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Development of a serological test for *Ixodes ricinus* bite in vertebrates

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Abstract

The research line of my PhD project was focused on parasitism supported by *Ixodes ricinus* tick, the most common tick species occurring in Europe infecting humans and animals. The importance of this tick species is due to its wide area of distribution (i.e. Europe and Northern Africa), to its low host specificity and to its central role in the transmission of multiple infectious agents (e.g. the causative agent of Lyme borreliosis, rickettsial diseases, tick-borne encephalitis (TBE) virus) to both humans and animals. In addition to these pathogens, *I. ricinus* hosts an intracellular bacterium named *Midichloria mitochondrii* to recall its unique intra-mitochondrial lifestyle. Indeed, this bacterium presents the peculiar capacity to invade and multiply inside mitochondria, where it has been found between the inner and outer membranes and consuming the mitochondrial matrix. The presence of *M. mitochondrii* has been detected in female reproductive tract, in salivary glands and rostrum of adult females of *I. ricinus,* where is present with a prevalence of 100%, while the prevalence in males is around 44%. A vertical transmission of the bacterium has been reported, as indicated by PCR on eggs and newly emerged larvae, together with its horizontal transmission to vertebrate host during *I. ricinus* blood meal, as demonstrated by molecular and serological evidences in different host species (including humans). Even though the transmission of *M. mitochondrii* into the vertebrate hosts during the *I. ricinus* bite is currently established, it is still unknown whether immature stages (larvae and nymphs) of this tick species transmit the symbiont during the blood meal. Additionally, its recently discovered replication into the vertebrate host does not necessarily imply a pathogenic role of this endosymbiont. A limit which characterizes tick parasitism in humans is represented by tick bite features: tick bites in humans often go unnoticed, because people may mistake the bite of another arthropod as that of a tick and components of tick saliva have anesthetic properties which inhibit host pain/hitch responses. Additionally, the predominant developmental stage of *I. ricinus* found to feed on humans are nymphs, which are difficult to detect due to their small size. For this reason, given the current lack of a reliable test for *I. ricinus* bite, the development of a diagnostic test for tick bite would be 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 diseases (Amyotrophic lateral sclerosis, rheumatologic and cardiovascular diseases). Moreover, the emergence of new tick-borne diseases and the re-emergence of existing ones, together with the rising dispersal of *I. ricinus*, increase the importance of this

public health issue. The present PhD project has been focused on five principal researches: *i)* investigation of *M. mitochondrii* presence in blood and sera of roe deer (*Capreolus capreolus*) individuals through molecular and serological analyses; *ii)* serological detection of anti-*M. mitochondrii* antibodies in humans suffering from symptoms referable to tick-borne diseases from several European countries to investigate whether a protein of *M. mitochondrii* (FliD) could represent a marker suitable for diagnosis of *I. ricinus* bite; *iii)* study of the time and kinetic of seroconversion against the flagellar FliD protein of *M. mitochondrii* and salivary glands crude protein extract of *I. ricinus* ticks in rabbits experimentally infested with this tick species; *iv)* detection of additional antigenic proteins belonging to *I. ricinus* and *M. mitochondrii* to use as potential biomarkers for tick bite in a peptide-based ELISA assay; *v)* determination of the genetic variability of a previously undescribed *Midichloria* strain found in African *Hyalomma marginatum* ticks collected from migratory birds on Ventotene Island (Central Italy) and the quantification of the amount of this bacterium in ticks using a novel Real Time approach.

Here is presented a brief description of each performed research:

i) M. mitochondrii is a tick-borne intracellular bacterium of the order Rickettsiales, found with high prevalence in *I. ricinus*. *M. mitochondrii* is capable of vertical transmission in the tick, but recently evidence of potential horizontal transmission to the tick hosts through the blood meal has been reported. We investigated the presence of the bacterium in the blood of roe deer (*C. capreolus*) collected from an area known to be highly infested with *I. ricinus* ticks. We collected blood and sera samples for 3 yr in Gardouch (Haute Garonne, France) and subjected them to molecular screening through PCR and to serological investigation using enzyme-linked immunosorbent assay and Western blot. Bacterial DNA was detected in the blood of four of seven animals, but only at one or two points in time, whereas all sera were positive for *M. mitochondrii* antigens at all times. Our results indicated that the presence of the bacterium in the blood is transient, but the antibody response appeared to be long-lasting, possibly due to constant exposure to tick bites, and thus to repeated injection of bacteria. The role of *M. mitochondrii* in the mammalian host, and its interaction with other tick-borne bacteria, remains unknown.

ii) The most common tick species parasitizing humans in Europe is *I. ricinus*, the main vector of the causative agent of Lyme disease *B. burgdorferi* in this continent. This tick species also hosts the endosymbiont *M. mitochondrii*, which can be transmitted to the vertebrate host during the tick blood meal. Since a high proportion of tick bites remains unnoticed due to rarity of immediate symptoms, and a reliable test for *I. ricinus* bite is still lacking, we

investigated whether seropositivity toward a protein of *M. mitochondrii* (rFliD) could represent a marker suitable for diagnosis of *I. ricinus* bite in humans, focusing on samples collected in several European countries at different risk of tick bite.

We screened 274 sera collected from patients suffering from different symptoms potentially referable to tick-borne diseases using an ELISA protocol to detect anti-*M. mitochondrii* antibodies. In the cohort of examined patients, the majority (179) had been collected from patients who remembered a tick bite, the remaining (95) from patients that did not recall a tick bite. Our results show a clear trend indicating that positivity to *M. mitochondrii* is higher where tick bite can be regarded as certain/almost certain, and lower where there is an uncertainty on the bite, with the highest positivity in Lyme patients (47.3%) and the lowest (2%) in negative controls. According to the obtained results, *M. mitochondrii* can be regarded as a useful source of antigens for the development of a serological test for *I. ricinus* bite. In prospect, additional antigens from *M. mitochondrii* and from the tick salivary glands could be incorporated in a multi-antigen test for the diagnosis of the tick bite.

iii) I. ricinus endosymbiont *M. mitochondrii*, localized in ovaries and in salivary glands is vertically transmitted and is present in 100% of wild adult females, while prevalence values drop after some generations under laboratory conditions. Given the transmission of this bacterium to the vertebrate host during *I. ricinus* blood meal, this work was focused on monitoring *M. mitochondrii* antigens and DNA in a vertebrate model after infestation with *I. ricinus* for a time-span of four months. Two groups of rabbits were infested with *I. ricinus* females, respectively from the wild (naturally infected with the symbiont) and laboratory strain (lab; considered devoid of *M. mitochondrii* after quantitative PCR investigations) and screened using molecular and serological assays at nine time points. *M. mitochondrii* presence was detected in rabbits infested with wild *I. ricinus* ticks, but surprisingly also in those infested with lab ticks, albeit at later time points. This result prompted a more sensitive molecular screening of lab ticks, which were found to harbor very low symbiont loads. Our results indicate that transmission of the bacterium occurs even at low bacterial loads, and that antibody response against *M. mitochondrii* antigens begins within one week post-infestation with wild *I. ricinus*. Circulating DNA was detected in the blood of rabbits belonging to both groups up to the end of the experiment, suggesting a replication of the symbiont inside the vertebrate host.

iv) I. ricinus bite could represent a risk factor for the onset of several chronic-degenerative diseases (amyotrophic lateral sclerosis, myocardiopathy, and multiple sclerosis). This hypothesis opens the necessity to obtain an effective test capable of identifying bites of this

tick species in human and animal populations. The limits of the tick parasitism (misinformation in human population, features of the tick bite such as absence of itch, pain and immediate symptoms) prompted us to detect antigenic proteins belonging to *I. ricinus* salivary glands and to its endosymbiont *M. mitochondrii* to use as markers for tick bite. Additional antigenic proteins have been detected after immunoblotting analyses using protein extracts of salivary glands collected from *I. ricinus* adult females, and sera samples collected from experimentally infested rabbits. Novel peptides were designed from the most antigenic epitopes of each protein identified to detect antigenic markers for *I. ricinus* bite through an ELISA assay. The aim of the present work was to evaluate the occurrence of *I. ricinus* bite in different vertebrate hosts (rabbits, humans and roe deer) testing, through an ELISA approach, selected peptides developed from the identification of *I. ricinus* and *M. mitochondrii* antigenic proteins. The percentage of positivity, albeit low, found in tested human sera collected from subjects parasitized by ticks in Europe, and found all negative to *M. mitochondrii*, contribute to increase the percentage of sensitivity to investigate tick exposure. Interestingly, results obtained in rabbit sera testing two peptides showed positivity up to 16 weeks after experimental infestation, reinforcing the good property of these peptides as tick exposure indicator, with the final aim to combine different peptides in a multi-antigen ELISA test as diagnostic assay for tick bite to enhance the sensitivity of the test.

v) Ticks can be easily transported over long-distances while feeding in their hosts, in particular avian species may contribute to tick dispersal across geographical barriers during migration. The role of migrating birds in the spread of ticks has epidemiological implications, since birds may contribute to the dispersal of tick-borne pathogens and microorganisms. Although the high number of actual pathogens that ticks can host, especially concerning the Ixodidae family ("hard ticks"), a variety of symbiotic bacteria have been detected including members of the *Rickettsiaceae*, *Anaplasmataceae* and *Midichloriaceae* families (order Rickettsiales). Since many bird species are common hosts of tick species harboring bacteria belonging to *Midichloriaceae* family (i.e. *Ixodes* and *Hyalomma* tick genera), in this work we determined the existence of a previously undescribed *Midichloria* bacteria strain carried by African *H. marginatum* nymphs collected on Italian Island of Ventotene from migratory birds travelling back from Africa. Additionally, we quantified through an innovative Real time PCR approach this bacterium in *H. marginatum* nymphs showing a higher bacteria load after the blood meal, similarly to what has been observed for the symbiont *M. mitochondrii* in *Ixodes ricinus* ticks. Future studies should investigate the presence of this *Midichloria* bacterium in

adult *H. marginatum* ticks, in order to increase the knowledge concerning its role in the biology of this tick species and its transmission mode during the tick bite.

