in the experimental infestation) were tested in quantitative real time PCR (qPCR; as described in Sassera et al., 2008) in order to assess the absence of the symbiont. DNA was extracted with Qiagen DNeasy Blood and Tissue Kit following manufacturer instructions with proteinase K incubation at 56 °C overnight. DNA was eluted in 30 µl of sterile water and stored at -80 °C until use.

Six 10-weeks-old New Zealand White female rabbits (healthy and free of any pathogens and/or pathology), kept in single well-separated cages, fed *ad libitum* and used for the experimental infestation. The rabbits were divided in two groups composed by three individuals: i) R1, R2, R3 were parasitized by 20 lab strain *I. ricinus* females each; ii) R4, R5, R6 were parasitized with 20 wild *I. ricinus* females each. Few days before the experimental infestation, each group of 20 ticks were put together with 10 males in order to promote mating and obtain the complete repletion during the feeding on the host. Wild females were coupled with wild males, while lab strain females were coupled with males coming from the same colony. The infestation was performed by putting ticks only on one ear for each rabbit, in order to leave the other one free for subsequent blood withdrawals. Before the infestation, each ear was shaved (after topical administration of lidocaine) and E-collars were put on each rabbit in order to avoid scratching during the blood meal of ticks. A cotton ball was placed in the inner ear in order to prevent ticks to fall into it. Female ticks (together with males) were put on each rabbit and then each ear was isolated using a cotton stockinette sleeve fixed at the base of the ear with surgical tape (3M Micropore). Optimal humidity on the ear was kept

through daily water vaporization. Rabbits were weighted each week in order to control their health and their feeding behavior during the tick infestation.

In order to monitor the kinetic of the antibody response against both *M. mitochondrii* and *I. ricinus* salivary glands (SG) antigens and the presence of circulating bacteria in the bloodstream, nine blood samplings were performed, starting from T_0 (one week before experimental infestation) until T_8 (16 weeks after infestation), following the scheme shown in Table 1.

T_0	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8
-1 week	+1 week	+2 weeks	+3 weeks	+4 weeks	+6 weeks	+8 weeks	+12 weeks	+16 weeks

Table 1 Timeline of serum and blood samplings during the experimental infestation. The thicker line between T_0 and T_1 indicates the infestation of rabbits with ticks.

During each sampling, ~500 µl of EDTA uncoagulated blood and ~2 ml of serum were obtained from the marginal auricular vein. Samples were stored at -80°C until subsequent molecular and serological investigations.

The workflow of the experiment is shown in Figure 1.

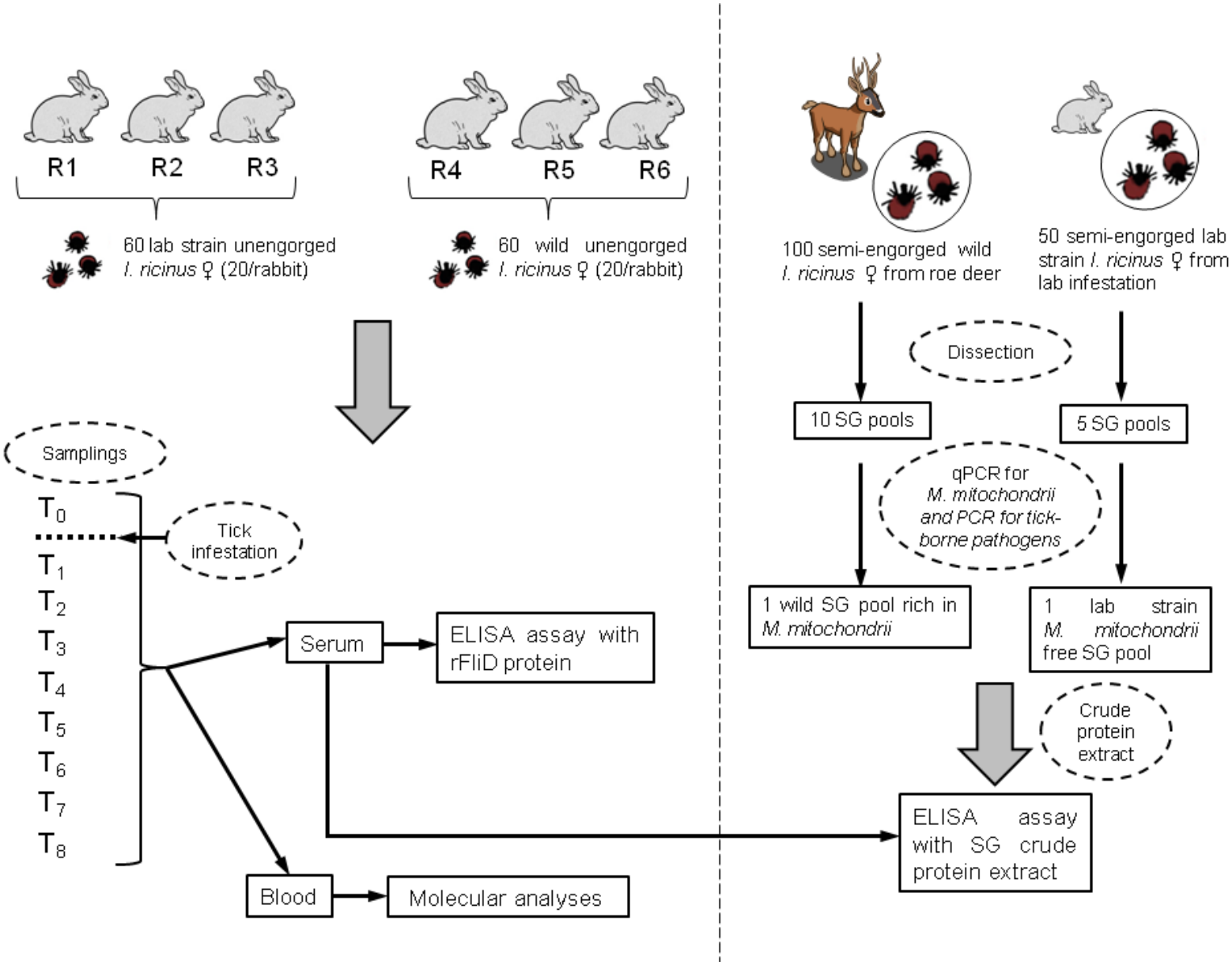

Figure 1 Workflow of the experimental infestation and samples collection. (SG = salivary glands)

Tick dissection for salivary glands recovery

In order to obtain antigens for subsequent serological tests, ticks were gathered for dissection for SG recovery and crude protein extract preparation:

In particular:

- 100 wild semi-engorged *I. ricinus* females (*M. mitochondrii*-positive) collected from roe deer (*C. capreolus*) in the Chizé forest (France);
- 50 *M. mitochondrii*-negative semi-engorged lab strain *I. ricinus* females (from the same colony as the others previously cited) that fed on New Zealand White female rabbits (healthy and free of any pathogens and/or pathology).

Ticks were removed avoiding mechanical damages to the mouthparts with the aim of a tick remover (Tick Twister®, H3D). The semi-engorged stage represent the best repletion stage, as SG reach the optimal size for dissection avoiding degenerative processes typical of SG of fully engorged females (Sauer et al., 1995).

Ticks were manually dissected in a drop of 1X PBS at pH 7.4 (Edwards et al., 2009) with the aid of a stereomicroscope (Leica) and SG were grouped in pools of organs from ten ticks each; each pool was preserved in 100 µl 1X PBS with 1 µl of 100X protease inhibitor (Sigma) and stored at -20°C until use. A total of 10 pools of SG from wild *I. ricinus* females and 5 pools from lab strain *I. ricinus* females were obtained.

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. Each sample was then centrifuged at maximum speed for 10 min and supernatants were recovered, quantified (Nanodrop 1000 Spectrophotometer) and stored at -80°C until use.

The remaining 20 µl were used for molecular analyses: DNA from each sample was extracted using the Qiagen DNeasy Blood and Tissue Kit following manufacturer instructions. DNA was eluted in 30 µl of sterile water and stored at -20°C until molecular analysis.

See Figure 1 for the workflow of the experiment concerning the collection of salivary glands.

PCRs for common tick-borne pathogens

The presence/absence of common tick-borne pathogenic bacteria (Sassera et al., 2011) was assessed in all the extracted DNA from SG pools using previously published PCR protocols

for *B. burgdorferi*, *Anaplasma spp.*, *Ehrlichia spp.* and *Rickettsia spp.* (Pesquera et al., 2015; Michelet et al., 2014; Pistone et al., 2010). The representative sample of 20 unengorged lab strain ticks belonging to the same population used for the experimental infestation was also tested in qualitative PCR to assess the absence of the aforementioned tick-borne pathogens.

qPCR for *M. mitochondrii*

In order to confirm the absence of *M. mitochondrii* from lab strain ticks used in the experimental infestation, DNA extracted from 20 representative individuals of the lab strain population were tested. A previously described Sybr green qPCR approach developed for the detection and quantification of *M. mitochondrii* based on the amplifications of a fragment of *M. mitochondrii gyrB* gene (coding for the protein gyrase B) and a fragment of *I. ricinus* nuclear gene *cal* (coding for the protein calreticulin) was performed (Sassera et al., 2008). The absence of the symbiont was also assessed in the extracted DNA from lab strain SG pools. The same approach was followed for the quantification of *M. mitochondrii* in wild SG pools and for the confirmation of *M. mitochondrii* absence from lab strain SG pools. In order to proceed with specific analyses focused on the seroconversion against *M. mitochondrii* antigens, only SG pools resulting negative for the presence of common tick-borne pathogens were used for subsequent analyses and tested in qPCR for the presence/absence of the endosymbiont.

ELISA assays

ELISA assays were performed on rabbit sera obtained from the experimental infestation using the following antigens: i) *M. mitochondrii* rFliD protein, ii) lab strain *I. ricinus* SG crude protein extract, iii) wild *I. ricinus* SG crude protein extract. The ELISA assays with SG crude protein extracts (from wild and lab strain ticks) as complex of antigens were set up in order to highlight an overall differential antibody response against *M. mitochondrii* antigens. Sera were then tested in a third ELISA using a specific antigen belonging to *M. mitochondrii* in order to detect the antibody response against the endosymbiont of *I. ricinus*.

The antibody response against the complex of antigens obtained from SG crude protein extracts (from both lab strain and wild *I. ricinus*) was determined using a 96-well microtiter plates coated with 1 µg of crude protein extract per well in carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C. Plates were then washed three times with 200 µl of wash

solution (PBS and 0,1% Tween-20) for 5 min and then treated with 100 µl of blocking solution (3% BSA in PBS) for 1 h at 37 °C. After further rinsing of the wells with wash solution, each sample (tested in duplicate) was diluted 1:100 in PBS supplemented with 1% BSA and 100 µl were incubated for 1 h at 37 °C. After three additional 5 min rinses with wash solution, peroxidase-conjugated Goat anti-Rabbit IgG secondary antibody (Sigma) diluted 1:2000 in PBS and 1% BSA was added and incubated for 45 min at 37 °C. After three more washes with wash solution, 100 µl of OPD-substrate (staining solution pH 5, prepared with OPD dissolved in buffer to a concentration of 0.5 mg/ml, 25 mM citric acid, 45 mM sodium phosphate dibasic and peroxide at final concentration of 0.04%) were added and the plates were incubated for 10 min at room temperature, in the dark. The absorbances of the not-stopped reactions were measured at 450 nm. Threshold value was established as the mean optical density $O.D._{450/630}$ of the T0 sera from all the six rabbits used for the experimental infestation plus three times the standard deviation (i.e. mean $O.D._{450/630}$ + 3 standard deviations).

A third ELISA assay was set up in order to detect a specific antibody response against the bacterial symbiont. The aim of this last assay was to evaluate the antibody response against *M. mitochondrii* antigens using the recombinant form of the flagellar protein FliD of *M. mitochondrii* (38 kDa). The recombinant protein was previously obtained as described in Mariconti et al. (2012b). The 96-well microtiter plates were coated with 100 ng of rFliD protein in carbonate-bicarbonate buffer (pH 9.6) and the same protocol explained above was followed.

Detection of *M. mitochondrii* in blood samples

DNA was extracted from EDTA-preserved blood (sampled from all the six rabbits at all time points) using QIAamp DNA Blood Midi Kit (Qiagen) and following manufacturer instructions. Elution was performed in sterile water and stored at -80 °C until use. Quality of the extraction was checked by amplifying *GAPDH* rabbit gene as described in Liang et al. (2008).

A qualitative PCR approach was used in order to detect *M. mitochondrii* circulating in blood from infested rabbits. We used two sets of primers targeting the *gyrB* gene of *M. mitochondrii*: the first set of primers (GyrB-F1: 5'-AAGCTAAGAATTTGGCGTGATG-3', gyrB-R6: 5'-GTTTTGGCTTCATTTGGATTTTC-3'; primers final concentration: 1 µM; amplification size: 776 bp) was used for a first round of amplification. PCR was performed under the

following protocol: 95 °C for 3 min, then 95 °C for 30 s, 57 °C for 30 s and 72 °C for 45 s for 40 times. The reamplification was performed using primers gyrB-F (5'-CTTGAGAGCAGAACCACCTA) and gyrB-R (5'-CAAGCTCTGCCGAAATATCTT-3') (final primer concentration 1 μM; amplification size 125 bp) (Sassera et al., 2008) under the following thermal profile: 95 °C for 3 min, then 40 cycles at 95 °C for 15 s and at 60 °C for 30 s. A total of four replicates from each starting sample were obtained. Blood samples obtained from rabbits infested with lab strain *I. ricinus* were processed separately from bloods of rabbits infested with wild *I. ricinus*; separate analyses on extracted DNA was followed as well, in order to avoid any kind of contamination. The obtained PCR products were separated by electrophoresis on agarose gel, excised, purified and sequenced in order to confirm the specificity of the amplification.