Riassunto

La linea di ricerca del mio progetto di dottorato è incentrata sul parassitismo esercitato dalla zecca dura *Ixodes ricinus,* la specie più comune in Europa. L'importanza rivestita da *I. ricinus* è dovuta alla sua ampia area di distribuzione (Europa e Africa settentrionale), alla bassa specificità d'ospite e al suo ruolo centrale nella trasmissione di molteplici agenti infettivi sia all'uomo che agli animali (ad esempio l'agente causativo della malattia di Lyme, diverse malattie supportate da rickettsie, l'encefalite virale da zecche). Oltre agli agenti eziologici sopra menzionati, *I. ricinus* ospita un batterio simbionte intramitocondriale chiamato *Midichloria mitochondrii* per richiamare la sua peculiare caratteristica di vivere all'interno dei mitochondri. Questo batterio è infatti in grado di invadere e moltiplicarsi all'interno dei mitocondri, dove si localizza nello spazio intermembrana compreso tra la membrana interna e quella esterna, consumando la matrice mitocondriale. La prevalenza di *M. mitochondrii* nelle zecche femmine è pari al 100%, e il batterio si trova in varie tipologie cellulari dell'ovario, mentre nelle zecche maschio la prevalenza scende al 44%. L'identificazione molecolare di questo batterio in uova e larve effettuata mediante PCR dimostra la trasmissione verticale di *M. mitochondrii* alla progenie, così come è stata dimostrata la sua trasmissione orizzontale all'ospite vertebrato durante il pasto di sangue mediante analisi molecolari e sierologiche. Sebbene la trasmissione del batterio all'ospite vertebrato sia ormai accertata, rimane ancora da indagare se questo simbionte viene trasmesso anche dagli stadi immaturi di *I. ricinus* (larva e ninfa). Inoltre, la replicazione recentemente dimostrata del batterio all'interno dell'ospite, non implica in alcun modo che questo batterio eserciti un ruolo patogeno. Un importante limite che caratterizza il parassitismo esercitato dalle zecche sull'uomo è rappresentato in primo luogo dalle caratteristiche proprie del morso della zecca: spesso passa inosservato in quanto può essere scambiato con il morso di altri insetti e inoltre le componenti presenti nella saliva delle zecche hanno proprietà anestetizzanti che inibiscono le sensazioni di dolore e prurito. Un altro aspetto da tenere presente è che l'uomo viene morso principalmente da larve e ninfe che, a causa delle piccole dimensioni, sono difficilmente visibili. Per questo motivo, data l'attuale mancanza di un test attendibile che identifichi il morso di *I. ricinus*, sarebbe essenziale lo sviluppo di un marcatore diagnostico per la puntura di zecca sia per quanto riguarda lo studio del suo ruolo clinico ed epidemiologico, sia per valutare l'ipotesi del morso di zecca come fattore di rischio per l'insorgenza di malattie cronico-degenerative (ad esempio sclerosi laterale amiotrofica, malattie cardiovascolari e reumatiche). L'insorgenza di nuove

malattie legate al morso di zecca (TBD) e la ricomparsa di TBD esistenti, insieme alla crescente diffusione di *I. ricinus,* concorrono ad aggravare questo importante problema di salute pubblica.

Questo progetto di dottorato si è focalizzato principalmente su cinque argomenti: *i)* lo studio della presenza di *M. mitochondri* circolante nel sangue e nel siero di alcuni caprioli (*Capreolus capreolus*) attraverso indagini molecolari e sierologiche; *ii)* l'identificazione sierologica di *M. mitochondrii* circolante in soggetti umani affetti da sintomi correlati a TBD provenienti da diversi paesi europei, al fine di verificare se la proteina batterica FliD possa rappresentare un marker adatto alla diagnosi del morso di *I. ricinus; iii)* lo studio del tempo di sieroconversione e della cinetica della risposta anticorpale contro la proteina batterica FliD e proteine di ghiandole salivari di *I. ricinus* in conigli infestati sperimentalmente; *iv)* l'indentificazione di proteine antigeniche di *I. ricinus* e *M. mitochondrii* da utilizzare come potenziali marcatori per rilevare la puntura di zecca mediante un test ELISA; *v)* la determinazione della variabilità genetica di un nuovo batterio del genere *Midichloria* identificato in zecche della specie *Hyalomma marginatum* raccolte da uccelli migratori sull'isola di Ventotene (Italia centrale), e la determinazione della quantità di questo batterio nelle zecche mediante un approccio di PCR Real time.

Qui di seguito viene riportata una breve descrizione di ciascun argomento:

i) M. mitochondrii è un batterio intracellulare dell'ordine Rickettsiales tramesso dalle zecche, che si trova con una elevata prevalenza nella zecca dura *I. ricinus*. *M. mitochondrii* viene trasmesso verticalmente alla progenie, e negli ultimi anni sono state riportate diverse evidenze di trasmissione all'ospite vertebrato durante il pasto di sangue della zecca. In questo lavoro sperimentale abbiamo indagato la presenza del batterio in campioni di sangue e siero di capriolo (*C. capreolus*) raccolti da un'area nota per essere altamente infestata dalla zecca *I. ricinus* (Gardouch, Francia). Nella primavera del 2014, 2015 e 2016 sono stati raccolti campioni di sangue e siero nella regione francese sopra menzionata e analizzati mediante indagine molecolare (PCR) e sierologica (test ELISA e Western blot). Il DNA batterico è stato rilevato nel sangue di quattro caprioli, ma solo in uno o due anni di campionamento, mentre i campioni di siero sono risultati positivi per l'antigene di *M. mitochondrii* in tutti e tre gli anni di campionamento. I nostri risultati indicano che la presenza del batterio nel sangue è temporanea, mentre la risposta anticorpale sembra essere di lunga durata, probabilmente a causa della costante esposizione dei caprioli alle punture di zecca, e quindi alla ripetuta trasmissione del batterio. Rimane ancora sconosciuto il ruolo di *M. mitochondrii* nell'ospite vertebrato e la sua interazione con altri batteri trasmessi da zecche.

ii) I. ricinus è la più comune specie di zecca che parassita l'uomo in Europa, e rappresenta anche il principale vettore dell'agente eziologico della malattia di Lyme, il batterio *Borrelia burgdorferi*. Questa specie di zecca ospita anche il batterio simbionte *M. mitochondrii*, che può essere trasmesso all'ospite vertebrato durante il morso della zecca. Poiché un elevato numero di morsi di zecca nell'uomo rimane sconosciuto a causa della assenza di sintomi, e al momento non è ancora disponibile un test affidabile che diagnostichi il morso di zecca, in questo lavoro sperimentale è stato valutato se la sieropositività verso la proteina batterica FliD possa rappresentare un valido marker per il morso di *I. ricinus* in campioni di soggetti umani provenienti da diversi paesi europei a diverso rischio di esposizione al morso di zecca. In questo lavoro sperimentale sono stati analizzati, mediante un test ELISA, 274 sieri prelevati da pazienti umani che presentavano sintomi correlati a TBD per valutare la presenza di anticorpi diretti verso il batterio *M. mitochondrii*. La maggioranza dei campioni (179) analizzati è stata prelevata da soggetti umani che ricordavano il morso da zecca, mentre i rimanenti (95) da soggetti che non avevano memoria di morso. I risultati ottenuti hanno mostrato che la positività a M. mitochondrii è più alta quando il morso di zecca è certo, mentre è più basso quando non si è sicuri della presenza del morso. La positività più alta si verifica nei pazienti affetti da Lyme (47,3%) e quella più bassa si ritrova nei controlli negativi (2%). Alla luce di questi risultati, il batterio *M. mitochondrii* può essere considerato un utile "pacchetto" di antigeni per lo sviluppo di test sierologici in grado di diagnosticare il morso della zecca *I. ricinus*. In futuro, potranno essere incorporati in un test multi-antigenico per la diagnosi di morso da zecca ulteriori antigeni provenienti sia dal batterio che dalle ghiandole salivari di zecca.

iii) Il simbionte *M. mitochondrii,* localizzato nell'ovario e nelle ghiandole salivari della zecca *I. ricinus*, viene trasmesso verticalmente alla progenie ed è presente nelle zecche femmine di campo con una prevalenza del 100%, mentre tale prevalenza diminuisce dopo alcune generazioni di zecche allevate in laboratorio. Dal momento che questo batterio viene trasmesso all'ospite vertebrato durante il pasto di sangue di *I. ricinus*, questo lavoro sperimentale si è focalizzato sul monitoraggio della presenza di anticorpi diretti verso *M. mitochondrii* e di DNA batterico in conigli infestati sperimentalmente per un periodo di tempo di quattro mesi. Brevemente, due gruppi di conigli sono stati infestati rispettivamente con zecche *I. ricinus* femmine di campo (quindi naturalmente infettate da *M. mitochondrii*) e con zecche allevate in laboratorio (quindi prive del batterio); i campioni di sangue e siero sono stati prelevati da ciascun coniglio nove volte (partendo da una settimana prima dell'inizio dell'infestazione (T0) fino a quattro mesi dopo l'inizio dell'infestazione (T8)) e analizzati

mediante PCR e test ELISA per valutare la presenza del batterio. La presenza di *M. mitochondrii* è stata rilevata sia nei conigli infestati con zecche di campo che in quelli infestati con zecche di laboratorio, spingendoci ad analizzare queste ultime mediante uno screening molecolare più sensibile che ha permesso di identificare un carico batterico molto basso anche in questo strain di zecche. I nostri risultati mostrano che la trasmissione batterica si verifica anche a bassi carichi batterici e che la risposta anticorpale verso *M. mitochondrii* inizia entro la prima settimana di infestazione con zecche *I. ricinus* di campo. Inoltre è stato rilevato il DNA batterico circolante nel sangue dei conigli appartenenti a entrambi i gruppi fino alla fine dell'esperimento, suggerendo una replicazione del simbionte all'interno dell'ospite vertebrato.

iv) L'ipotesi che il morso della zecca *I. ricinus* possa rappresentare un fattore di rischio per l'insorgenza di diverse malattie cronico-degenerative quali la sclerosi laterale amiotrofica, la miocardiopatia e la sclerosi multipla, apre la necessità di produrre un test diagnostico affidabile in grado di identificare i morsi di questa specie di zecca sia nell'uomo che negli animali. I limiti rappresentati dal parassitismo supportato dalle zecche (disinformazione nella popolazione, le caratteristiche del morso di zecca quali assenza di prurito, dolore e sintomi immediati) ci hanno spinto a identificare proteine antigeniche provenienti dalle ghiandole salivari di *I. ricinus* e dal suo simbionte *M. mitochondrii* da usare come marcatore per la puntura di zecca. A questo proposito, sono state identificate proteine antigeniche dopo aver effettuato analisi di immunoblotting utilizzando estratti proteici di ghiandole salivari di zecche femmine di *I. ricinus* e campioni di siero raccolti da conigli infestati sperimentalmente. Sono stati disegnati diversi peptidi partendo dagli epitopi maggiormente antigenici di ciascuna proteina d'interesse, al fine di identificare marcatori antigenici per il morso di *I. ricinus* attraverso un saggio ELISA. Lo scopo di questo lavoro sperimentale è stato quello di indagare il parassitismo di *I. ricinus* in diverse specie di vertebrati (coniglio, uomo e capriolo) testando, mediante un test ELISA, i peptidi sviluppati dalle proteine antigeniche di questa specie di zecca e del batterio *M. mitochondrii*. La percentuale di positività, seppur bassa, riscontrata nei sieri umani raccolti da soggetti parassitati da zecche e precedentemente risultati negativi per *M. mitochondrii*, contribuisce ad aumentare la percentuale di sensibilità del test per identificare il morso di zecca. È inoltre interessante notare che i sieri di coniglio testati usando due tra i peptidi prodotti come antigene del saggio ELISA, hanno mostrato positività fino a quattro mesi dopo l'infestazione sperimentale, rafforzando la buona proprietà di questi peptidi come indicatore del morso di zecca.

v) Le zecche possono essere facilmente trasportate su lunghe distanze mentre si nutrono sui loro ospiti; in particolare, durante le migrazioni, le specie aviarie possono contribuire alla dispersione delle zecche attraverso le barriere geografiche. Il ruolo degli uccelli migratori nella diffusione delle zecche presenta implicazioni epidemiologiche, dal momento che gli uccelli possono contribuire alla dispersione dei vari agenti patogeni e dei microrganismi trasmessi dalle zecche. Poiché diverse specie di uccelli rappresentano comuni ospiti di zecche che ospitano batteri appartenenti alla famiglia delle *Midichloriaceae* (ad esempio i generi *Ixodes* e *Hyalomma*), in questo lavoro abbiamo identificato un nuovo batterio del genere *Midichloria* in ninfe della specie *Hyalomma marginatum* raccolte sull'isola di Ventotene (Italia centrale) da uccelli migratori provenienti dall'Africa. Abbiamo inoltre quantificato questo batterio nelle ninfe mediante un approccio di PCR Real time, osservando un aumento del carico batterico in seguito al pasto di sangue, similmente a quanto osservato per *M. mitochondrii* nella zecca *I. ricinus*. Studi futuri mireranno a investigare la presenza di questo batterio in zecche adulte di *H. marginatum*, con lo scopo di determinarne la localizzazione accrescendo così le conoscenze riguardanti il suo ruolo nella biologia di questa specie di zecca, e la sua modalità di trasmissione durante il pasto di sangue.

1 Introduction

1.1 Ticks: a general overview

Ticks are obligate blood-sucking arthropods found throughout most regions of the world (Sonenshine and Roe, 2014). Ticks are chelicerates classified as a subgroup of the subclass Acari, in the order Ixodida of the superorder Parasitiformes (Krantz and Walter, 2009; Nicholson et al., 2009). They can be recognized by their flattened body shape (when unfed), their four pairs of walking legs (three pairs in larvae), and the presence of a hypostome with numerous recurved teeth. At present, almost 900 species have been described (Barker and Murrell, 2008), and are subdivided into three families: Ixodidae, Argasidae and Nuttalliellidae (Nava et al., 2009). Approximately 80% of the world's tick fauna are ixodid ticks (known as "hard ticks" due to the presence of a sclerotized dorsal plate and a protruding capitulum) and, with the exception of one species in the family Nuttalliellidae, the remainder are argasid ticks ("soft ticks", characterized by the absence of dorsal plate and the capitulum, except in larvae, positioned sub-terminally) (Jongejan and Uilenberg, 2004). This thesis work will focus on the Ixodidae family, since is the most involved taxon in human and animal tick parasitosis. The world's ixodid tick fauna consists of 241 species in the genus *Ixodes* and 442 species in the remaining genera (the most important genera are *Amblyomma, Dermacentor, Haemaphysalis, Hyalomma, Ixodes, Rhipicephalus* and *Boophilus;* the five species of the genus *Boophilus* have been placed in the genus *Rhipicephalus* by Horak et al. in 2002 to reflect their close phylogenetic relationship). The species belonging to this family are arranged into two major groups (Prostriata and Metastriata; Oliver 1989).

Generally, the body of ticks is divided into three regions: the idiosoma (body region), the capitulum, and the legs. 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.

The life cycle of all hard ticks consist of four developmental stages, the egg and three active parasitic stages (larva, nymph, and adult [male and female]; Figure 1) (Apanaskevich and Oliver, 2014).

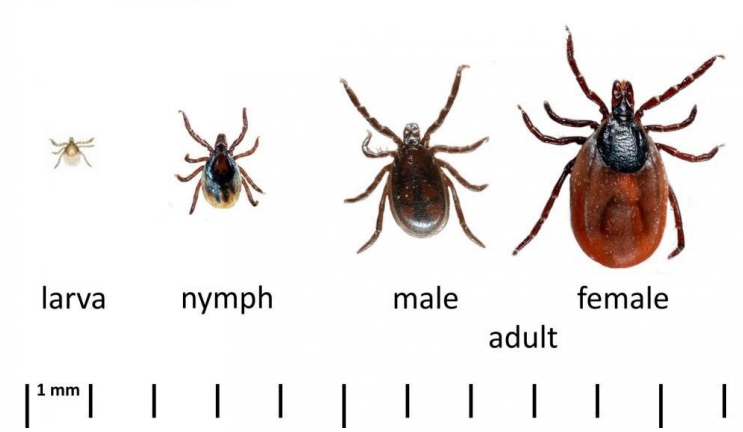

Figure 1: Tick developmental stages of the genus *Ixodes.* (www.merckvetmanual.com)

One of the most outstanding characteristics of ticks is their long life cycle. Most ixodid tick species have life spans that last at least one year; many live for two or even three years. Each active stage feeds only once in its life, although females forcibly detached from their hosts are able to reattach and continue feeding. For instance, females of *I. ricinus* were able to reattach to another host and finish their blood meal after five days of feeding and attaining a weight of 50 mg (Apanaskevich and Oliver, 2014).

Ixodidae may be one-, two- or three-host species depending on the number of host animals they attach to during their life cycle. The vast majority of ixodid ticks are characterized by a three-host life cycle (Figure 2). In a three-host life cycle, ticks do not moult on the host; the larvae drop off from the host after feeding and molt to the nymphal stage. Subsequently, the unfed nymphs quest for a host and may attach to the same or different hosts, feed, drop off, and molt to the adult stage. The adults then find their host and females mate and feed, drop off the host, lay a large batch of eggs, and die (Apanaskevich and Oliver, 2014). Males of most Metastriata need to feed on hosts in order to promote the maturation of their sperm (Oliver

1982), while in the majority of Prostriata, males do not require a blood meal and are able to copulate with the females off or on the host. The longevity of *Ixodes* males (Prostriata) is much shorter than that of the male metastriate ticks.

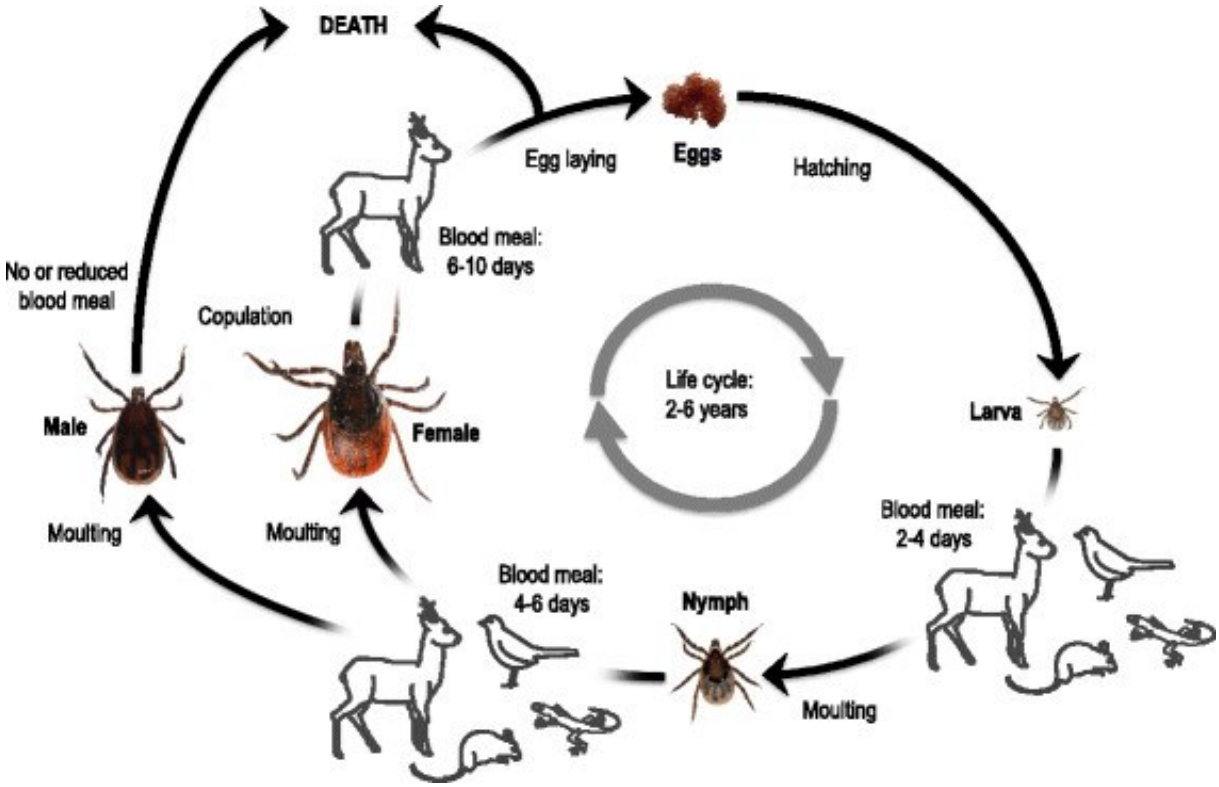

Figure 2: Typical three-host Ixodidae life cycle. (www.researchgate.net)