3.5.3 RESULTS

The experimental infestation was carried out without any particular problem for the rabbits. All rabbits maintained approximately the same weight from T_0 to T_8 (data not shown). Wild ticks completed the blood meal in ~10 days, while lab strain ticks showed a delay in completing the engorgement, which lasted ~14 days.

PCR for the presence of common tick-borne pathogens

The presence of tick-borne pathogens was assessed in all DNA samples extracted from SG pools obtained from semi-engorged females (10 pools from wild ticks and 5 pools from the lab strain ticks). Three out of ten wild *I. ricinus* SG pools resulted positive to tick-borne pathogens (i.e. *Borrelia* spp.) and were thus excluded from subsequent analyses, while the remaining seven pools resulted free of these pathogens. The 5 SG pools from the lab strain ticks were all negative for the presence of any tested pathogen.

The 20 unengorged *I. ricinus* from the lab strain (belonging to the same colony of the ticks used for the experimental infestation) were also negative to tick-borne pathogens.

qPCR for *M. mitochondrii*

qPCR analyses were performed on DNA extracted from seven wild SG pools DNA samples that were negative to tick-borne pathogens and on DNA extracted from five lab strain SG pools, in order to respectively determine the richest *M. mitochondrii* wild SG pool (data not shown) and to evaluate the *M. mitochondrii* load (expected to be null) in the lab strain SG pools. Crude extract proteins obtained from the pool with the highest *M. mitochondrii* load was chosen for the serological analyses. Lab strain SG pools resulted negative to the presence of *M. mitochondrii*.

ELISA assays

ELISA assays were performed on all the sera sampled from the six rabbits, from T_0 to T_8. Three types of antigens were tested on the sera: i) wild SG crude protein extract, ii) lab strain SG crude protein extract, iii) *M. mitochondrii* rFliD protein. Sera sampled at T_0 from all rabbits (R1-R6) were used in order to determine the cut-off threshold of each ELISA test.

i) ELISA assay with wild *I. ricinus* SG crude protein extract

The results of the ELISA assay using wild SG crude protein extract that were made reacting with all rabbit sera are indicated in Table 2.

	T0	T1	T2	T3	T4	T5	T6	T7	T8
R1	0.136	0.161	1.747	1.433	1.397	1.240	1.338	1.717	1.620
R2	0.177	0.157	1.267	1.482	1.819	1.782	1.694	1.410	1.240
R3	0.107	0.181	1.859	1.715	1.395	1.267	1.614	2.099	2.022
R4	0.254	1.257	3.258	3.212	3.272	3.270	4.000	3.235	3.139
R5	0.194	1.833	3.251	3.279	3.233	3.237	3.018	3.083	3.258
R6	0.178	1.204	3.424	4.000	4.000	4.000	4.000	4.000	4.000

Table 2 $O.D._{450/630}$ values for IgG antibodies reacting with wild *I. ricinus* salivary glands crude protein extract in rabbits infested with both lab strain and wild *I. ricinus*.

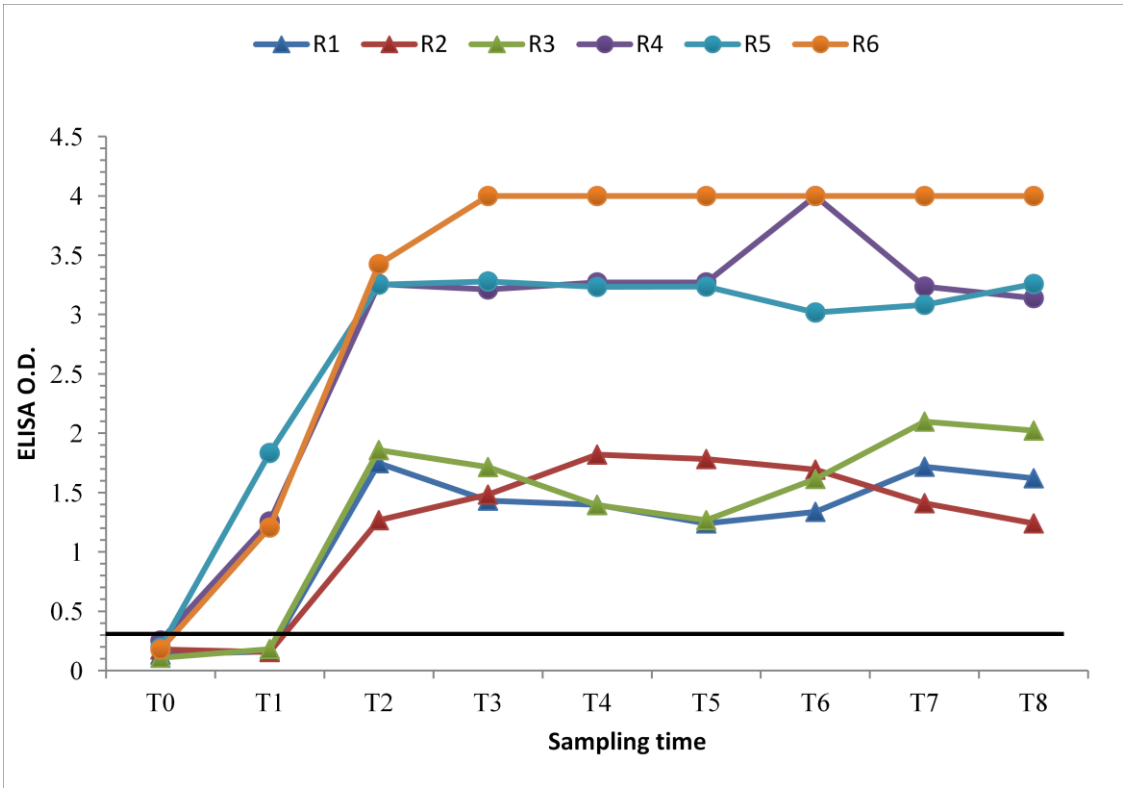

Figure 2 Antibody response, detected by ELISA, against wild *I. ricinus* salivary glands antigens in rabbits infested with both lab strain (▲ R1-R3) and wild (● R4-R6) *I. ricinus* (cut-off threshold set at O.D. 0.32).

The cut-off of the reaction was established at 0.32. The trend of absorbance values from all the sampled rabbit sera reacting with wild *I. ricinus* SG crude protein extract is shown in Figure 2. A clear distinction between O.D. measurements can be observed between the two groups of rabbits infested respectively with wild *I. ricinus* and lab strain *I. ricinus*. Indeed, rabbits infested with lab strain ticks presented lower O.D. values compared to the ones with wild *I. ricinus*. Positivity in rabbits infested with wild *I. ricinus* is observed starting from one week after infestation (T_1) and reaches a plateau in antibody response from T_2. Rabbits infested with lab strain ticks show positivity to wild SG antigens starting from T_1 and show a similar pattern of response among each other.

ii) ELISA assay with lab strain *I. ricinus* SG crude protein extract

The results from the second ELISA assay, using the lab strain SG crude protein extract, are reported in Table 3; the cut-off value was established at 0.22.

	T0	T1	T2	T3	T4	T5	T6	T7	T8
R1	0.138	0.204	1.766	1.816	1.588	1.315	1.137	1.298	1.440
R2	0.155	0.267	1.215	1.143	1.250	1.418	1.784	1.419	1.239
R3	0.109	0.160	1.337	1.575	1.482	1.305	1.241	1.343	1.410
R4	0.166	0.740	1.814	1.806	1.740	1.264	1.313	1.435	1.482
R5	0.143	0.693	0.926	1.355	1.414	1.610	1.130	1.399	1.299
R6	0.089	0.423	1.803	1.877	1.617	1.486	1.514	1.941	1.910

Table 3 $O.D._{450/630}$ values for IgG antibodies reacting with lab strain *I. ricinus* salivary glands crude protein extract in rabbits infested with both lab strain and wild *I. ricinus*.

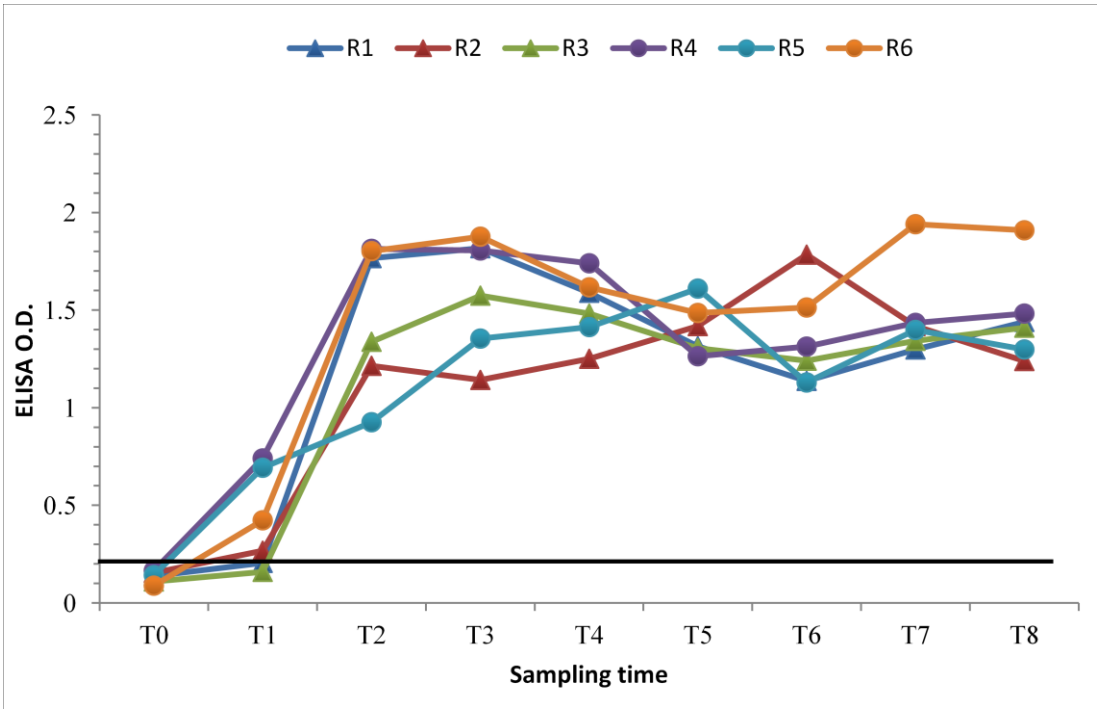

Figure 3 Antibody response, detected by ELISA, against lab strain *I. ricinus* salivary glands antigens in rabbits infested with both lab strain (▲ R1-R3) and wild (● R4-R6) *I. ricinus* (cut-off threshold set at O.D. 0.22).

The kinetic of the antibody response appears similar for the two groups of infested rabbits (Figure 3), no difference was observable between lab strain *I. ricinus* and wild *I. ricinus* infested rabbits. Seropositivity against lab strain SG antigens rises between the first and the second week after infestation.

iii) ELISA with rFliD protein

A third ELISA assay was performed on sera from rabbits R1-R6 using *M. mitochondrii* rFliD protein as antigen. O.D.$_{450/630}$ values are reported below (Table 4).

The cut-off of the reaction was established at 0.16. The kinetic of the antibody response is shown in Figure 4. Rabbits infested with lab strain ticks showed an overall seronegativity to *M. mitochondrii* rFliD (a total of 18 out of 27 time points O.D. values under the threshold and seven out of 27 borderline values). Two measurements over the threshold were detectable around 8-12 weeks after infestation with lab strain *I. ricinus* in R2.

	T0	T1	T2	T3	T4	T5	T6	T7	T8
R1	0.121	0.114	0.151	0.122	0.138	0.115	0.132	0.140	0.152
R2	0.138	0.107	0.118	0.159	0.160	0.189	0.194	0.149	0.137
R3	0.111	0.122	0.161	0.162	0.126	0.099	0.149	0.126	0.158
R4	0.102	0.168	0.202	0.192	0.163	0.159	0.160	0.160	0.160
R5	0.1035	0.16	0.154	0.163	0.215	0.176	0.161	0.163	0.156
R6	0.0912	0.193	0.161	0.148	0.131	0.131	0.148	0.147	0.170

Table 4 O.D.$_{450/630}$ values for IgG antibodies reacting with rFliD in rabbits infested with lab strain and wild *I. ricinus*.