The majority of ixodid tick larvae feed during a 3- to 6-day period, nymphs during a 3- to 10-day period, and females during a 6- to 12-day period. The extension of the feeding period of ixodid females is dependent on whether they copulate with a male or not; unmated females can stay attached to hosts for up to several weeks. The longevity of feeding also can depend on the host (for example, ticks feed longer on reptile hosts). Other factors such as locality on the host and immune status of the host are also important for the duration of feeding (Apanaskevich and Oliver, 2014). For successful blood feeding, ticks have evolved mechanisms to disrupt the natural hemostatic mechanisms of the host. The ticks must also maintain blood flow and prevent or minimize pain, inflammation, itching, and immune rejection (Sonenshine and Roe, 2014). Recent discoveries concerning the molecular biology of tick salivary glands function have revealed hundreds of different proteins, including many that have no known function and many that are unique to ticks. Especially prominent are those adapted to prevent blood coagulation, inhibit platelet aggregation (e.g., lipocalins) and

histamine secretion, suppress inflammation, and bind host immunoglobulins (Sonenshine and Roe, 2014).

As in the case with many other arthropods, ticks are very sensitive to climate. Since ticks spend most of their life cycles in the environment, host availability and vegetation significantly modulate the dynamics of tick populations (Estrada-Peña, 2009). The geographical ranges of tick populations are not static, and will shift as a consequence of changes in climate and in human activities (changes in vegetation, land use or host displacement). It is assumed that climate warming will produce the inexorable extension of the range of disease-transmitting arthropods, including ticks (Estrada-Peña, 2009). Climate change will produce an expansion in the geographical range of any tick species, and species currently found in warmer environments will be introduced to areas beyond their range, specially by migratory birds, that can act as carriers of immature stages of ticks (Hoggstraal and Kaiser, 1961). There are several evidences of the slow but continuous expansion of the historical frontiers of some tick species into areas where they were previously absent. Warmer temperatures are the main divers of these range changes (Estrada-Peña, 2009).

1.2 Ticks and their microbial communities

1.2.1 Tick-borne pathogens and symbionts

Ticks are widely feared because of their role in the transmission of human and animal diseases. Approximately 10% of the 900 currently known tick species are of significant importance both in medical and veterinary field (de la Fuente et al., 2017). Ticks infest every class of terrestrial vertebrates, including mammals, birds, reptiles, and (in a few cases) even amphibians, transmitting a greater variety of disease-causing agents than any other group of arthropods (Sonenshine and Roe, 2014). Although mosquitoes transmit pathogens that infect far more people and cause more severe diseases of humans and animals (e.g., malaria, dengue fever, yellow fever, heartworm), ticks transmit a greater variety of pathogenic organisms (hereafter tick-borne pathogens-TBP), including fungi, viruses, bacteria, and protozoa (Sonenshine and Roe, 2014). The list of known or potential TBP is constantly evolving (de la Fuente et al., 2008a; Heyman et al., 2010; Dantas-Torres et al., 2012; Rizzoli et al., 2014). Currently, more than 16 specific tick-borne (or tick-caused) diseases of humans and more than 19 tick-borne diseases of livestock and companion animals have been described (Nicholson et al., 2009; Dantas-Torres et al., 2012). Examples of human diseases caused by these tick-borne

pathogens include Lyme disease, Rocky Mountain spotted fever, Mediterranean spotted fever, human granulocytic anaplasmosis, human monocytic anaplasmosis, tularemia, Colorado tick fever, and tick-borne encephalitis. Examples of tick-borne diseases of livestock and companion animals include babesiosis, theileriosis, heartwater, anaplasmosis, Lyme disease, and ehrlichiosis (Sonenshine and Roe, 2014). Moreover, ticks can cause severe toxic conditions such as paralysis and toxicosis, irritation and allergy (Jongejan and Uilenberg, 2004).

Lyme disease, the most commonly reported tick-borne disease in Europe and North America, was first recognized as a reportable human disease in the United States in the 1980s, with only a few hundred cases per year. Its incidence has since increased to more than 28.000 cases per year in 2008 (Anonymous 2008); thousands of cases per year also occur in Europe and northern Asia (i.e., throughout much of the holoarctic region of the world). Moreover, rather than a single genotype, *Borrelia burgdorferi* s.l. (transmitted by ticks of the *I. ricinus* complex), a number of 19 different genospecies (e.g., *B. garinii, B. bissettii*) are now known to cause this disease, with significant differences in clinical symptoms in different regions of its vast zoogeographic range (Sonenshine and Roe, 2014; Casjens et al., 2011; Stanek et al., 2011). Another example is represented by Rocky Mountain spotted fever, whose frequency is also increasing; this is a severe and life-threatening disease widespread throughout most of the United States and much of Latin America, caused by the intracellular bacterium *Rickettsia rickettsii* transmitted by ticks of the *Dermacentor* species (Sonenshine and Roe, 2014).

Currently it is clear that ticks carry complex microbial communities that include TBP, but also non-pathogenic microorganisms, such as commensal and mutualistic microbes (Andreotti et al., 2011; Carpi et al., 2011; Williams-Newkirk et al., 2014; Duron et al., 2015, 2017). Before 1990, their existence was largely ignored and all bacteria found in ticks were usually considered to be potential TBP, but the advent of PCR assays led to an increasing understanding that a few intracellular bacteria (e.g. *Coxiella* and *Francisella*-like endosymbiont) are non-pathogenic microorganisms hosted by ticks (Niebylski et al., 1997; Noda et al., 1997). It is now clear that ticks carry complex microbial communities that are largely dominated by non-pathogenic microorganisms. The composition of these microbial communities could vary depending on the examined tick species (Lalzar et al., 2012), the season during which ticks were collected (Lalzar et al., 2012), the examined geographical regions (van Overbeek et al., 2008; Carpi et al., 2011), the examined tick life stage (Moreno et al., 2006; Clay et al., 2008; Williams-Newkirk et al., 2014), and between different feeding statuses (Heise et al., 2010; Menchaca et al., 2013; Zhang et al., 2014).

As vertebrate pathogens, TBP normally spread via horizontal transmission during the tick bite, but a few TBP can also be vertically transmitted in ticks, and thus be maintained throughout each generation as observed for *Babesia* species (Chauvin et al., 2009), *R. rickettsii* (Burgdorfer et al., 1981) or viruses (Xia et al., 2016). Other tick microorganisms are highly specialized intracellular symbionts depending almost on transovarial transmission to ensure their persistence in tick populations (Lo et al., 2006; Sassera et al., 2006; Klyachko et al., 2007; Felsheim et al., 2009; Machado-Ferreira et al., 2011; Lalzar et al., 2014; Duron et al., 2015; Kurtti et al., 2015). In most cases, the function of tick endosymbionts in relation to their host has not been determined. Many of these endosymbionts have obligate intracellular life cycles or are difficult to cultivate, which may explain the gaps in current knowledge (Tully et al., 1995; Kurtti et al., 1996; Niebylski et al., 1997; Duron et al., 2015). However, for some bacteria, tissue-specific localization has been defined, leading us to better understand bacterial impact on both tick biology and pathogen transmission (Noda et al., 1997; Klyachko et al., 2007; Lalzar et al., 2014; Narasimhan and Fikrig, 2015).

Maternally-inherited symbionts are well-known to use specific adaptive strategies to spread and persist within arthropod populations, conferring crucial and diverse benefits to their hosts, playing nutritional roles, or affecting fitness, development, reproduction and immunity (Ahantarig et al., 2013). At least 10 distinct genera of maternally-inherited symbionts have been reported in ticks over the last decade (Duron et al., 2017). These symbionts comprise *Coxiella*-like endosymbionts (hereafter *Coxiella*-LE), *Rickettsiella*-LE, *Arsenophonus*, *Francisella*-LE, *Cardinium*, *Spiroplasma*, *Lariskella*, *Midichloria*, *Rickettsia*, *Wolbachia*. *Coxiella*-LE and *Francisella*-LE are only found in ticks, while the remaining symbiont genera are more or less found in other arthropod groups. *Coxiella*-LE are believed to be the most common vertically transmitted agents in hard ticks (Bernasconi et al., 2002; Lee et al., 2004; Clay et al., 2008; Bonnet et al., 2013; Cooper et al., 2013). These symbionts have been reported as essential for tick survival and reproduction in the *Amblyomma americanum* lone star tick, showing a reduction in tick offspring production and an increased time to oviposition after their removal following antibiotic treatment (Zhong et al., 2007). This symbiont typically infects the ovaries and the distal part of Malpighian tubules, suggesting a possible role in nutrition, osmoregulation, or excretion (Klyachko et al., 2007; Machado-Ferreira et al., 2011; Lalzar et al., 2014). The presence of major B vitamin synthetizing pathways in *Coxiella*-LE genome enables ticks to utilize an unbalanced dietary resource becoming hematophagy specialists, since these vitamins are not obtainable in sufficient quantities from a uniquely blood-based diet (Gottlieb et al., 2015; Smith et al., 2015).