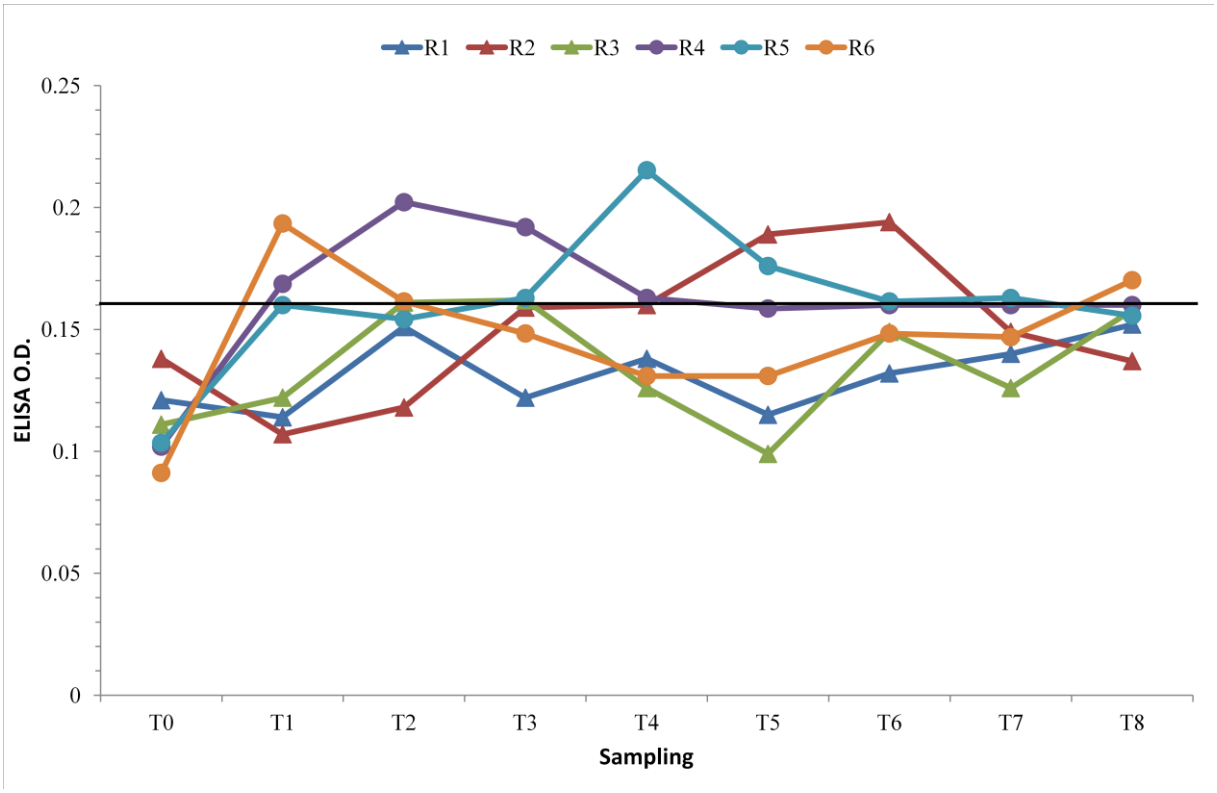

Figure 4 Antibody response, detected by ELISA, against rFliD protein in rabbits infested with lab strain (▲ R1-R3) and wild (● R4-R6) *I. ricinus* (cut-off threshold set at O.D. 0.16).

Seroconversion against rFliD can be observed in Figure 4 at one week after the infestation with wild *I. ricinus* (rabbits R4-R6). For two out of three rabbits infested with wild ticks, a weak, borderline seropositivity against *M. mitochondrii* rFliD protein can still be observed until the end of the experiment (16 weeks after the tick infestation). The antibody response against the flagellar protein appeared to be higher between the first and the fifth week after tick infestation, with a decrease reaching the cut-off threshold by the end of the screening. One rabbit (R6) showed an initial increase in the antibody response against rFliD, but was followed by decreasing and negative O.D. values. However, an increase of the antibody response over the cut-off threshold can be than observed at T_8.

Presence of circulating *M. mitochondrii* DNA in blood samples

Results from *M. mitochondrii* detection in rabbit blood samples are summarized in Table 5. Four replicates were obtained starting from each sample, the results were expressed as "+" when one or two out of four replicates resulted positive to *M. mitochondrii* detection, "++" when three or all replicates were positive. Some serum samples from rabbits infested with lab strain ticks resulted weakly positive at some time points after tick infestation (R1: 4/8 positive

time points; R2: 2/8 positive time points; R3: 2/8 positive time points). PCR positivity at almost all time points after T_0 was observed in two out of three rabbits infested with wild *I. ricinus* (R4: 8/8 positive time points; 6/8 positive time points). For one rabbit (R6), the one that showed a weak seroconversion against rFliD protein, blood positivity for *M. mitochondrii* was detected only for three out of eight time points after tick infestation.

	R1	R2	R3	R4	R5	R6
T_0	-	-	-	-	-	-
T_1	-	-	-	+	+	+
T_2	-	-	-	++	+	-
T_3	+	-	+	+	+	-
T_4	-	+	-	++	++	-
T_5	-	++	++	+	+	-
T_6	+	-	-	+	+	+
T_7	+	-	-	+	-	+
T_8	+	-	-	+	-	-

Table 5 *M. mitochondrii* positivity in blood samples.

3.5.4 DISCUSSION AND CONCLUSIONS

In this work serological and molecular analyses were performed in order to determine the time and the duration of seroconversion of a vertebrate model against antigens of *M. mitochondrii.*

ELISA assays

The comparison of ELISA test results performed using the pack of antigens of SG crude protein extracts obtained from wild and lab strain *I. ricinus* showed an interesting O.D. values trend for the serological titers. When using wild SG crude protein extract, higher above-threshold O.D. values are observable for sera of rabbits infested with wild *I. ricinus* ticks compared to sera of rabbits infested with lab strain ticks. This is probably due to the presence of an antibody response produced against tick SG antigens coupled with *M. mitochondrii* antigens in sera from rabbits infested with wild ticks (and possibly against antigens from other

microorganisms harbored by lab strain *I. ricinus* and not tested in this work). This result was confirmed by testing sera against lab strain SG crude protein extract. In this case, the obtained O.D. values showed a similar trend for both sera from rabbits infested with wild and lab strain ticks, as the only antigenic molecules were the ones belonging to *I. ricinus* SG (and potentially to other microorganisms present both in wild and in lab strain ticks and not tested in this work).

Immunoplates coated with *M. mitochondrii* rFliD protein can evidence antibodies produced against *M. mitochondrii* flagellar cap antigen in humans (Mariconti et al., 2012b) and animals (i.e. dogs; Bazzocchi et al., 2013) exposed to tick bites. rFliD protein was firstly chosen as *M. mitochondrii* antigenic marker due to its characteristic as surface protein, its high specificity (Mariconti et al., 2012a) and lacking of cross-reactivity observed between this recombinant protein and other proteins belonging to other tick-borne bacteria (for example *B. burgdorferi*). Sera from rabbits infested with wild *I. ricinus* showed the expected positive response against *M. mitochondrii* antigen rFliD, but, even though a positive antibody response was still observed until the end of the screening, the highest O.D. values were observed approximately within one month after the infestation. A decrease of the O.D. values under the cut-off threshold could be due to the elimination of bacteria by the host immune defense or could be caused by a difference in the flagellar proteins expression in different phases of the cycle of *M. mitochondrii*. The weak antibody response of one rabbit infested with wild ticks (and thus harboring *M. mitochondrii*), could be attributed to inter-individual differences in *M. mitochondrii* load (as observed in Sassera et al., 2008) or to some inter-individual differences in the ability of conveying *M. mitochondrii* into the vertebrate host. In some cases, inhibition of endosymbiont transovarial transmission can be blocked by the presence of a second bacterial species. This is observed, for example, in *Dermacentor andersoni* ticks infected with *Rickettsia montana* and/or *Rickettsia rhipicephali*, which could acquire secondary infection with *Rickettsia rickettsii*, but are unable to maintain both infections via transovarial transmission (Felsheim et al., 2009). A difference in the innate immune response among the infested rabbits could also be considered as one of the possible variables in the under-/above-threshold O.D. values against rFliD protein.

PCR analyses on blood samples

In agreement with the previous results based on the ELISA tests using rFLID protein, suggesting an antibody reaction against *M. mitochondrii* antigens in rabbits infested with lab strain ticks (R1-R3), PCR analyses showed that R1-R3 rabbits presented *M. mitochondrii* DNA in their blood, at least during some time points of sampling. Those results suggest that at least some lab strain ticks can still harbor *M. mitochondrii* at low percentage and are able to covey the symbiont, so that the bacterial DNA can still be detected in the vertebrate host even 6-12 weeks after infestation. The lab strain ticks used for the experimental infestation (at least few of them), supposed to be completely free of *M. mitochondrii*, were able to transmit *M. mitochondrii* to the vertebrate host. Despite the long time maintaining of the ticks in laboratory conditions, a documented reduction to 18% of positive females observed after five generations raised in a laboratory colony (Lo et al., 2006) and *M. mitochondrii* qPCR negativity for 20 representative individuals of this population, the symbiont was not completely depleted. It is plausible that the number of *M. mitochondrii* bacteria harbored by these lab strain *I. ricinus* could have been reduced over the generations and the qPCR method was not sensitive enough (lower qPCR limit is 10 *gyrB* copies/µl) to detect the low bacterial load. Another explanation could be the presence of *M. mitochondrii* only in some isolated individuals belonging to the lab strain colony and not present in the individuals used for the qPCR. Positive PCR results for *M. mitochondrii* in rabbits infested with lab strain ticks, together with seropositivity to rFliD for those rabbits, is compatible with the fact that, in humans, a single tick bite can induce antibody response against *M. mitochondrii* (Mariconti et al., 2012b). We can assume that low *M. mitochondrii* load in some (or all) ticks used for the infestation can induce an antibody response against the flagellar protein FliD in rabbits infested with a total amount of 20 ticks. Concerning this topic, more studies should be performed in order to obtain the complete depletion of the symbiont from the tick host.

PCR replicates for the detection of *M. mitochondrii* showed that the number of bacteria is probably not particularly high. We do not know if this can be due to the relatively small volume of sampled blood (which might not allow to retrieve enough circulating bacteria) or if it is caused by a reduced transmission from the tick bite under laboratory experimental conditions. This aspect needs to be better investigated in the future. At the same time, replication/survival of the bacteria inside the vertebrate host is still not clear as well as the possible ability of *M. mitochondrii* invading specific cells/organs and thus allowing it to hide from the detection into the blood stream.

In conclusion, with this experimental infestation work we were able to show the first seroconversion of a vertebrate model against *M. mitochondrii*. The seroconversion occurs approximately around the first and the second week after tick infestation. The flagellar protein FliD can be considered as an interesting marker for *I. ricinus* bite, although the kinetic of the antibody response against this protein suggests that a possible screening for this marker should be considered only within six month after tick infestation. Additional markers both from *M. mitochondrii* and *I. ricinus* should be considered in view of developing a possible marker for *I. ricinus* bite detection. In addition, no information are available concerning the presence of *M. mitochondrii* in the salivary glands (and its consequent transmission to the vertebrate hosts) of larval and nymphal stages of *I. ricinus*, thus FliD might be considered only in case of adult tick infestation.

This study has shown that *M. mitochondrii* represents an actual pack of antigens that can persist in the vertebrate host for a prolonged time and that its DNA can be detected into the vertebrate up to four month after tick infestation , although only further and in-depth studies will be able to assess if this bacterium could be the cause of specific diseases or degeneration in the host.

3.6 Blood circulation and presence in the subcutaneous muscle layer of a *Midichloria*-like organism (MLO) associated with red mark syndrome in the rainbow trout *Oncorhynchus mykiss* (Walbaum)

In preparation for submission.

3.6.1 AIM OF THE STUDY

In order to better understand the spread of the MLO associated with RMS in skin of affected *O. mykiss* specimens, we performed molecular detection of this bacterium on the epidermis-dermis layer and underlying muscle layer of the skin lesion, comparing the bacterial loads between the two layers. We performed, as well, molecular analyses on organs and blood samples in order to better understand the circulation of the bacterium. Finally, entering and exiting water from a RMS-affected fish pond were filtered and analyzed in order to determine if the MLO is present in the environment.

3.6.2 MATERIALS AND METHODS

Fish samples

A total of eleven rainbow trout were collected in a commercial fish farm in Northern Italy in 2016. Two samplings were performed at different RMS outbreak stages. During the first sampling (March, middle-stage of the condition, affecting ~50% of farmed fish population; hereafter "S1") a total of six individuals were collected from the same pond: two apparently healthy individuals (F1-F2) were picked as negative controls and four specimens (F3-F6) affected by RMS were selected. During the second sampling (April, when the overall farmed fish population was recovering from the condition; hereafter "S2"), a total of five individuals were collected: two negative controls (F7-F8) and three individuals (F9-F11) presenting scar tissue deriving from healing RMS skin lesions (pale yellow/grey patches with reduced redness; Oidtmann et al., 2013). Selected specimens were euthanized with a controlled overdose of MS-222 (tricaine methanesulfonate) before proceeding. Different sterilized scalpels were used for each dissection. For molecular analyses, portions of epidermis-dermis

from the lesion (when present), subcutaneous muscle underlying the skin lesion (when present), healthy skin, sections of liver, trunk kidney, spleen, gills, heart, intestine and brain were collected from each sample. Samples were then stored in RNAlater (Qiagen) at -80°C until use. From each specimen ~1.5 ml blood was drawn from cardinal vein and stored in lithium heparin tubes at -80°C until DNA extraction. For one individual of the first sampling only skin and blood samples were available.

For histological analyses, cross sections of affected skin with subcutaneous muscle, were excised and fixed in 4% paraformaldheyde solution, stored at 4°C for 24 hours and subsequently processed for paraffin inclusion.

Water filters sampling

Two liters of the fish pond entering and exiting water were filtered during each sampling (S1 and S2). Filtration was performed as following: a first filtration was performed using a bibulous paper filter, then a further filtration step was performed with a 0.45 µm filter. Bibulous paper filters and 0.45 µm filters were then stored separately in RNAlater at -80°C until use.

Histology

In order to confirm the diagnosis of the disease affecting *O. mykiss* individuals, histological observations were performed. Paraformaldheyde-fixed skin with subcutaneous muscle samples were dehydrated through graded ethanol processing and embedded in paraffin wax blocks (Paraplast) for histological analyses. Sections of 3 µm were obtained and subsequent staining with Harris' hematoxylin and eosin Y was performed. The confirmation of the diagnosis was also performed basing on RMS guidelines provided by Oidtmann et al. (2013).

DNA extraction from fish tissues and organs

RNAlater preserved skin and organs were rinsed in sterile 1X PBS before DNA extraction. DNA from these samples was extracted using the DNeasy Blood and Tissue Kit (Qiagen) following manufacturer instructions with an overnight proteinase K incubation step at 56°C. DNA from blood samples was extracted with the QIAamp DNA Blood Midi Kit (Qiagen)

following manufacturer instruction. The extracted DNA was eluted in sterile water and stored at -80°C until use.

DNA extraction from water filters

Crude DNA extraction from water filters was performed as follows: each filter was washed in a 50 ml falcon tube through vortexing for 90 s using a washing solution (1X sterile PBS and 0.2% Tween). The obtained washed sample was then collected and centrifuged at 20,000 g for 10 m at 4°C. The obtained pellet was resuspended in 60 µl of 1X sterile PBS and 1 µl of proteinase K (20 mg/ml) was added; incubation was carried out at 56°C for 2 hours. The lysed pellet was then centrifuged at 16,000 g for 10 minutes and surnatant was recovered and stored at -80°C for further molecular analyses.

PCR detection of the MLO in fish organs and tissues and in water filters

Molecular detection for the presence of the MLO in sampled skin and organs was performed following the protocol for a quantitative real time PCR (qPCR) approach described in Cafiso et al. (2016). Shortly, a fragment of the *16SrDNA* gene of the MLO associated with RMS and a fragment of the *igf1* gene of *O. mykiss* (a single copy gene coding for *insulin growth factor I*; Lloyd et al., 2011) were cloned into a pGEM-T easy plasmid vector (Promega) in order to produce standards for absolute quantification and PCR efficiency was assessed by serial dilutions of purified plasmid DNA, from 10^9 copies/µl to 10 copies/µl. *16SrDNA* and *igf1* gene copies were obtained through a comparison of the qPCR results of each sample with those of serial dilutions of purified plasmid (containing known copy numbers). Bacterial loads were expressed as *16 rDNA*/*igf1* x 1000 ratio.

The detection of the MLO DNA circulating in blood samples and the presence of the MLO in pellets from entering and exiting water filtering was performed with the real time PCR approach explained above (Cafiso et al., 2016). The detection was performed without quantification and amplifying only the bacterial *16SrDNA* gene fragment. The obtained melting curve peaks were compared to those of positive controls in order to verify the detection of the MLO in blood samples. PCR products were subsequently run in agarose gel, purified and sequenced to confirm the specificity of the amplification.

3.6.3 RESULTS

Histological observations

Macroscopically affected skin and muscle samples revealed severe lymphocytic-histiocytic dermatitis. In the central part of the lesion scale loss, scale fragmentation and resorption due to osteoclastic activity was observed (Fig. 1).

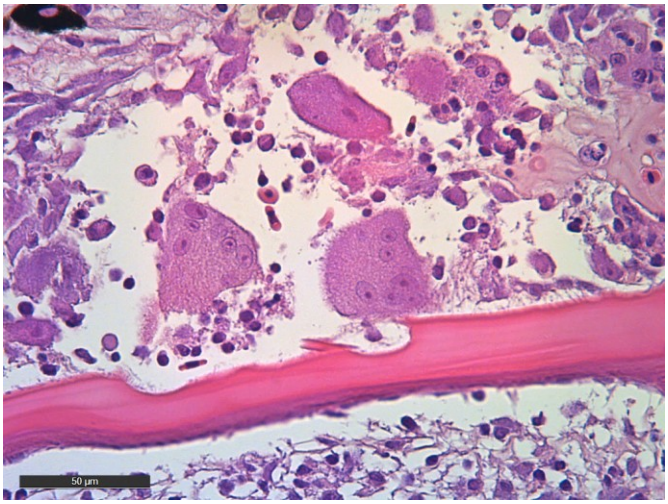

Fig. 1 Osteoclasts (giant multinucleated cells) in the scale pocket. Bar = 50 µm.

Lymphocitic infiltration was recorded also in the adipose tissue of the hypodermis and in the underlying red muscle. Extensive inflammation of the dense connective tissue of the dermis (stratum compactum) increased up to 300% the thickness of this layer in comparison to unaffected skin collected from the same specimens. In comparison to the dermal lesions, the epidermis is only mildly affected with moderate hyperplasia and lymphocytic infiltration. At histological level, no differences were observed between sections from S1 and S2. Examples of histological observations of affected and normal skin are shown in Fig. 2.

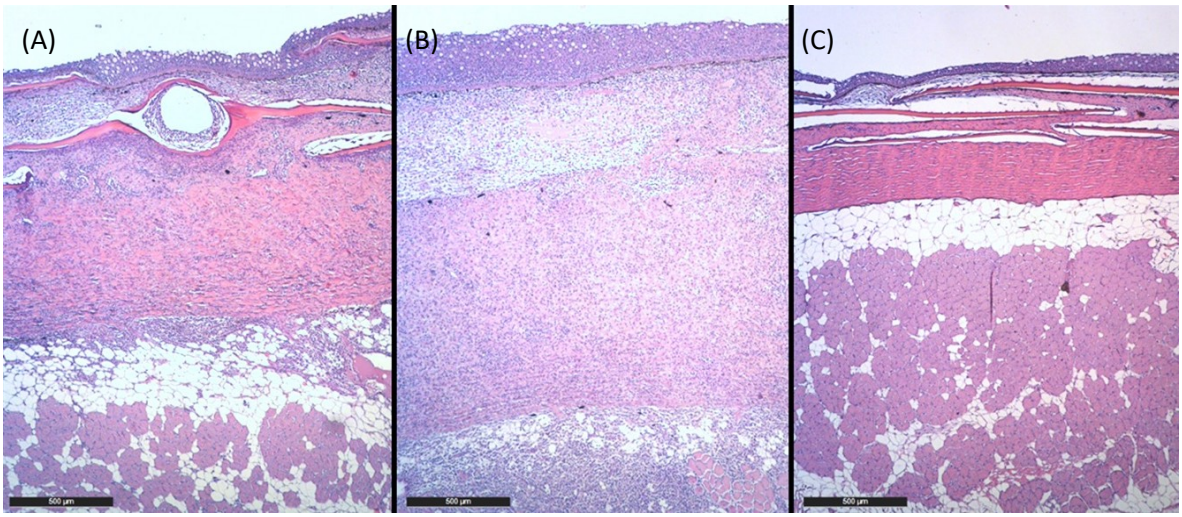

Fig. 2 Histological appearance of skin sections from sampled *O. mykiss* specimens. (A) and (B) Affected skin and muscle of two affected specimens (respectively from S1 and S2); (C) Normal skin. Bar = 500 µm.

Molecular analyses on fish samples

Results from molecular analyses for MLO in organs and tissues are summarized in Table 1. qPCR analyses showed that those specimens that appeared healthy at initial inspection (i.e. negative controls) were negative for the presence of the MLO in skin and organs in both sampling S1 and S2. Normal skin from affected individuals was free of MLO bacteria. Positive samples were found in three out of four RMS-affected fish from S1 (middle-stage of the condition). In these three positive samples, the subcutaneous muscle was always positive for the MLO. For one individual (F3), it was not possible to quantify the presence of bacteria, as the MLO was detected out of the dynamic range of our qPCR method. The result regarding this sample was thus considered only as "positive" to the MLO presence. The highest bacterial load (with a *16SrDNA*/*igf1* x 1000 ratio of 8.3 x 10^{-1}) was found in subcutaneous muscle from F2. For this specimen, positivity for the MLO was also found in the epidermis-dermis layer and in the kidney (4.1 x 10^{-2} ratio). No other organ sample was found positive for the presence of the MLO. For one out of four affected samples from S1 (F1), despite skin lesion was present, no qPCR detection for the presence of the MLO was obtained.

Fish sample	Sampling period	*16SrDNA/igf1* x 1000 ratio		Positive organ	*16SrDNA/igf1* x 1000 ratio in positive organs	Positivity in blood
		Dermis	Subcutaneous muscle			
F3	S1	ND	ND	ND	ND	-
F4	S1	5.0×10^{-1}	8.34×10^{-1}	Kidney	4.11×10^{-2}	+
F5	S1	ND	+	ND	ND	-
F6	S1	ND	2.36×10^{-1}	NA	ND	-
F9	S2	ND	ND	ND	ND	-
F10	S2	ND	ND	ND	ND	-
F11	S2	ND	5.64×10^{-1}	ND	ND	-

Table 1 Detection and quantification of the MLO based on *16SrDNA/igf1* x 1000 ratio in organs and tissues of RMS-affected trout. ND = no bacteria detected; NA = not available.

Among specimens presenting recovering skin tissue from RMS lesions (S2, late stage of the condition), only one RMS-affected individual was positive for the MLO at muscular level with a *16SrDNA/igf1* x 1000 ratio of 5.6×10^{-1}. No additional samples from RMS-affected fish resulted positive neither at skin lesion nor at organs level, and apparently normal skin was negative for the MLO presence. Control specimens were free of bacteria.

Circulating MLO in the bloodstream was assessed through real time PCR and only one specimen (F2) from S1 appeared to be positive. This individual was the one presenting real time PCR positivity in epidermis-dermis, subcutaneous muscle and kidney.

Molecular analyses on water filters

The bibulous paper filters used for exiting water filtration resulted positive for the MLO presence both in S1 and S2. The specificity of the PCR amplification was confirmed after electrophoresis run in agarose gel, band excision, purification and sequencing. Only one DNA sample extracted from exiting 0.45 μm filter in S2 resulted positive for the MLO presence. The DNA extracted from entering water filters was always negative for the presence of the MLO. Results of water filtration are shown in Table 2.

Samples	Bibulous paper	0.45 µm filter
Entering water S1	-	-
Exiting water S1	+	-
Entering water S2	-	-
Exiting water S2	+	+

Table 2 Positive results for presence of the MLO associated to RMS in DNA extracted from water samples subjected to filtration with bibulous paper and 0.45 µm filters. S1 = sampling performed at middle stage of the condition, S2 = sampling performed at late stage of the condition.

3.6.4 DISCUSSION

Although an overall gross appearance of RMS lesions between sampling S1 and S2 was observable, analyses showed that the histological status of the lesions was similar among the sampled individuals. Despite a recovering of the superficial layer of the skin, the underlying strata can still be damaged and subjected to inflammatory processes. Concerning molecular analyses, most of positive samples were observed in deeper lesions, as only subcutaneous muscle underlying the lesion was positive for the MLO. If the etiological agent of RMS could be attributed to the MLO, it is possible that these bacteria may preferentially affect muscular layer. Additionally, this result is in accordance with observations performed by other authors concerning the presence of inflammatory foci in deeper layers of the skin, especially at the beginning of the condition (Metselaar, 2012). It is possible that MLO associated with RMS could present the same tropism towards mitochondria as observed for *M. mitochondrii* in *I. ricinus* (Lo et al., 2006) and can be thus invade these organelles, leading to cell degeneration. This hypothesis is also compatible with RMS symptoms involving muscles of internal organs (i.e. myocarditis and acute inflammation of intestinal muscle; Oidtmann et al., 2013). We could thus hypothesize that these bacteria might invade cells that are rich in these organelles. Negativity for the presence of the MLO in healthy skin samples from unaffected specimens and the absence, as well, from healthy skin tissue of RMS-affected individuals support the hypothesis of the univocal connection between this member of the family *Midichloriaceae* and RMS skin lesions. In this work, positivity in organs (kidney and blood) was observed

only for one specimen, indeed, as already described, internal organs of RMS-affected specimens are not always involved (Cafiso et al., 2016; Oidtmann et al., 2013). The specimen showing a positive internal organ was also found positive for circulating MLO in the bloodstream. This represents the first detection of blood circulating MLO in RMS-affected fish and gives support to the hypothesis about the possible ability of this MLO to spread from skin lesions to internal organs through blood circulation. As the only positive organ was kidney, we can suppose that melano-macrophages, which are normally located in hematopoietic tissues of fish (i.e. spleen and kidney), can act as focal depositories for resistant intracellular bacteria and can perform antigen trapping and presentation to lymphocytes (Agius and Roberts, 2003). The negativity found in organs and blood samples from the other RMS-affected specimens confirmed this speculation. The spread was also observed during previous molecular analyses in RMS-affected trout from Scotland (Cafiso et al., 2016). The dynamics of the spread is still not clear, it might depend on some host characteristics or by some interactions involving the MLO and environmental/vertebrate host conditions. Skin and organ samples from one RMS-affected sample in S1 were negative for the presence of the MLO: these negative results must be considered also in the light of the sensitivity of the qPCR method (which is 10 copies/µl for the *16SrDNA* gene fragment); negative results can be thus be due to the low presence of bacteria in the examined tissue sections. It is plausible that this individual was already recovering from the condition, with a decrease of the bacterial load to undetectable levels. The same result was indeed observed in samples from S2, when skin lesions were healing, with only one specimen positive to the MLO.