Candidatus Midichloria mitochondrii (hearafter *Midichloria mitochondrii*) is an intramitochondrial bacterium found in the hard tick *I. ricinus* (Lo et al., 2006; Sassera et al., 2006; Montagna et al., 2013). Recent sequencing and analysis of the *M. mitochondrii* genome led to the hypothesis that the bacteria could serve as an additional ATP source for the host cell during the oogenesis (Sassera et al., 2011). In addiction, this symbiont has been ascribed a possible helper role in tick molting processes (Zchori-Fein and Bourtzis, 2011). Interestingly, the fact that *I. ricinus* adult males are less commonly infected than individual females by *M. mitochondrii* (Lo et al., 2006), suggests that this bacterium may be an important nutritional symbiont for this tick species, given that adult males from *Ixodes* species do not blood-feed, implying that they do not need nutritional symbionts (Duron et al., 2017). This topic concerning the presence of *M. mitochondrii* in ticks will be better explained in the next paragraphs.

Many of tick species commonly harbor facultative symbionts host more than one microorganism (Bonnet et al., 2017a). Indeed, six distinct genera of maternally inherited symbionts co-exist in *I. ricinus* (*Midichloria*, *Spiroplasma*, *Coxiella*-LE, *Wolbachia* and *Rickettsiella*; Duron et al., 2017), and in *R. decoloratus* populations (*Midichloria*, *Coxiella*-LE, *Francisella*-LE, *Rickettsia*, *Cardinium*, and *Spiroplasma*; Duron et al., 2017).

A fascinating aspect is that non-pathogenic microorganisms can also interfere with TBP replication and transmission by influencing TBP abundance and their transmission to vertebrate host (Bonnet et al., 2017a). An example is represented by the existence of defensive symbionts that protect their host against a variety of pathogens. Burgdorfer et al. were the first to report the presence of defensive symbionts in the tick *Dermacentor andersoni*, where the symbiont *Rickettsia peacockii* hampered the multiplication and transovarial transmission of the spotted fever agent *R. rickettsii* (Burgdorfer et al., 1981). In *Ixodes scapularis* it has also been reported that male ticks infected by *Rickettsia buchneri* have significantly lower rates of *B. burgdorferi* infection than symbiont-free males (Steiner et al., 2008).

Recent literature has reported that certain symbionts may be transmitted to vertebrates following tick bite (Shivaprasad et al., 2008; Woc-Colburn et al., 2008; Vapniarsky et al., 2012; Bazzocchi et al., 2013; Edouard et al., 2013; Angelakis et al., 2016; Seo et al., 2016; Bonnet et al., 2017b), with the potential to opportunistically infect these hosts, including humans. Among these symbionts, *Coxiella*-LE are the most commonly found microorganisms in vertebrates. It has recently been reported that tick transmitted *Coxiella*-LE cause mild infectious symptoms in humans from Europe (Angelakis et al., 2016). The ability of this

symbiont to infect vertebrates through tick bite could be explained by its presence in salivary glands of some tick species (Klyachko et al., 2007; Machado-Ferreira et al., 2011; Qiu et al., 2014), in addiction to ovaries and Malpighian tubules. Another example is represented by the *I. ricinus* intra-mitochondrial symbiont *M. mitochondrii*, whose presence has been detected in several vertebrate hosts after the tick bite (Mariconti et al., 2012a; Bazzocchi et al., 2013). This transmission could be explained by the localization of *M. mitochondrii* bacteria in the salivary glands and saliva of *I. ricinus* (Di Venere et al., 2015), aside from tick ovaries. In addiction, *M. mitochondrii* DNA and antibodies against a bacterium antigen (the flagellar protein FliD), were detected in the blood of several vertebrates species exposed to tick bites (Mariconti et al., 2012a; Bazzocchi et al., 2013). However, whether *M. mitochondrii* can cause a true infection and pathological alterations in mammalian hosts remains to be determined.

1.2.2 Bacteria of the family *Midichloriaceae* in ticks

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* (Taylor and Hoerauf, 1999; Werren et al., 2008). The order Rickettsiales is composed of three families of bacteria: *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*. The family *Midichloriaceae* was described by Montagna and colleagues 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 (Lewis, 1979; Fritsche et al., 1999). 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 (Perlman et al. 2006; Weinert et al. 2009), and in filarial nematodes (Taylor et al., 2005; Werren et al., 2008). In addition to the usual genera historically included in the Rickettsiales on the basis of phenotypic and/or genotypic data (Weisburg et al., 1989; Drancourt and Raoult, 1994; Werren et al., 1995; Roux et al., 1997), a large number of RLO that are associated with protozoa, insects and other invertebrates, and fungi are also described (Preer et al., 1984). Bacterial *16S rRNA* gene sequences recently obtained for ticks and amoebae showed high similarity to *16S rRNA* gene sequences from members of the Rickettsiales (Fritsche et al., 1999; Goddard et al., 2003; Parola et al., 2003; Beninati et al., 2004; Mediannikov et al., 2004). Phylogenetic analyses of these sequences revealed the existence of a novel clade within the order Rickettsiales (Beninati et al., 2004). The *Midichloriaceae* family is formed by bacteria associated with a wide range of hosts (from protists to Cnidaria and Porifera, from parasitic arthropods to mammals); until now, all the members studied by transmission electron microscopy are intracellular and with a typical Gram-negative cell wall. This widespread distribution, together with 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* (the most studied member of this family) to vertebrate hosts after *I. ricinus* bite, as mentioned in Paragraph 1.2.1. In addition, the presence of a bacterium belonging to the *Midichloriaceae* family has been associated with two chronic skin diseases of unknown aetiology affecting farmed rainbow trout (*Oncorhynchus mykiss)* in United States and Europe (i.e. strawberry disease and red mark syndrome; (Lloyd et al., 2008; Metselaar et al., 2010)).

Bacteria closely related to the type-representing species of the *Midichloriaceae* family (*M. mitochondrii*) have been detected in many tick genera distributed all over the world: *Ixodes, Rhipicephalus, Dermacentor, Haemaphysalis, Hyalomma, Amblyomma* (Beninati et al., 2004; Sacchi et al., 2004; Sassera et al., 2006; Lo et al., 2006; Sassera et al., 2008; Epis et al., 2008; Hornok et al., 2008; Venzal et al., 2008; Beninati et al., 2009). They have also been detected in several other haematophagous arthropods, such as mites of bats, bed bugs and two tabanid fly species. Recent reports are expanding the view of this family, now including numerous bacteria of great biological and medical interest, indicating a widespread distribution with an increasing range of hosts. In fact, several sequences have been obtained also from rather

different hosts, like amoebae, marine sponges, hydrae, and corals (Reeves et al., 2006; Richard et al., 2009; Hornok et al., 2008; Vannini et al., 2010). 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 study conducted in 2016 revealed the presence of bacteria belonging to the monophyletic group of the genus *Midichloria*, in 17 different tick species, including the first report of a representative of this genus, in a soft tick species, *Ornithodoros maritimus* (Cafiso et al., 2016). The study highlighted different prevalence levels and variable bacterial loads in the different analyzed tick species, including the tick *I. aulacodi*, where the bacterium *Midichloria* has been detected in 100% of the examined samples, suggesting a similar behavior to *M. mitochondrii* of *I. ricinus*.

Although the *16S rRNA* marker, due to limited genetic variability, is not suitable for a fine phylogenetic discrimination, the phylogenetic tree did not show evidence of co-cladogenesis between bacteria and their tick hosts (Epis et al., 2008). For example, *Midichloria* associated to different species of the genus *Ixodes* do not form a monophyletic group, but are scattered in various clusters of the phylogenetic tree, sometimes clustering with sequences associated to other tick genera or even other tick families (Cafiso et al., 2016). The wide distribution of this bacterium in haematophagous arthropods and the lack of concordance between the phylogenies of *M. mitochondrii* and its tick hosts suggest that, in addition to vertical transfer, *Midichloria* bacteria may undergo horizontal transfer between ticks through vertebrate hosts, possibly during the blood meal (Epis et al., 2008; Cafiso et al., 2016). This is also supported by evidences of the horizontal transmission of *M. mitochondrii* to several animal species after *I. ricinus* bite (Mariconti et al., 2012a; Bazzocchi et al., 2013) as illustrated in Paragraph 1.2.1.

A study conducted in 2018 detected for the first time the presence of *Midichloria* bacteria in African tick species (*Hyalomma marginatum*) collected from migrating birds on Ventotene Island ((Central Italy) Di Lecce et al., 2018)). Migratory birds often serve as carriers of pathogens of medical and veterinary importance, enhancing the dispersal of microorganisms and their vectors over long distances across countries and continents (Hahn et al., 2009; Bauer and Hoye, 2014; Altizer et al., 2011). Since birds are common hosts of ticks harboring bacteria, viruses and protozoa, they may contribute to tick and tick-borne microorganisms dispersal across geographical barriers, such as oceans and deserts.

For this reason, the study of the role of migratory birds as reservoir host of *Midichloria* bacteria, which has never been previously addressed, may help us to obtain valuable information to define the geographic distribution of these bacteria, and to increase the knowledge of their potential infectious role.

1.2.3 *Ixodes ricinus* and its endosymbiont *Midichloria mitochondrii*

The hard tick *I. ricinus*, known as the "sheep tick" or the "castor bean tick", is the most common tick species occurring in Europe, and infecting humans. The importance of *I. ricinus* is due to its wide area of distribution (i.e. Europe and Northern Africa), to its low host specificity and capacity to parasitize humans and to its central role in the transmission of multiple infectious agents (Socolovski et al., 2009). This tick species is primarily associated with shrubs and deciduous and mixed forests. However, as a consequence of changing land use and wildlife management, persistent tick populations and high prevalence of infections with tick-borne pathogens have also been observed in urban and peri-urban sites in many European countries (Gern et al., 1997; Junttila et al., 1999; Ogden et al., 2000; Maetzel et al., 2005; Wielinga et al., 2006; Schorn et al., 2011). *I. ricinus* is a generalist exophilic tick species that is able to feed on over 300 different vertebrate species (Bowmann and Nuttall, 2008). *I. ricinus* has a long-lasting life cycle, involving three active life stages (larvae, nymphs, and adults) that quest and attach to a host and feed on blood for a few days before detachment and subsequent molting or laying eggs (females). Populations of large animals like roe deer and wild boar have become more abundant in peri-urban areas around European villages and cities, leading to establishment of tick populations, shift of natural transmission cycles of some pathogens, and increase of the disease risk for humans and domestic animals (Pfäffle et al., 2013).