Concerning water filtration, it seems that bibulous paper filters represented the best way to recover the MLO DNA from water. The fact that only one 0.45 filter used for S2 exiting water was positive to the MLO presence, while the presence of the MLO was detected after filtration of exiting water with bibulous paper in both samplings, can be explained by the association of these bacteria with bigger cells. The screening of water entering and exiting the fish pond with two different types of filters showed that the MLO is probably and mostly associated with cells which are retrieved during the first step of filtration with bibulous paper. It is thus possible that only few bacterial cells could have slipped away from the first filtration step and could have been recovered after the second step filtration (0.45 µm filter). Negativity for entering water does not mean the absence of the MLO in water outside the pond, but it is more plausible that fish ponds could represent a place where the MLO can accumulate. It is also possible that the low amount of filtered water could have affected the possibility of

obtaining positivity for the MLO in entering water. It could also be possible that applying a third step of filtration using a 0.22 μm filter, free MLO could be gathered.

These results suggest that the MLO associated with RMS might be brought to fish ponds from entering water and it could be harbored by some eukaryotic cells, such as aquatic protists. Ciliates and amoebae are already known to harbor different members of the family *Midichloriaceae* (Szokoli et al., 2016; Boscaro et al., 2013a,b), so it is possible that these unicellular eukaryotes can represent the vector or, at least, a temporary host, of the putative etiological agent of RMS or even being its environmental reservoir. Further analyses focused on studying protists inhabiting fish ponds, free in the water or in the sediment, could be able to shed light on this topic.

4 Conclusion of the PhD project and future perspectives

Midichloriaceae is a bacterial family of high molecular biodiversity and extremely varied in terms of the ecological and taxonomic diversity of their hosts. Therefore, the family *Midichloriaceae* can be seen as a wide bacterial group, still to be investigated under several aspects. The association of members of the family *Midichloriaceae* with some parasites (such as ticks, fleas and amoebae) suggests that these bacteria could be potentially infectious to vertebrates (including humans) and even responsible for medical and veterinarian diseases.

Our results suggest that members of the genus *Midichloria*, in which all midichloriaceae retrieved from ticks are grouped, could have horizontally spread during the evolution through different tick species and genera (until reaching taxonomic level of family; *id est* from *Ixodidae* to *Argasidae*). *Midichloria* bacteria in ticks could have crossed into one tick to another, using vertebrates as a sort of bridge for the horizontal transmission, resulting in different types of relationships in their arthropod hosts (*i.e.* in one case a low prevalence with low bacterial load and horizontal transmission or a stronger relationship, in which the bacterium can assume a mutualistic role within the host, with a strong increase in load per individual, higher prevalence and possibly vertical transmission in the other).

The transmission of *Midichloriaceae* from ticks to vertebrates and their possible infectivity was already reported in literature, especially concerning *M. mitochondrii* in *I. ricinus*. We performed an experimental infestation of a vertebrate model (rabbit) showing how the antibody response of the host against FliD protein of the bacterium is produced soon after the tick bite. The antibody response against the flagellar protein of *M. mitochondrii* is maintained for at least four months after infestation. The presence of circulating DNA of *M. mitochondrii* in bloodstream gives support to these observations. Despite these results, it is still not clear if the bacteria can replicate inside the host or if they can accumulate in some body district. Moreover, comparing the antibody response against *M. mitochondrii* free *I. ricinus* (lab strain) and *M. mitochondrii* positive *I. ricinus* (wild) ticks, we observed that *M. mitochondrii* actually represents an additional package of antigens transmitted from tick to the vertebrate hosts (in addition to specific tick antigens). We cannot conclude that *M. mitochondrii* can cause an overt disease in the vertebrate, but this gives support regarding the possible role of this bacterium in diseases characterized by mitochondrial damage or by alterations in the apoptotic cascade. On the other hand, human diseases suspected to be linked with tick

parasitism (or other ectoparasites) could be re-interpreted and subjected to further investigation. In addition, clinical cases compatible with rickettsiosis or ehrlichiosis in humans/animals parasitized by *I. ricinus*, but characterized by the absence of serological titers for known pathogenic rickettsiae, could be re-investigated for the presence of signs of *Midichloriaceae* infections. Starting from the obtained results concerning the experimental infestation, it will be possible to investigate and develop markers to identify the bite of *I. ricinus*, which is essential not only for the study of the clinical and epidemiological role of this vector, but also to evaluate the hypothesis of the tick bite as a risk factor for the onset of chronic degenerative disease mentioned above. Antigens produced by the symbiont represent an advantage compared to antigens of the tick, as they are indeed conveyed into the host and the bacterium represents a producer of an immunogenic structure that might potentially replicate on site. The use of a combination of antigens from symbiont (and eventually from the tick) could reduce the possibility of cross-reactivity without decreasing the sensitivity, and could enable the development of a multi-band Western blot test, if the ELISA method, that represent the first choice, encountered problems of specificity.

Although a long term rearing in lab strain conditions has showed a significant decrease in the *M. mitochondrii* load in *I. ricinus* (Lo et al., 2006), the results obtained in this PhD work suggest that that a ten-year-captivity is not enough for the complete removal of *M. mitochondrii* from its host. The complete depletion of the symbiont should be pursued through different approaches.

The systemic spread of the MLO associated with RMS in affected *O. mykiss* through blood, together with the detection of this bacterium in different organs, supports the strong association observed between the MLO and the disease. Further studies pursued with the use of specific techniques for the detection of bacteria in tissues and cells, such as detection of the bacterium with FISH or ultrastructural analyses using electronic microscopy. Investigations should be focused also in analyzing the environmental habitat of farmed rainbow trout affected by RMS, with a special consideration for the presence of protists inhabiting water and or/sediment of fish ponds. Considering the presence of Midichloriaceae in different protist species, the vector of the MLO associated with RMS might be found in these eukaryotic hosts.

5 References

Adam, K. (2009). A retrospective epidemiological study of red mark syndrome in Scottish farmed rainbow trout (*Oncorhynchus mykiss*). Marine Scotland Science, Internal Report No 14/09.

Agius, C., and R. J. Roberts. "Melano-Macrophage Centres and Their Role in Fish Pathology." J Fish Dis *26*, 499–509.

Anderson, J.F., and Magnarelli, L.A. (2008). Biology of ticks. Infect Dis Clin North Am *22*, 195–215.

Arthur, D.R. (1965) Ticks of the Genus *Ixodes* in Africa. The Athlone press, London.

Bandi, C., McCall, J.W., Genchi, C., Corona, S., Venco, L., and Sacchi, L. (1999). Effects of tetracycline on the filarial worms *Brugia pahangi* and *Dirofilaria immitis* and their bacterial endosymbionts *Wolbachia* fn1. Int J Par *29*, 357–364.

Baneth, G. (2014). Tick-borne infections of animals and humans: a common ground. Int J Parasitol *44*, 591–596.

Batut, J., Andersson, S.G.E., and O'Callaghan, D. (2004). The evolution of chronic infection strategies in the α-proteobacteria. Nat Rev Micro *2*, 933–945.

Bazzocchi, C., Mariconti, M., Sassera, D., Rinaldi, L., Martin, E., Cringoli, G., Urbanelli, S., Genchi, C., Bandi, C., and Epis, S. (2013). Molecular and serological evidence for the circulation of the tick symbiont *Midichloria* (*Rickettsiales*: *Midichloriaceae*) in different mammalian species. Parasit Vectors *6*, 350.

Beninati, T., Lo, N., Sacchi, L., Genchi, C., Noda, H., and Bandi, C. (2004). A Novel Alpha-Proteobacterium Resides in the Mitochondria of Ovarian Cells of the Tick *Ixodes ricinus*. Appl Environ Microbiol *70*, 2596–2602.

Beninati, T., Riegler, M., Vilcins, I.-M.E., Sacchi, L., McFadyen, R., Krockenberger, M., Bandi, C., O'Neill, S.L., and Lo, N. (2009). Absence of the symbiont *Candidatus* Midichloria mitochondrii in the mitochondria of the tick *Ixodes holocyclus*. FEMS Microbiol Letters *299*, 241–247.

Bhavsar, A.P., Guttman, J.A., and Finlay, B.B. (2007). Manipulation of host-cell pathways by bacterial pathogens. Nature *449*, 827–834.

Bonneau, S., Reymond, N., Gupta, S., and Navarro, C. (2015). Efficacy of a fixed combination of permethrin 54.5% and fipronil 6.1% (Effitix) in dogs experimentally infested with *Ixodes ricinus*. Parasit Vectors *8*, 204.

Boscaro, V., Petroni, G., Ristori, A., Verni, F., and Vannini, C. (2013a). "*Candidatus* Defluviella procrastinata" and "*Candidatus* Cyrtobacter zanobii", two novel ciliate endosymbionts belonging to the "*Midichloria* clade". Microbial Ecology *65*, 302–310.

Boscaro, V., Schrallhammer, M., Benken, K.A., Krenek, S., Szokoli, F., Berendonk, T.U.,

Schweikert, M., Verni, F., Sabaneyeva, E.V., and Petroni, G. (2013b). Rediscovering the genus *Lyticum*, multiflagellated symbionts of the order *Rickettsiales*. Scientific Reports *3*, 3305.

Brenner, D.J., O'Connor, S.P., Winkler, H.H., and Steigerwalt, A.G. (1993). Proposals To Unify the Genera *Bartonella* and *Rochalimaea*, with Descriptions of *Bartonella quintana* comb. nov., *Bartonella vinsonii* comb. nov., *Bartonella henselae* comb. nov., and *Bartonella elizabethae* comb. nov., and To Remove the Family *Bartonellaceae* from the Order *Rickettsiales*. Int J Syst Evol Microbiol *43*, 777–786.

Busby, A.T., Ayllón, N., Kocan, K.M., Blouin, E.F., de la Fuente, G., Galindo, R.C., Villar, M., and de la Fuente, J. (2012). Expression of heat shock proteins and subolesin affects stress responses, *Anaplasma phagocytophilum* infection and questing behaviour in the tick, *Ixodes scapularis*. Med Vet Entomol *26*, 92–102.

Cafiso, A., Sassera, D., Serra, V., Bandi, C., McCarthy, U., and Bazzocchi, C. (2016) Molecular evidence for a bacterium of the family *Midichloriaceae* (order *Rickettsiales*) in skin and organs of the rainbow trout *Oncorhynchus mykiss* (Walbaum) affected by red mark syndrome. J Fish Dis *39*, 497–501.

Cameron, L.A., Giardini, P.A., Soo, F.S., and Theriot, J.A. (2000). Secrets of actin-based motility revealed by a bacterial pathogen. Nature Reviews. Mol Cell Biol *1*, 110–119.

Candiano, G., Bruschi, M., Musante, L., Santucci, L., Ghiggeri, G.M., Carnemolla, B., Orecchia, P., Zardi, L., and Righetti, P.G. (2004). Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. Electrophoresis *25*, 1327–1333.

Carvalho, D.S., Andrade, R.F.S., Pinho, S.T.R., Góes-Neto, A., Lobão, T.C.P., Bomfim, G.C., and El-Hani, C.N. (2015). What are the Evolutionary Origins of Mitochondria? A Complex Network Approach. PLoS One *10*, e0134988.

Chomel, B. (2015). Lyme disease. Revue Scientifique Et Technique (International Office of Epizootics) *34*, 569–576.

Cotté, V., Sabatier, L., Schnell, G., Carmi-Leroy, A., Rousselle, J.-C., Arsène-Ploetze, F., Malandrin, L., Sertour, N., Namane, A., Ferquel, E., et al. (2014). Differential expression of *Ixodes ricinus* salivary gland proteins in the presence of the *Borrelia burgdorferi* sensu lato complex. J Proteomics *96*, 29–43.

Dantas-Torres, F., Oliveira-Filho, E.F., Soares, F.A.M., Souza, B.O.F., Valença, R.B.P., and Sá, F.B. (2008). Ticks infesting amphibians and reptiles in Pernambuco, Northeastern Brazil. Revista Brasileira De Parasitologia Veterinaria = Brazilian Journal of Veterinary Parasitology: Orgao Oficial Do Colegio Brasileiro De Parasitologia Veterinaria *17*, 218–221.