As mentioned above, *I. ricinus* is capable of transmitting multiple infectious agents, both bacterial (e.g. causative agents of Lyme borreliosis and rickettsial diseases), and viral [e.g. tick-borne encephalitis (TBE) virus] pathogens (Medlock et al., 2013); *I. ricinus* vector-borne diseases are spreading throughout Europe (Altpeter et al., 2013; Hubalek and Rudolf, 2012; Rizzoli et al., 2011).

In addition to the pathogens mentioned above, *I. ricinus* hosts an intracellular bacterium whose pathogenic role is still to be investigated: *M. mitochondrii* (order Rickettsiales, family *Midichloriaceae*). *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 *M. mitochondrii* replicated in the mitochondria, which then burst and released bacteria into the cytoplasm (Lo et al., 2006). 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; Figure 3).

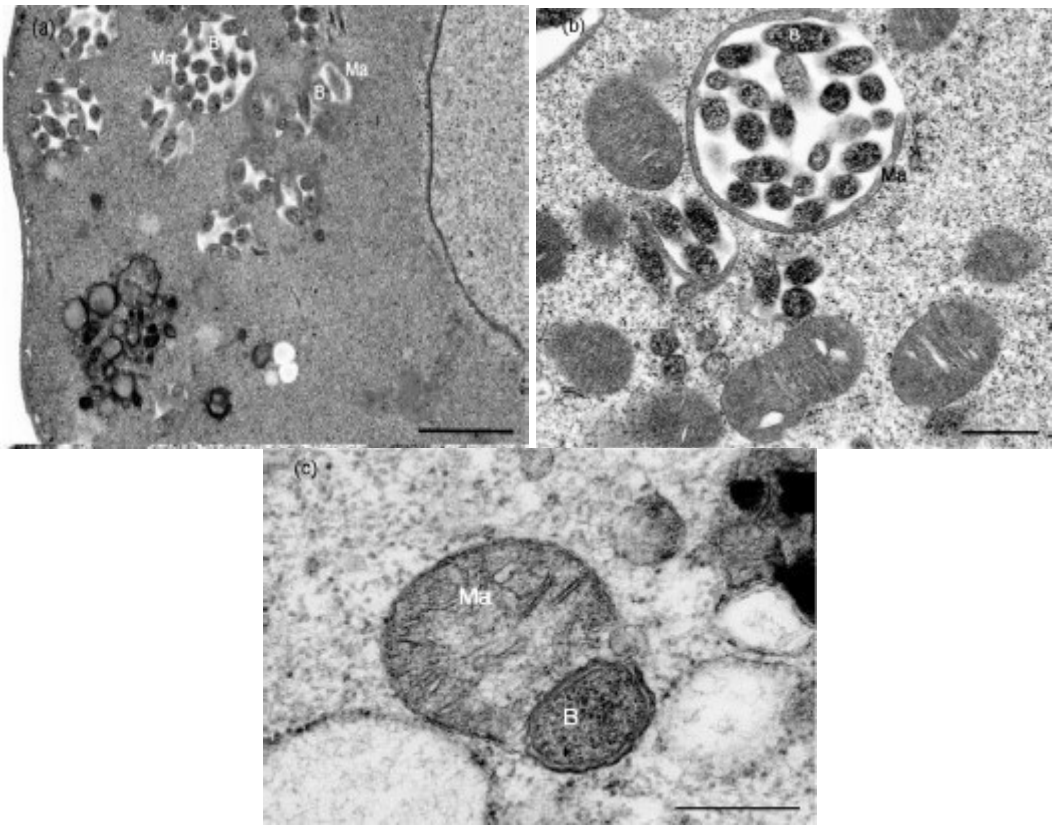

Figure 3: Electron micrographs of ovarian cells of *I. ricinus* containing "*Candidatus* M. mitochondrii" (B) within intact or degraded mitochondrial matrix (Ma). Bars, 2.4 mm (a), 1.2 mm (b) and 0.45 mm (c). (Sassera et al., 2006)

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). Given its peculiar capacity of colonizing mitochondria (Beninati et al., 2004), the symbiont was then proposed to be renamed "*Candidatus* Midichloria mitochondrii" (Sassera et al., 2006) with a name able to recall its unique intra-mitochondrial lifestyle. *M. mitochondrii* has so far only

been observed within the ovary of *I. ricinus* with 100% prevalence in female ticks, while the prevalence in males is around 44%. This ubiquitous presence suggests that the symbiont plays a role in the biology of female ticks, considering that evidence for efficient vertical transmission of the bacterium has been reported, as indicated by PCR on eggs and newly-emerged larvae (Sassera et al., 2006). This prevalence is observed throughout the entire geographical distribution of *I. ricinus* and no alterations of the reproduction have been observed in this tick species so far (Sassera et al., 2008). Little is known about the role of *M. mitochondrii* in its arthropod host. The population dynamics of the symbiont during the host's life cycle has been followed using quantitative real-time PCR (qPCR): bacterial load increase after blood meal of the tick and decrease after the molting, indicating that bacterial growth is linked to the blood meal. Whether this coincides with the production of metabolites by *M. mitochondrii* for its host, or if it may reflect competition among symbionts for transmission to the next stage of the tick remains to be determined (Sassera et al., 2008). qPCR has also shown that a single tick ovary can contains several millions of genome copies of this symbiont, and up to 10^5-10^6 genome copies can be detected in single eggs (Sassera et al., 2008). Based on the relatively low variance of bacterial numbers in larvae, it appears that the symbiont is transferred to both males and females, but the higher variance in nymphs and the large difference between adults, suggests a specialization toward females during the nymph stage (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). As mentioned in Paragraph 1.2.1, there are several hypotheses about some possible roles of *M. mitochondrii* in its arthropod host: helper role during the tick molting processes (Zchori-Fein and Bourtzis, 2011), heme detoxification (Sassera et al., 2011), nutritional role (Duron et al., 2017), and to confer protection toward different types of pathogens/parasites (Pistone et al., 2011).

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. After the genome sequencing of *M. mitochondrii*, the presence of 26 genes

involved in flagellar synthesis was described (Sassera et al., 2011). *M. mitochondrii* was the first member of the Rickettsiales described to present a complete set of genes coding for a putative flagellar apparatus (Mariconti et al., 2012b). Following this discovery, a fragment of the flagellar protein "FliD" (i.e. the external cap) was produced in recombinant form and purified. The molecular mass of the rFLID is 38 kDa while the native molecular mass is 100 kDa. This protein was used in a study conducted in 2012 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) that have posed the basis for the development of novel markers for investigating the exposure of humans and animals to this tick species (Mariconti et al., 2012a). Another study reported indirect signs of the presence of *M. mitochondrii* bacteria in sera of dogs exposed to *I. ricinus* bite through an ELISA test that evaluated the presence of antibodies against the rFliD protein (Bazzocchi et al., 2013). In this context *M. mitochondrii* can thus be considered not only as a symbiont of the *I. ricinus* tick, but also as a potential infectious agent or, at least, as a package of antigens that ticks can inoculate into vertebrate hosts during the blood meal (Mariconti et al., 2012a).

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, sheep and dogs exposed to the risk of tick bite; Bazzocchi et al., 2013), in roe deer specimens, which are known to be highly parasitized by this tick species (Skarphédinsson et al., 2005).

Cafiso et al. (2019) firstly reported a study on the onset of the antibody response against *M. mitochondrii* flagellar protein FliD in a vertebrate model (rabbit) after infestation with *I. ricinus*, and its kinetic during a time-span of four months. The circulation of *M. mitochondrii* DNA in blood was also evaluated by PCR assay for the duration of the experiment. This study showed that the seroconversion against *M. mitochondrii* rFliD in rabbits infested with *I. ricinus* was observed to occur one week post-infestation. Furthermore, the symbiont seems to replicate inside the vertebrate host, as revealed by molecular and serological analyses showing positivity up to 16 weeks after experimental infestation (Cafiso et al., 2019). Additionally, this work provides evidence for a positive antibody response against rFliD protein even when the vertebrate host is parasitized by *I. ricinus* ticks harboring reduced bacterial loads ((*I. ricinus* ticks maintained under laboratory conditions at for over 10 generations) Cafiso et al., 2019).

Even though the transmission of *M. mitochondrii* into the vertebrate hosts during the *I. ricinus* bite is currently established, it is still unknown whether immature stages (larvae and nymphs)

of this tick species transmit the symbiont during the blood meal. The fate of this symbiont in the vertebrate host after the tick bite is still unexplored, in fact, the transmission and replication of *M. mitochondrii* into the vertebrate host does not necessarily imply a pathogenic role of this endosymbiont. *M. mitochondrii* might play a primary or secondary role in pathologies deriving from the tick bite, indeed there are several hypotheses on the existence of a correlation between the *I. ricinus* bite and the development of chronic-degenerative diseases with complex aetiology (Hansel et al., 1995; Halperin et al., 1990; Harvey and Martz, 2007; Hsieh et al., 2007; Lelovas et al., 2008). These hypotheses could be taking into account considering the ability of *M. mitochondrii* to invade mitochondria and to be transmitted to vertebrate host during the tick blood meal.

1.3 Tick immunobiology and the problem of tick bite

1.3.1 Monitoring tick bite exposure: identification of potential candidate vaccine antigens

Ticks are of vast medical and veterinary public health importance due to direct damage caused by feeding and their roles in transmitting infectious agents (Brossard and Wikel, 2004). Indeed, these arthropods are currently considered the second most important vectors for pathogens in human and animal populations after mosquitoes (Raoult and Parola, 2007; Beugnet and Marie, 2009). Tick control has been primarily based on the use of acaricides resulting in partial efficacy and enhanced selection of resistant tick populations (de la Fuente and Contreras, 2015).