Dergousoff, S.J., and Chilton, N.B. (2011). Novel genotypes of *Anaplasma bovis*, "*Candidatus* Midichloria" sp. and *Ignatzschineria* sp. in the Rocky Mountain wood tick, *Dermacentor andersoni*. Vet Microbiol *150*, 100–106.

Dumler, J.S., and Walker, D.H. (2005). Rocky Mountain spotted fever--changing ecology and persisting virulence. N Engl J Med *353*, 551–553.

Dumler, J.S., Barbet, A.F., Bekker, C.P., Dasch, G.A., Palmer, G.H., Ray, S.C., Rikihisa, Y., and Rurangirwa, F.R. (2001). Reorganization of genera in the families *Rickettsiaceae* and

Anaplasmataceae in the order *Rickettsiales*: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia* equi and "HGE agent" as subjective synonyms of *Ehrlichia phagocytophila*. Int J Syst Evol Microbiol *51*, 2145–2165.

Dumler, J.S., Choi, K.-S., Garcia-Garcia, J.C., Barat, N.S., Scorpio, D.G., Garyu, J.W., Grab, D.J., and Bakken, J.S. (2005). Human granulocytic anaplasmosis and *Anaplasma phagocytophilum*. Emerging Infect. Dis. *11*, 1828–1834.

Dunning Hotopp, J.C., Clark, M.E., Oliveira, D.C., Foster, J.M., Fischer, P., Muñoz Torres, M.C., Giebel, J.D., Kumar, N., Ishmael, N., Wang, S., Ingram, J., Nene, R.V., Shepard, J., Tomkins, J., Richards, S., Spiro, D.J., Ghedin, E., Slatko, B.E., Tettelin, H., and Werren J.H. (2007). Widespread lateral gene transfer from intracellular bacteria to multicellular eukaryotes. Science *317*, 1753–6.

Duron, O., Bouchon, D., Boutin, S., Bellamy, L., Zhou, L., Engelstädter, J., and Hurst, G.D. (2008). The diversity of reproductive parasites among arthropods: *Wolbachia* do not walk alone. BMC Biol *6*, 27.

Edgar, R.C. (2004). MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics *5*, 113.

Edwards, K. T., Goddard, J., and Varela-Stokes, A. S. (2009). Examination of the internal morphology of the ixodid tick, *Amblyomma maculatum* Koch, (Acari: Ixodidae); a "how-to" pictorial dissection guide. Midsouth Entomol *2*, 28–39.

Epis, S., Mandrioli, M., Genchi, M., Montagna, M., Sacchi, L., Pistone, D., and Sassera, D. (2013). Localization of the bacterial symbiont *Candidatus* Midichloria mitochondrii within the hard tick *Ixodes ricinus* by whole-mount FISH staining. Ticks Tick Borne Dis *4*, 39–45.

Epis, S., Sassera, D., Beninati, T., Lo, N., Beati, L., Piesman, J., Rinaldi, L., McCoy, K.D., Torina, A., Sacchi, L., et al. (2008). *Midichloria mitochondrii* is widespread in hard ticks (Ixodidae) and resides in the mitochondria of phylogenetically diverse species. Parasitology *135*, 485–494.

Erickson, D. (1969). An investigation on Strawberry Disease in trout. American Fishes and U. S. Trout News, p. 26.

Erickson, D.L., Anderson, N.E., Cromar, L.M., and Jolley, A. (2009). Bacterial Communities Associated With Flea Vectors of Plague. J Med Entomol *46*, 1532–1536.

Estrada-Peña, A., Daniel, M., Frandsen, F., Gern, L., Gettinby, G., Gray, J.S., Jaenson, T.G., Jongejan, F., Kahl, O., Korenberg, E., et al. (1998). *Ixodes ricinus* strains in Europe. Zentralbl Bakteriol *287*, 185–189.

Felsheim, R.F., Kurtti, T.J., and Munderloh, U.G. (2009). Genome sequence of the endosymbiont *Rickettsia peacockii* and comparison with virulent *Rickettsia rickettsii*: identification of virulence factors. PLoS One *4*, e8361.

Ferguson, H.W., Girons, A., Rizgalla, G., LaPatra, S., Branson, E.J., MacKenzie, K., Davies, M., Collins, R.O., Diab, A., and Crumlish, M. (2006). Strawberry disease in rainbow trout in Scotland: pathology and association with *Flavobacterium psychrophilum*. Vet Rec *158*, 630–

632.

Ferla, M.P., Thrash, J.C., Giovannoni, S.J., and Patrick, W.M. (2013). New rRNA Gene-Based Phylogenies of the Alphaproteobacteria Provide Perspective on Major Groups, Mitochondrial Ancestry and Phylogenetic Instability. PLoS One *8*, e83383.

Flynn, P.C., and Kaufman, W.R. (2015). Mechanical properties of the cuticle of the tick *Amblyomma hebraeum* (Acari: Ixodidae). J Exp Biol *218*, 2806–2814.

Fraune, S., and Bosch, T.C.G. (2007). Long-term maintenance of species-specific bacterial microbiota in the basal metazoan *Hydra*. PNAS *104*, 13146–13151.

Fritsche, T.R., Horn, M., Seyedirashti, S., Gautom, R.K., Schleifer, K.-H., and Wagner, M. (1999). In Situ Detection of Novel Bacterial Endosymbionts of *Acanthamoeba* spp. Phylogenetically Related to Members of the Order *Rickettsiales*. Appl Environ Microbiol *65*, 206–212.

de la Fuente, J., Estrada-Pena, A., Venzal, J.M., Kocan, K.M., and Sonenshine, D.E. (2008). Overview: Ticks as vectors of pathogens that cause disease in humans and animals. Front Biosci: A Journal and Virtual Library *13*, 6938–6946.

Fujita, H., Takada, N., Kawabata, H., Ishiguro, F., Yamamoto, S., et al. (2007). Some suggestive records of rickettsiae isolated from ticks in Korea and central China. Annual Report of Ohara General Hospital *47*, 21–4.

Gern, L., and Rais, O. (1996). Efficient transmission of *Borrelia burgdorferi* between cofeeding *Ixodes ricinus* ticks (Acari: Ixodidae). J Med Entomol *33*, 189–92.

Ghittino, C., Latini, M., Agnetti, F., Panzieri, C., Lauro, L., Ciappelloni, R., and Petracca, G. (2003). Emerging pathologies in aquaculture: effects on production and food safety. Vet Res Commun *27*, 471–479.

Giuliano, S., Agresta, A.M., De Palma, A., Viglio, S., Mauri, P., Fumagalli, M., Iadarola, P., Montalbetti, L., Salvini, R., and Bardoni, A. (2014). Proteomic analysis of lymphoblastoid cells from Nasu-Hakola patients: a step forward in our understanding of this neurodegenerative disorder. PLoS One *9*, e110073.

Goddard, J., Sumner, J.W., Nicholson, W.L., Paddock, C.D., Shen, J., and Piesman, J. (2003). Survey of ticks collected in Mississippi for *Rickettsia*, *Ehrlichia*, and *Borrelia* species. J Vector Ecol *28*, 184–189.

Goebel, W., and Gross, R. (2001). Intracellular survival strategies of mutualistic and parasitic prokaryotes. Trends Microbiol *9*, 267–273.

Gofton, A.W., Oskam, C.L., Lo, N., Beninati, T., Wei, H., McCarl, V., Murray, D.C., Paparini, A., Greay, T.L., Holmes, A.J., et al. (2015). Inhibition of the endosymbiont "*Candidatus* Midichloria mitochondrii" during *16S rRNA* gene profiling reveals potential pathogens in *Ixodes* ticks from Australia. Parasit Vectors *8*, 345.

Gouin, E., Welch, M.D., and Cossart, P. (2005). Actin-based motility of intracellular pathogens. Curr Opin Microbiol *8*, 35–45.

Halperin, J.J., Kaplan, G.P., Brazinsky, S., Tsai, T.F., Cheng, T., Ironside, A., Wu, P., Delfiner, J., Golightly, M., and Brown, R.H. (1990). Immunologic reactivity against *Borrelia burgdorferi* in patients with motor neuron disease. Arch Neurol *47*, 586–594.

Hänsel, Y., Ackerl, M., and Stanek, G. (1995). [ALS-like sequelae in chronic neuroborreliosis]. Wien Med Wochenschr *145*, 186–188.

Harvey, W.T., and Martz, D. (2007). Motor neuron disease recovery associated with IV ceftriaxone and anti-*Babesia* therapy. Acta Neurol Scand *115*, 129–131.

Hillyard, P.D. (1997). Ticks of North-West Europet. The Dorset Press, Dorchester.

Horigane, M., Ogihara, K., Nakajima, Y., Honda, H., and Taylor, D. (2007). Identification and expression analysis of an actin gene from the soft tick, *Ornithodoros moubata* (Acari: Argasidae). Arch Insect Biochem Physiol *64*, 186–199.

Hsieh, Y.-F., Liu, H.-W., Hsu, T.-C., Wei, J.C.-C., Shih, C.-M., Krause, P.J., and Tsay, G.J. (2007). Serum reactivity against *Borrelia burgdorferi OspA* in patients with rheumatoid arthritis. Clin Vaccine Immunol *14*, 1437–1441.

Jongejan, F., and Uilenberg, G. (2004). The global importance of ticks. Parasitology *129* Suppl, S3–S14.

Karbowiak, G., Biernat, B., Stańczak, J., Szewczyk, T., and Werszko, J. (2016). The role of particular tick developmental stages in the circulation of tick-borne pathogens affecting humans in Central Europe. 3. Rickettsiae. Ann Parasitol *62*, 89–100.

Klompen, J.S., and Oliver, J.H. (1993). Haller's organ in the tick family Argasidae (Acari: Parasitiformes: Ixodida). J Parasitol *79*, 591–603.

Kubilay, A., Ciftci, S., Yildirim, P., Didinen, B.I., Metin, S., Dmirkan, T., ... & Oidtmann, B. (2014). First observation of red mark syndrome (RMS) in cultured Rainbow Trout (*Oncorhynchus mykiss* Walbaum, 1972) in Turkey. B Eur Assoc of Fish Pat *34*, 95–101.

Labuda, M., Danielová, V., Jones, L.D., and Nuttall, P.A. (1993) Amplification of tick-borne encephalitis virus infection during co-feeding of ticks. Med Vet Entomol *7*, 339–342.

Lacroix, B., and Citovsky, V. (2016). Transfer of DNA from Bacteria to Eukaryotes. mBio *7*, e00863-16.

Lelovas, P., Dontas, I., Bassiakou, E., and Xanthos, T. (2008). Cardiac implications of Lyme disease, diagnosis and therapeutic approach. Int J Cardiol *129*, 15–21.

Leung, T.L.F., and Poulin, R. (2008). Parasitism, commensalism, and mutualism: exploring the many shades of symbioses. Vie Milieu *58*, 107–115.

Lewis, D. (1979). The detection of *Rickettsia*-like microorganisms within the ovaries of female *Ixodes ricinus* ticks. Z Parasitenkd *59*, 295–298.

Liang, R., Woo, S.L., Nguyen, T.D., Liu, P.C., and Almarza, A. (2008). Effects of a bioscaffold on collagen fibrillogenesis in healing medial collateral ligament in rabbits. J Orthop Res *26***,** 1098–104.

Liao, M., Boldbaatar, D., Gong, H., Huang, P., Umemiya, R., Harnnoi, T., Zhou, J., Tanaka, T., Suzuki, H., Xuan, X., et al. (2008). Functional analysis of protein disulfide isomerases in blood feeding, viability and oocyte development in *Haemaphysalis longicornis* ticks. Insect Biochem Mol Biol *38*, 285–295.

Lloyd, S.J., LaPatra, S.E., Snekvik, K.R., St-Hilaire, S., Cain, K.D., and Call, D.R. (2008). Strawberry disease lesions in rainbow trout from southern Idaho are associated with DNA from a *Rickettsia*-like organism. Dis Aquat Organ *82*, 111–118.

Lloyd, S.J., LaPatra, S.E., Snekvik, K.R., Cain, K.D., and Call, D.R. (2011). Quantitative PCR demonstrates a positive correlation between a *Rickettsia*-like organism and severity of strawberry disease lesions in rainbow trout, *Oncorhynchus mykiss* (Walbaum). J Fish Dis *34*, 701–709.

Lo, N., Beninati, T., Sassera, D., Bouman, E. a. P., Santagati, S., Gern, L., Sambri, V., Masuzawa, T., Gray, J.S., Jaenson, T.G.T., et al. (2006). Widespread distribution and high prevalence of an alpha-proteobacterial symbiont in the tick *Ixodes ricinus*. Environ Microbiol *8*, 1280–1287.

Loftis, A.D., Reeves, W.K., Szumlas, D.E., Abbassy, M.M., Helmy, I.M., Moriarity, J.R., and Dasch, G.A. (2006). Rickettsial agents in Egyptian ticks collected from domestic animals. Exp Appl Acarol *40*, 67–81.

Manilla, G. (1998). Acari: Ixodida. Vol. XXXVI. Fauna D'Italia. Bologna: Ed.

Mans, B.J., de Klerk, D., Pienaar, R., de Castro, M.H., and Latif, A.A. (2015). Next-generation sequencing as means to retrieve tick systematic markers, with the focus on *Nuttalliella namaqua* (Ixodoidea: Nuttalliellidae). Ticks Tick Borne Dis *6*, 450–462.

Mariconti, M., Epis, S., Sacchi, L., Biggiogera, M., Sassera, D., Genchi, M., Alberti, E., Montagna, M., Bandi, C., and Bazzocchi, C. (2012a). A study on the presence of flagella in the order *Rickettsiales*: the case of "*Candidatus* Midichloria mitochondrii." Microbiology *158*, 1677–1683.

Mariconti, M., Epis, S., Gaibani, P., Valle, C.D., Sassera, D., Tomao, P., Fabbi, M., Castelli, F., Marone, P., Sambri, V., et al. (2012b). Humans parasitized by the hard tick *Ixodes ricinus* are seropositive to *Midichloria mitochondrii*: is *Midichloria* a novel pathogen, or just a marker of tick bite? Pathog Glob Health *106*, 391–396.

Maritz-Olivier, C., Stutzer, C., Jongejan, F., Neitz, A.W.H., and Gaspar, A.R.M. (2007). Tick anti-hemostatics: targets for future vaccines and therapeutics. Trends Parasitol *23*, 397–407.

Matsuura, Y., Kikuchi, Y., Meng, X.Y., Koga, R., and Fukatsu, T. (2012). Novel Clade of Alphaproteobacterial Endosymbionts Associated with Stinkbugs and Other Arthropods. Appl Environ Microbiol *78*, 4149–4156.

Mayer, M.P., and Bukau, B. (2005). Hsp70 chaperones: cellular functions and molecular mechanism. Cell Mol Life Sci *62*, 670–684.

McCutcheon, J.P. (2016) From microbiology to cell biology: when an intracellular bacterium becomes part of its host cell. Curr Opin Cell Biol *41*, 132–136.

Mediannikov, O., and Fenollar, F. (2014). Looking in ticks for human bacterial pathogens. Microb Pathog *77*, 142–148.

Mediannikov, O.I., Ivanov, L.I., Nishikawa, M., Saito, R., Sidel'nikov, I.N., Zdanovskaia, N.I., Mokretsova, E.V., Tarasevich, I.V., and Suzuki, H. (2004). [Microorganism "Montezuma" of the order *Rickettsiales*: the potential causative agent of tick-borne disease in the Far East of Russia]. Zh Mikrobiol Epidemiol Immunobiol *1*, 7–13.

Medlock, J.M., and Leach, S.A. (2015). Effect of climate change on vector-borne disease risk in the UK. Lancet Infect Dis *15*, 721–730.

Metselaar, M. (2012). Aetiology of red mark syndrome inrainbow trout (*Oncorhynchus mykiss*). PhD Thesis, Institute of Aquaculture, University of Stirling, Scotland.

Metselaar, M., Thompson, K.D., Gratacap, R.M.L., Kik, M.J.L., LaPatra, S.E., Lloyd, S.J., Call, D.R., Smith, P.D., and Adams, A. (2010). Association of red-mark syndrome with a *Rickettsia*-like organism and its connection with strawberry disease in the USA. J Fish Dis *33*, 849–858.

Michelet, L., Delannoy, S., Devillers, E., Umhang, G., Aspan, A., Juremalm, M., Chirico, J., Wal, V.D., J, F., Sprong, H., et al. (2014). High-throughput screening of tick-borne pathogens in Europe. Front Cell Infect Microbiol *4*, 103.

Montagna, M., Sassera, D., Epis, S., Bazzocchi, C., Vannini, C., Lo, N., Sacchi, L., Fukatsu, T., Petroni, G., and Bandi, C. (2013). "*Candidatus* Midichloriaceae" fam. nov. (*Rickettsiales*), an Ecologically Widespread Clade of Intracellular Alphaproteobacteria. Appl Environ Microbiol *79*, 3241–3248.

Moraes, J., Galina, A., Alvarenga, P.H., Rezende, G.L., Masuda, A., da Silva Vaz, I., and Logullo, C. (2007). Glucose metabolism during embryogenesis of the hard tick *Boophilus microplus*. Comp Biochem Physiol A Mol Integr Physiol *146*, 528–533.

Najm, N.A., Silaghi, C., Bell-Sakyi, L., Pfister, K., and Passos, L.M.F. (2012). Detection of bacteria related to *Candidatus* Midichloria mitochondrii in tick cell lines. Parasitol Res *110*, 437–442.

Narasimhan, S., Montgomery, R.R., DePonte, K., Tschudi, C., Marcantonio, N., Anderson, J.F., Sauer, J.R., Cappello, M., Kantor, F.S., and Fikrig, E. (2004). Disruption of *Ixodes scapularis* anticoagulation by using RNA interference. Proc Natl Acad Sci U.S.A. *101*, 1141–1146.

Noguera, P., Pendrey, D., Norris, M., McIntosh, A., and Bruno, D.W. (2007). Investigations into "red mark syndrome" (RMS) in Scotland. In: *Flavobacterium* 2007 Workshop, National Conservation Training Center, May 2-4th Shepherdstown, WV

Oidtmann, B., Lapatra, S.E., Verner-Jeffreys, D., Pond, M., Peeler, E.J., Noguera, P.A., Bruno, D.W., St-Hilaire, S., Schubiger, C.B., Snekvik, K., et al. (2013). Differential characterization of emerging skin diseases of rainbow trout--a standardized approach to capturing disease characteristics and development of case definitions. J Fish Dis *36*, 921–937.

Oliver, J.H. (1989). Biology and Systematics of Ticks (Acari:Ixodida). Annu Rev Ecol Syst *20*, 397–430.

Oliver, K.M., Degnan, P.H., Burke, G.R., and Moran, N.A. (2009). Facultative Symbionts in Aphids and the Horizontal Transfer of Ecologically Important Traits. Annu Rev Entomol *55*, 247–266.

Olson, D.P., Beleau, M.H., Busch, R.A., Roberts, S. and Krieger, R.I. (1985) Strawberry disease in rainbow trout, *Salmo gairdneri* Richardson. J Fish Dis *8*, 103–111.

Oman, E.M. (1990) Strawberry Disease in Salmonids. MS thesis, University of Idaho, USA.

Otranto, D., Cantacessi, C., Pfeffer, M., Dantas-Torres, F., Brianti, E., Deplazes, P., Genchi, C., Guberti, V., and Capelli, G. (2015). The role of wild canids and felids in spreading parasites to dogs and cats in Europe. Part I: Protozoa and tick-borne agents. Vet Parasitol *213*, 12–23.

Palomar, A.M., Portillo, A., Santibáñez, P., Mazuelas, D., Roncero, L., García-Álvarez, L., Santibáñez, S., Gutiérrez, Ó., and Oteo, J.A. (2015). Detection of tick-borne *Anaplasma bovis*, *Anaplasma phagocytophilum* and *Anaplasma centrale* in Spain. Med Vet Entomol *29*, 349–353.

Pancholi, V. (2001). Multifunctional alpha-enolase: its role in diseases. Cell Mol Life Sci *58*, 902–920.

Parola, P., and Raoult, D. (2001). Tick-borne bacterial diseases emerging in Europe. Clin. Microbiol. Infect. *7*, 80–83.

Parola, P., Cornet, J.P., Sanogo, Y.O., Miller, R.S., Thien, H.V., Gonzalez, J.P., Raoult, D., Telford III, S.R., and Wongsrichanalai, C. (2003). Detection of *Ehrlichia* spp., *Anaplasma* spp., *Rickettsia* spp., and other eubacteria in ticks from the Thai-Myanmar border and Vietnam. J Clin Microbiol *41*, 1600–1608.

Parola, P., Paddock, C.D., and Raoult, D. (2005). Tick-borne rickettsioses around the world: emerging diseases challenging old concepts. Clin Microbiol Rev *18*, 719–756.

Pérez-Eid, C. (2007). Les Tiques - Identification, Biologie, Importance médicale Etvétérinaire. Tec and Doc Lavoisier, Paris.

Perlman, S.J., Hunter, M.S., and Zchori-Fein, E. (2006). The emerging diversity of *Rickettsia*. Proc R Soc Lond B: Biological Sciences *273*, 2097–2106.

Pesquera, C., Portillo, A., Palomar, A.M., and Oteo, J.A. (2015). Investigation of tick-borne bacteria (*Rickettsia* spp., *Anaplasma* spp., *Ehrlichia* spp. and *Borrelia* spp.) in ticks collected from Andean tapirs, cattle and vegetation from a protected area in Ecuador. Parasit Vectors *8*, 46.

Pinheiro, J., and Bates, D. (2006). Mixed-Effects Models in S and S-PLUS. Springer Science & Business Media.

Pistone, D., Pajoro, M., Fabbi, M., Vicari, N., Marone, P., Genchi, C., Novati, S., Sassera, D., Epis, S., and Bandi C. (2010) Lyme borreliosis, Po River Valley, Italy. Emerg Infect Dis. *16*, 1289–1291.

Pistone, D., Sacchi, L., Lo, N., Epis, S., Pajoro, M., Favia, G., Mandrioli, M., Bandi, C., and

Sassera D. (2011). *Candidatus* Midichloria mitochondrii: symbiont or parasite of tick mitochondria. In: Manipulative Tenants: Bacteria associated with Arthropods. Publisher Academic Press.

de Puytorac, P., and Grain, J. (1972). [Intramitochondrial bacteria and peculiarities of cytostomopharyngeal ultrastructure in the ciliate, *Urotricha ovata* Kahl (Ciliata)]. Comptes Rendus Des Seances De La Societe De Biologie Et De Ses Filiales *166*, 604–607.

Qiu, Y., Nakao, R., Ohnuma, A., Kawamori, F., and Sugimoto, C. (2014). Microbial population analysis of the salivary glands of ticks; a possible strategy for the surveillance of bacterial pathogens. PLoS One *9*, e103961.

Radulović, Ž.M., Kim, T.K., Porter, L.M., Sze, S.-H., Lewis, L., and Mulenga, A. (2014). A 24-48 h fed *Amblyomma americanum* tick saliva immuno-proteome. BMC Genomics *15*, 518.

Raoult, D., and Roux, V. (1997). Rickettsioses as paradigms of new or emerging infectious diseases. Clin Microbiol Rev *10*, 694–719.

Renvoisé, A., Merhej, V., Georgiades, K., and Raoult, D. (2011). Intracellular *Rickettsiales*: Insights into manipulators of eukaryotic cells. Trends Mol Med *17*, 573–583.

Rinehart, J.P., Li, A., Yocum, G.D., Robich, R.M., Hayward, S.A.L., and Denlinger, D.L. (2007). Up-regulation of heat shock proteins is essential for cold survival during insect diapause. Proc Natl Acad Sci U.S.A. *104*, 11130–11137.

Rodger, H. (2008). Red mark syndrome (RMS). Finfish News *6*, Summer/Autumn 2008, pp. 8–10.

Rollend, L., Fish, D., and Childs, J.E. (2013). Transovarial transmission of *Borrelia* spirochetes by *Ixodes scapularis*: A summary of the literature and recent observations. Ticks Tick Borne Dis *4*, 46–51.

Rynkiewicz, E.C., Hemmerich, C., Rusch, D.B., Fuqua, C., and Clay, K. (2015). Concordance of bacterial communities of two tick species and blood of their shared rodent host. Mol Ecol *24*, 2566–2579.

Sacchi, L., Bigliardi, E., Corona, S., Beninati, T., Lo, N., and Franceschi, A. (2004). A symbiont of the tick *Ixodes ricinus* invades and consumes mitochondria in a mode similar to that of the parasitic bacterium *Bdellovibrio bacteriovorus*. Tissue Cell *36*, 43–53.

Sasani, F., Shokrpoor, S., Rahmati-Holasoo, H., and Zargar, A. (2016). Appearance of red mark syndrome (RMS) in cultured rainbow trout (*Oncorhynchus mykiss* Walbaum, 1972) in Iran. B Eur Assoc Fish Pat *36*, 90–94.

Sassera, D., Beninati, T., Bandi, C., Bouman, E.A.P., Sacchi, L., Fabbi, M., and Lo, N. (2006). "*Candidatus* Midichloria mitochondrii", an endosymbiont of the tick *Ixodes ricinus* with a unique intramitochondrial lifestyle. Int J Syst Evol Microbiol *56*, 2535–2540.

Sassera, D., Lo, N., Bouman, E.A.P., Epis, S., Mortarino, M., and Bandi, C. (2008). "*Candidatus* Midichloria" endosymbionts bloom after the blood meal of the host, the hard tick Ixodes ricinus. Appl Environ Microbiol *74*, 6138–40.

Sassera, D., Lo, N., Epis, S., D'Auria, G., Montagna, M., Comandatore, F., Horner, D., Peretó, J., Luciano, A.M., Franciosi, F., et al. (2011). Phylogenomic evidence for the presence of a flagellum and *cbb(3) oxidase* in the free-living mitochondrial ancestor. Mol. Biol. Evol. *28*, 3285–3296.

Sauer, J.R., McSwain, J.L., Bowman, A.S., and Essenberg, R.C. (1995). Tick salivary gland physiology. Annu Rev Entomol *40*, 245–267.