As already mentioned in Paragraph 1.1, tick bites in humans often go unnoticed, because people may mistake the bite of another arthropod as that of a tick (Alarcon-Chaidez et al., 2006) and components of tick saliva have anesthetic properties which inhibit host pain/hitch responses (Francischetti al., 2009). Additionally, the predominant developmental stage of *I. ricinus* found to feed on humans are nymphs (Wilhelmsson et al., 2013), which are difficult to detect due to their small size. For this reason, especially for patients suspicious to have tick-borne diseases without a record of the tick bite, it is necessary to develop a diagnostic test for tick bite and new approaches are needed for effective control of ticks and their associated tick-borne diseases (de la Fuente and Contreras, 2015). The characterization of the immunobiology of the tick-host interface is essential to understand tick feeding, pathogen transmission and development of anti-tick vaccines (Brossard and Wikel, 2004). The only commercially available vaccines against ectoparasites were developed in the early 1990s for

the control of cattle tick infestations, and were based on recombinant *Rhipicephalus microplus* BM86/BM95 antigens (Cobon et al., 1995; de la Fuente et al., 1995; Massard et al., 1995; Vanegas et al., 1995; Willadsen et al., 1995; Canales et al., 1997). The application of these vaccines resulted in reduction of the number, weight and reproductive capacity of female ticks, resulting in a gradual reduction in tick infestations in subsequent generations (de la Fuente et al., 2007). A vaccine against Lyme borreliosis was approved in 1999 by the US Food and Drug Administration, but was withdrawn from the market in 2002 (Piesman and Eisen, 2008). Despite recent advances in the identification of candidate tick vaccine antigens, new tick and/or pathogen-derived antigens need to be identified to increase vaccine efficacy for the control of tick-borne diseases (de la Fuente and Contreras, 2015; Schetters et al., 2016). Multi-directional interactions occur between ticks, hosts, and transmitted pathogens in both the tick and host environments, affecting all three members (Chmelař et al., 2016; Figure 4). During the acquisition of a blood meal, ticks alternate blood feeding with the secretion of saliva into the wound.

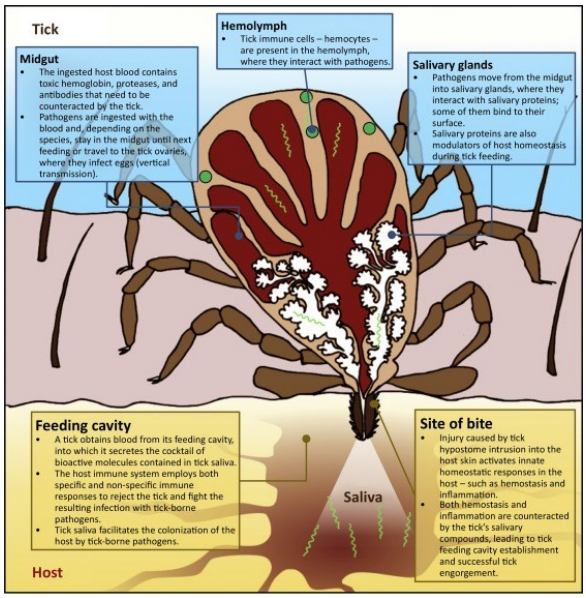

Figure 4: The complex interactions between ticks, hosts and tick-borne pathogens (Chmelař et al., 2016)

Tick saliva is a rich cocktail of bioactive compounds that facilitates an undisturbed blood feeding on the host for several days (de la Fuente et al., 2016). Ticks inject pharmacological compounds that include immunomodulators, inhibitors of pain/itch response, anticoagulants, inhibitors of platelet aggregation, and vasodilatory molecules, all of which contribute to both successful blood feeding and production of an antibody response directed against arthropod salivary proteins (Ribeiro et al., 1985; Ferreira et al., 2003; Steen et al., 2006; Frauenschuh et

al., 2007; Juncadella et al., 2007; Francischetti et al., 2009; Oliveira et al., 2010; Vančová et al., 2010). This serological response was demonstrated for numerous vector species, including Ixodidae (Steen et al., 2006; Francischetti et al., 2009), and has been proposed for use as a biomarker of host exposure to tick bites. The first demonstration of an association between exposure to arthropods and an antibody response was reported in 1990, using the sera from outdoor workers in New Jersey (USA) exposed to *I. scapularis* (Schwartz et al., 1990). Furthermore, a correlation between host antibody response and Lyme disease seropositivity has been demonstrated (Schwartz et al., 1990). Additionally, a decrease in the antibody response was observed in the absence of tick exposure for several months, demonstrating that the host immune response is transitory and is useful for following seasonal exposure to tick (Schwartz et al., 1991). These studies highlight the potential for the host immune response against salivary vector proteins to be used as a biomarker of tick bite exposure. The use of whole salivary gland protein extracts from vectors as a source of antigens presents numerous limitations, including low protein extract yields, with numerous tick dissections needed, inter-individual heterogeneity of salivary components, which prevents standardization, possibility of cross-reactions among salivary proteins of other arthropods (Vu Hai et al., 2014). A study conducted in 1998 demonstrated that *A. americanum* calreticulin induces an antibody response in human exposed to tick bite (Sanders et al., 1998). This protein was identified as one of the most abundant salivary gland proteins and contributes to blood meal success (Jaworski et al., 1995; Michalak et al., 2009). Calreticulin is an endoplasmic reticulum protein with multifunctional properties that participates in the folding of newly synthetizes proteins and in the regulation of intracellular Ca^{2+} homeostasis (Michalak et al., 2009). In the case of *Ixodes* tick species, antibody responses against *I. scapularis* calreticulin were found in humans exposed to this tick species using an ELISA test. The use of *Ixodes* calreticulin ELISA as a diagnostic indicator of *I. scapularis* exposure has been considered useful in developing a vaccine against tick-borne infections (Alarcon-Chaidez et al., 2006). In 2013 the first study examining the host immunological response to identified potential antigenic salivary biomarkers of *I. ricinus* bite exposure has been performed, using rabbits experimentally infested with *I. ricinus* ticks (and *R. sanguineus and D. reticulatus* ticks as controls) (Vu Hai et al., 2013). Immunoblot analyses were performed using protein salivary gland extracts from these three tick species to cross-compare the IgG profiles, highlighting a singularity in the immune patterns according to tick species exposure and the protein salivary gland extracts antigen source. This work demonstrated the high antigenicity of the calreticulin

protein, which could be used as a potential biomarker for tick exposure and also as a potential candidate for anti-tick vaccine development (Vu Hai et al., 2013).

Concerning *I. ricinus*, another salivary protein has been proposed as a potential tick bite marker in a study conducted in 2017 by Mihaljica et al. This is the case of the IrAV422 protein, previously described in *A. americanum* species (Mulenga et al., 2013), whose expression is up-regulated by feeding of the tick (Mulenga et al., 2007), and which is conserved across tick species, with no mammalian or other arthropod homologues, making it a unique tick saliva protein (Mulenga et al., 2013). In the study mentioned above, differences in sera reactivity among experimental infested animals (rats) were observed, casting doubt on its efficacy in different situations (Mihaljica et al., 2017).

Concerning *I. ricinus*-borne disease prophylaxis, current vaccination strategies seem to be insufficient to prevent such diseases, such as Lyme borreliosis. Indeed, given the broad spectrum and antigenic variability of *Borrelia* surface lipoproteins associated with different *Borrelia* species, no licensed vaccine against *B. burgdorferi* sensu latu is currently available (Schuijt et al., 2011). Another example is represented by the rather poor coverage of vaccination against tick-borne encephalitis (TBE) in almost all endemic European countries (Heinz et al., 2013). For this reason, the identification of additional tick-protective antigens to create anti-tick vaccines seems to be a promising strategy (Almazan et al., 2003; Carreon et al., 2012).

1.3.2 Identification of potential biomarkers for *I. ricinus* exposure

Tick-borne microorganisms can be transmitted by ticks from stage to stage (transstadially) or from one generation to the next (transovarially). Most tick-borne pathogens are acquired when ticks ingest an infective blood meal; therefore, the tick midgut serves as both barrier and gateway to pathogen invasion (Alarcon-Chaidez, 2014). Bioactive agents present in tick saliva potentiate transmission of several microorganisms, by modifying the host immune defenses to make the tick-host interface a less hostile environment during blood feeding (Alarcon-Chaidez, 2014).

As already mentioned in Paragraph 1.2.3, the high importance of the hard tick *I. ricinus* is due to its wide area of distribution, to its low host specificity and capacity to parasitize humans and to its central role in the transmission of multiple infectious agents (Socolovski et al., 2009).

Most tick-borne diseases do not show specific clinical manifestations, resulting in flu-like symptoms that can easily go unnoticed or misdiagnosed in humans as well as in other animals

(Mihaljica et al., 2017). The specific characteristics of the tick bite (absence of pain, rarity of immediate symptoms, and misinformation in the population) going to complicate this scenario, making difficult to estimate the actual health impact of this parasite, and leading to high risk of occult chronic infections. The impact on human health of these occult infections is a matter of debate, but many authors agree in hypothesizing a correlation between the *I. ricinus* bite and the development of chronic-degenerative diseases with complex etiology. Probably the most discussed correlation is the one between tick bite and Amyotrophic Lateral Sclerosis (ALS), for which there are several lines of evidence (Hansel et al., 1995; Halperin et al., 1990; Harvey and Martz, 2007). 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). Furthermore, the emergence of new tick-borne diseases and the re-emergence of existing ones, together with the increasing dispersal of *I. ricinus*, increase the importance of this public health issue.

Given the current lack of a reliable test for *I. ricinus* bite, the development of markers to identify the bite of this tick species would be 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 diseases mentioned above. The development of these markers would allow to achieve several important results, such as the determination of the incidence of occult tick bites in human and animal populations, the contribution to the diagnosis of many tick-borne diseases, especially in cases where the anamnestic information on exposure to ticks is not available.

As already discussed in Paragraph 1.3.1, some *I. ricinus* antigens have already been proposed as potential tick bite markers, i.e. calreticulin and IrAV422 proteins, but given the obtained results, there are concerns about their effectiveness as a valuable tick bite marker (Vu Hai et al., 2013; Mihaljica et al., 2017). The inoculation of *I. ricinus* symbiont *M. mitochondrii* during the blood meal, has now been demonstrated by several molecular and serological evidences, as previously mentioned (Mariconti et al., 2012a; Bazzocchi et al., 2013). The transmission of *M. mitochondrii*, together with the seropositivity against the bacterial flagellar protein FliD (Mariconti et al., 2012a; Bazzocchi et al., 2013), makes this bacterium a potential novel marker to diagnose *I. ricinus* bite, especially in those areas endemic for this tick species and considered a high-risk areas for the transmission of several *I. ricinus*-borne pathogens. Even though several evidences currently demonstrate the transmission of *M. mitochondrii* into the vertebrate host during *I. ricinus* blood meal, the transmission of this symbiont by immature developmental stages of *I. ricinus* (larvae and nymphs) has yet to be demonstrated.