Schmidt-Posthaus, H., Bergmann, W., Knüsel, R., Heistinger, H., and Licek, E. (2009). Appearance of red mark syndrome/ cold water strawberry disease in Switzerland and Austria. Dis Aquat Org *88*, 65–68.

Schulz, F., Martijn, J., Wascher, F., Lagkouvardos, I., Kostanjšek, R., Ettema, T.J.G., and Horn, M. (2016). A *Rickettsiales* symbiont of amoebae with ancient features. Environ Microbiol *18*, 2326–2342.

Schwan, T.G. (1996). Ticks and *Borrelia*: model systems for investigating pathogen-arthropod interactions. Infect Agents Dis *5*, 167–181.

Schwarz, A., Tenzer, S., Hackenberg, M., Erhart, J., Gerhold-Ay, A., Mazur, J., Kuharev, J., Ribeiro, J.M.C., and Kotsyfakis, M. (2014). A Systems Level Analysis Reveals Transcriptomic and Proteomic Complexity in *Ixodes ricinus* Midgut and Salivary Glands During Early Attachment and Feeding. Mol Cell Proteomics *13*, 2725–2735.

Senra, M.V.X., Dias, R.J.P., Castelli, M., Silva-Neto, I.D., Verni, F., Soares, C.A.G., and Petroni, G. (2016). A House for Two--Double Bacterial Infection in *Euplotes woodruffi* Sq1 (Ciliophora, Euplotia) Sampled in Southeastern Brazil. Microb Ecol *71*, 505–517.

Serra, V., Fokin, S.I., Castelli, M., Basuri, C.K., Nitla, V., Verni, F., Sandeep, B.V., Kalavati, C., and Petroni, G. (2016). "*Candidatus* Gortzia shahrazadis", a Novel Endosymbiont of *Paramecium multimicronucleatum* and a Revision of the Biogeographical Distribution of *Holospora*-Like Bacteria. Front Microbiol *7*, 1704.

da Silva, R.M., Noce, B.D., Waltero, C.F., Costa, E.P., de Abreu, L.A., Githaka, N.W., Moraes, J., Gomes, H.F., Konnai, S., Vaz, I. da S., et al. (2015). Non-classical gluconeogenesis-dependent glucose metabolism in *Rhipicephalus microplus* embryonic cell line BME26. Int J Mol Sci *16*, 1821–1839.

Skarphédinsson, S., Jensen, P.M., and Kristiansen, K. (2005). Survey of tickborne infections in Denmark. Emerg Infect Dis *11*, 1055–1061.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C. (1985). Measurement of protein using bicinchoninic acid. Anal Biochem *150*, 76–85.

Socolovschi, C., Mediannikov, O., Raoult, D., and Parola, P. (2009). The relationship between spotted fever group *Rickettsiae* and Ixodid ticks. Vet Res *40*, 1, 34–20.

Sonenshine, D.E., and Hynes, W.L. (2008). Molecular characterization and related aspects of the innate immune response in ticks. Front Biosci *13*, 7046–7063.

Stamatakis, A., Hoover, P., and Rougemont, J. (2008). A Rapid Bootstrap Algorithm for the

RAxML Web Servers. Syst Biol 57, 758–771.

Stańczak, J., Gabre, R.M., Kruminis-Łozowska, W., Racewicz, M., and Kubica-Biernat, B. (2004). *Ixodes ricinus* as a vector of *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum* and *Babesia microti* in urban and suburban forests. Ann Agric Environ Med *11*, 109–114.

Stevens, J.M., Galyov, E.E., and Stevens, M.P. (2006). Actin-dependent movement of bacterial pathogens. Nat Rev Microbiol *4*, 91–101.

Sunagawa, S., Woodley, C.M., and Medina, M. (2010). Threatened Corals Provide Underexplored Microbial Habitats. PLoS One *5*, e9554.

Szokoli, F., Sabaneyeva, E., Castelli, M., Krenek, S., Schrallhammer, M., Soares, C.A.G., da Silva-Neto, I.D., Berendonk, T.U., and Petroni, G. (2016). "*Candidatus* Fokinia solitaria", a Novel "Stand-Alone" Symbiotic Lineage of *Midichloriaceae* (*Rickettsiales*). PLoS One *11*, e0145743.

Taylor, M.J., and Hoerauf, A. (1999). *Wolbachia* Bacteria of Filarial Nematodes. Parasitol Today *15*, 437–442.

Taylor, M.J., Bandi, C., and Hoerauf, A. (2005). *Wolbachia*. Bacterial Endosymbionts of Filarial Nematodes. In Advances in Parasitology, R.M. and D.R. J.R. Baker. pp. 245–284. Ed. Academic Press.

Vannini, C., Ferrantini, F., Schleifer, K.-H., Ludwig, W., Verni, F., and Petroni, G. (2010). "*Candidatus* anadelfobacter veles" and "*Candidatus* Cyrtobacter comes," two new *Rickettsiales* species hosted by the protist ciliate *Euplotes harpa* (Ciliophora, Spirotrichea). Appl Environ Microbiol *76*, 4047–4054.

Verner-Jeffreys, D.W., Pond, M.J., Peeler, E.J., Rimmer, G.S.E., Oidtmann, B., Way, K., Mewett, J., Jeffrey, K., Bateman, K., Reese, R.A., et al. (2008). Emergence of cold water strawberry disease of rainbow trout *Oncorhynchus mykiss* in England and Wales: outbreak investigations and transmission studies. Dis Aquat Org *79*, 207–218.

Vu Hai, V., Pages, F., Boulanger, N., Audebert, S., Parola, P., and Almeras, L. (2013). Immunoproteomic identification of antigenic salivary biomarkers detected by *Ixodes ricinus*-exposed rabbit sera. Ticks Tick Borne Dis *4*, 459–468.

Wang, H., Tsai, M., Tu, M., and Lee, S. (2000). Universal primers for amplification of the complete mitochondrial *12S rRNA* gene in vertebrates. Zool Stud *39,* 61–66.

Weinert, L.A., Werren, J.H., Aebi, A., Stone, G.N., and Jiggins, F.M. (2009). Evolution and diversity of *Rickettsia* bacteria. BMC Biology *7*, 6.

Werren, J.H. (1997). Biology of *Wolbachia*. Annu Rev Entomol *42*, 587–609.

Werren, J.H., Baldo, L., and Clark, M.E. (2008). *Wolbachia*: master manipulators of invertebrate biology. Nat Rev Micro *6*, 741–751.

Williams, K.P., Sobral, B.W., and Dickerman, A.W. (2007). A Robust Species Tree for the Alphaproteobacteria. J Bacteriol *189*, 4578–4586.

Williams-Newkirk, A.J., Rowe, L.A., Mixson-Hayden, T.R., and Dasch, G.A. (2012). Presence, genetic variability, and potential significance of "*Candidatus* Midichloria mitochondrii" in the lone star tick *Amblyomma americanum*. Exp Appl Acarol *58*, 291–300.

Yamataka, S., and Hayashi, R. (1970). Electron microscopic studies on the mitochondria and intramitochondrial microorganisms of *Halteria geleiana*. J Electron Microsc *19*, 50–62.

Zhong, J., Jasinskas, A., and Barbour, A.G. (2007). Antibiotic treatment of the tick vector *Amblyomma americanum* reduced reproductive fitness. PLoS One *2*, e405.

Zhu, Z., Aeschlimann, A., and Gern, L. (1992). *Rickettsia*-like microorganisms in the ovarian primordial of molting *Ixodes ricinus* (acari : ixodidae) larvae and nymphs. Ann Parasitol Hum Comp *67*, 99–110.

Zug, R., and Hammerstein, P. (2015). Bad guys turned nice? A critical assessment of *Wolbachia* mutualisms in arthropod hosts. Biol Rev Camb Philos Soc *90*, 89–111.

6 List of publications

Published papers

Cafiso, A., Bazzocchi, C., De Marco, L., Opara, M.N., Sassera, D., and Plantard, O. (2016). Molecular screening for *Midichloria* in hard and soft ticks reveals variable prevalence levels and bacterial loads in different tick species. Ticks Tick Borne Dis *7*, 1186–1192.

Cafiso, A., Sassera, D., Serra, V., Bandi, C., McCarthy, U., and Bazzocchi, C. (2016). Molecular evidence for a bacterium of the family *Midichloriaceae* (order *Rickettsiales*) in skin and organs of the rainbow trout *Oncorhynchus mykiss* (Walbaum) affected by red mark syndrome. J Fish Dis *39*, 497–501.

Di Venere, M., Fumagalli, M., Cafiso, A., De Marco, L., Epis, S., Plantard, O., Bardoni, A., Salvini, R., Viglio, S., Bazzocchi, C., Iadarola, P., and Sassera, D. (2015) *Ixodes ricinus* and Its Endosymbiont *Midichloria mitochondrii*: A Comparative Proteomic Analysis of Salivary Glands and Ovaries. PLoS One *23*, 10(9):e0138842.

Ninio, C., Plantard, O., Serra, V., Pollera, C., Ferrari, N., Cafiso, A., Sassera, D., and Bazzocchi, C. (2015) Antibiotic treatment of the hard tick *Ixodes ricinus*: Influence on *Midichloria mitochondrii* load following blood meal. Ticks Tick Borne Dis *6*, 653–657.

Abstracts and posters for National and International Conferences

Cafiso, A., Serra, V., Bersani, M., De Marco, L., Opara, M.N., Sassera, D., Plantard, O., Bazzocchi, C. (2016). Molecular screening for *Midichloria* bacteria in hard and soft ticks (acari: Ixodida). At: XXIX Congresso Nazionale SoIPa. Bari (Italy).

Cafiso, A., Serra, V., Petroni, G., Comandatore, F., Bazzocchi, C. Batteri della famiglia *Midichloriaceae* (ordine *Rickettsiales*): possibili patogeni emergenti per i vertebrati?. (2016). At: XXII Convegno nazionale S.I.P.I. – Società Italiana Patologia Ittica. San Michele All'Adig, TN (Italy).

Serra, V., Cafiso, A., Parisio, G., Sassera, D., Formenti, N., Verheyden, H., Plantard, O., Bazzocchi, C. (2016) Molecular and serological evidences of circulating *Midichloria mitochondrii* in roe deer (*Capreolus capreolus*) after Ixodes ricinus bite. At: XXIX Congresso Nazionale SoIPa. Bari (Italy).

Cafiso, A., Serra, V., Petroni, G., Bazzocchi, C. (2015). Batteri della famiglia *Midichloriaceae* (ordine *Rickettsiales*): agente eziologico della red mark syndrome in *Oncorhynchus mykiss*?. At: XXI Convegno Nazionale S.I.P.I. – Società Italiana Patologia Ittica. Chioggia (Italy).

Cafiso, A., Serra, V., Plantard, O., Sassera, D., Floriano, A.M., Bazzocchi, C. (2015). Emerging pathogens in vertebrates: bacteria of the *Midichloriaceae* family (order *Rickettsiales*). At: Pathobiome: pathogens in microbiota in hosts. Maisons-Alfort (France).

Di Venere, M., Fumagalli, M., De Marco, M., Cafiso, A., Serra, V., Plantard, O., Salvini, R., Sassera, D. (2015), Profiling the proteome of salivary glands and ovaries of the tick *Ixodes*

ricinus. At: IV International Conference on Analytical Proteomics. Lisboa (Portugal).

Serra, V., Cafiso, A., Ninio, C., Verheyden, H., Plantard, O., Sassera, D., Epis, S., Bazzocchi, C. (2015). *Midichloria mitochondrii*, an intracellular alphaproteobacteria considered as a symbiont of the tick *Ixodes ricinus*, found circulating in roe-deer (*Capreolus capreolus*) through molecular and serological evidences. At: Pathobiome: pathogens in microbiota in hosts. Maisons-Alfort (France).

Cafiso, A., Serra, V., Bazzocchi, C., Epis, S., Gaiarsa, S., Bandi, C., Mariconti, M., Plantard, O., Sassera, D. (2014). Antibiotic treatment of the tick vector *Ixodes ricinus*: effects on *Midichloria mitochondrii*. At: XXVIII Congresso Nazionale SoIPa. Roma (Italy).

Bazzocchi, C., Cafiso, A., Petroni, G., Lanzoni, O., Sassera, D., Epis, S., Mariconti, M., Bandi, C. (2014). Emerging pathogens in vertebrates: biology, genomics and infectivity of bacteria ascribed to the family *Midichloriaceae*. At: XXVIII Congresso Nazionale SoIPa. Roma (Italia).

Martin, E., Cafiso, A., Mereghetti, V., Chouaia, B., Bazzocchi, C., Bandi, C., Epis, S., Montagna, M. (2014). At: Widespread presence of *Wolbachia* in an Alpine population of the viviparous lead beetle *Oreina cacaliae* (Coleoptera: Chrysomelidae). At: 8th International *Wolbachia* Conference. Igls (Austria).

Sassera, D., Bazzocchi, C., Mariconti, M., Epis, S., Cafiso, A., Bandi, C. (2014). *Midichloria mitochondrii*: a novel tick-borne infectious agent? At: 9th Conference Louis Pasteur – Emerging Infectious Diseases. Paris (France).