For this reason, other antigens from *M. mitochondrii*, as well as from the tick itself, could be investigated and finally incorporated into a multi-antigen test for the diagnosis of the tick bite. The work conducted by Di Venere et al. (2015) has the aim to investigate, from a proteomic point of view, the tick *I. ricinus* and its symbiont, with the goals of better understanding the interaction between the bacterium and the tick, and to pose the basis of immunoproteomic approaches useful for detecting antigenic proteins for innovative diagnostic and vaccination approaches. The aim has been achieved through the comparison of the proteomic profiles of the ovary tissue and of salivary glands collected from tick adult females, by identifying tissue-specific proteins and proteins involved in the tick-bacterium interaction using two-dimensional electrophoresis. Most of the antigenic spots detected after immunoblotting analyses have been identified as *I. ricinus* proteins (Di Venere et al., 2015).

Additional antigenic proteins have been detected after immunoblotting analyses using protein extracts of salivary glands collected from *I. ricinus* adult females, and sera samples collected from experimentally infested rabbits according to the protocol described in Di Venere et al., 2015. Novel peptides were designed from the most antigenic epitopes of each protein identified to detect antigenic markers for *I. ricinus* bite through an enzyme-linked immunosorbent assay (ELISA). A novel ELISA approach based on the use of new antigens will allow the identification of new and more suitable markers for investigating the exposure of humans and animals to *I. ricinus* bite. It has to be reminded that a marker based on *M. mitochondrii* antigens could represents a potential problem regarding the transmission of the bacterium which may not occur in 100% of cases of tick bite (the bacterium is inoculated into the vertebrate host during the blood meal of *I. ricinus* adult females, but this fact has not yet been demonstrated in larval and nymphal stages, as already mentioned).

A procedure with the simplicity of an ELISA and based on synthetic antigens would in principle circumvent the technical complexity and potential antigen variability associated with immunoblot diagnosis. Synthetic peptides used as antigens have the added advantage of avoiding contamination with low-level impurities derived from *E. coli* or other cloning vectors required to produce recombinant proteins.

2 Aims of the PhD project

Ticks are obligate haematophagous ectoparasites of mammals, amphibians, reptiles and birds and globally distributed from the tropics to the Polar regions. Given the impressive variety of infectious agents (bacteria, viruses, protozoa) they can transmit, they are considered to be the second most important vectors for pathogen that cause diseases in humans and animals after mosquitoes (Beugnet and Marie, 2009). Approximately 900 tick species have been described, of which nearly 10% have an impact on human and animal health (Jongejan and Uilenberg, 2004). Ticks do not only carry pathogens, but several commensal and symbiotic microorganisms are also present in these arthropods, of which biology and effects on ticks remain unexplored.

The tick family of Ixodidae includes 692 species, and hosts a variety of symbiotic bacteria belonging to the *Rickettsiaceae*, *Anaplasmataceae* and *Midichloriaceae* families. The bacterial family *Midichloriaceae* is formed by bacteria associated with a wide range of phylogenetically diverse hosts, from protists to Cnidaria and Porifera, from parasitic arthropods to mammals. Several lines of evidence indicate that this bacterial family includes agents transmissible to vertebrate hosts (including humans), and possibly involved in the development of pathological alterations. These data obviously need to be better investigated to evaluate the effective diffusion of these bacteria inside vertebrates and the possible pathogenic effect. For example, the biology of the representative member of *Midichloriaceae* family (*M. mitochondrii* associated with the hard tick *I. ricinus)* has yet to be understood, although several lines of evidence indicate its transmission during the tick bite with a modulation of the host immune response (humans and other animals infested by *I. ricinus* have been found seropositive to FliD protein of *M. mitochondrii*; Mariconti et al., 2012a; Bazzocchi et al., 2013). The seroconversion time against *M. mitochondrii* rFliD has been studied in rabbits experimentally infested with *I. ricinus* for a time-span of four months, showing to occur starting from one week post-infestation up to 16 weeks after experimental infestation (see work conducted by Cafiso et al., 2019 in the Chapter 3). The replication of *M. mitochondrii* inside the vertebrate host has also been demonstrated for the first time in this vertebrate model, since bacterial DNA has been detected in blood samples up to 16 weeks post-infestation, suggesting the bacterial capacity of survive and replicate into the host after its inoculation (Cafiso et al., 2019). Additionally, *M. mitochondrii* transmission has also been demonstrated when the symbiont load is reduced in case of tick laboratory strains (where a

bacterial decrease in prevalence after some generations under laboratory conditions it is well known).

The detection of bacteria closely related to *M. mitochondrii* has been showed in different tick genera all over the world, such as *Ixodes*, *Rhipicephalus*, *Dermacentor*, *Haemaphysalis*, *Amblyomma*, *Hyalomma*, as well as in other haematophagous arthropods (Beninati et al., 2004; Sacchi et al., 2004; Sassera et al., 2006; Lo et al., 2006; Sassera et al., 2008; Beninati et al., 2009; Epis et al., 2008; Hornok et al., 2008; Venzal et al., 2008). The first detection of *Midichloria* bacteria in *H. marginatum* ticks collected from migratory birds travelling back to Africa (Di Lecce et al., 2018), may contribute to obtain valuable information to define the geographic distribution of this bacterium, as well as to increase the knowledge of its potential infectious role, given the important role of avian migration in the spread of tick-borne microorganisms.

As mentioned above, *Midichloriaceae* family includes bacteria transmissible to vertebrates whose involvement in pathological alterations is still to be investigated. The specific features of the tick bite, characterized by the absence of pain and itch (blocked by anesthetic components of the tick saliva) and rare immediate symptoms, along with misinformation in the population, often leading to undetected tick bites, lack of a prompt therapy with the risk of occult chronic infections. The suspected correlation between the development of several animal and human diseases and tick bites, together with the lack of a currently reliable test capable of detecting *I. ricinus* bite, make the development of markers to identify tick parasitism essential to determine the incidence of occult tick bites in human and animal populations. Furthermore, the development of tick bite markers could be useful to evaluate the debated role of tick bite as a risk factor for the onset of different chronic degenerative disease (Hansel et al., 1995; Halperin et al., 1990; Harvey and Martz, 2007; Hsieh et al., 2007; Lelovas et al., 2008). The development of a method based on a serological test to detect *I. ricinus* bite in humans and animals represents the main aim of my PhD project.

The principal aims of my PhD project were:

1) To investigate the presence of *M. mitochondrii* in blood and sera collected from some roe deer (*Capreolus capreolus*) individuals, different in age and sex, from a specific and defined area in France, collected during the spring of 2014, 2015 and 2016. Blood and sera samples were subjected to molecular screening through PCR and to serological investigation using enzyme-linked immunosorbent assay (ELISA) and Western-blot. All samples have been analyzed in three different times to investigate how the bacterial

pattern changes during the years;

2) To investigate whether humans suffering from several symptoms correlated to the tick bite from several European countries at different risk of *I. ricinus* bite, produce antibodies against the flagellar FliD protein of *M. mitochondrii* using an ELISA approach. This part of the project was conducted at the BCA-clinic of Augsburg (Germany), where I conducted a period of research with the focus to validate a serological marker to detect *I. ricinus* bite in humans exposed to the bite of this tick species;

3) The study of the time and kinetic of seroconversion against the flagellar FliD protein of *M. mitochondrii* and salivary glands crude protein extract of *I. ricinus* ticks in rabbits experimentally infested with this tick species (wild and laboratory strain *I. ricinus*);

4) To detect new antigenic proteins belonging to *I. ricinus* tick and its symbiont *M. mitochondrii* to use as potential biomarkers for tick bite. This aim represents an attractive tool for producing serological tests for tick bite and/or possible vaccines against the parasite, since nowadays a method capable of determine the true risk associated with tick exposure is not available. The use of a combination of antigens from symbiont and 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;

5) To determine the genetic variability of a previously undescribed *Midichloria* strain within the *Midichloriaceae* family found in *H. marginatum* ticks collected from migratory birds on Ventotene Island (Central Italy) and to quantify the amount of this bacterium in ticks using a novel Real Time approach. The present study represents the first investigation of *Midichloria* bacteria harbored by ticks collected from trans-Saharan migratory birds travelling back from Africa. Since avian migration represents an important mechanism for the spread of ticks and their microorganisms, the study of the potential emerging infectious role of *Midichloria* bacteria in these hosts could be considered an interesting future perspective to study their geographic distribution and their role in the tick biology.

During my PhD course, I attended on other research activities concerning the study of *M. mitochondrii* and bacteria related to *Midichloriaceae* family to expand the knowledge about

their biological role and to investigate their fate in the host:

6) The study of the relationship between a bacterium of the family *Midichloriaceae* (*Midichloria*-like organisms; MLO) and the red mark syndrome (RMS) in rainbow trout (*Oncorhynchus mykiss*) through a novel quantitative PCR approach. This molecular method allowed to investigate and quantify the presence of the MLO associated with RMS in affected *O. mykiss* skin and organs (heart, liver, spleen, intestine, kidney);

7) The study of *M. mitochondrii* life cycle within the host oocytes using a multidisciplinary approach (electron microscopy, molecular biology, statistics and system biology) in order to test the hypothesis that the symbiont could move within the mitochondrial network, passing from a mitochondrion to another. I focused my attention on the molecular analyses (Real time PCR) to quantify *M. mitochondrii* and mitochondria organelles in *I. ricinus* oocytes.

I have also collaborated with PhD colleagues in two other research activities concerning topics different from my main PhD project aim:

1) The study of the differential expression of Leukotriene A4 Hydrolase (*LTA4H*) and Fragile X Mental Retardation, Autosomal Homolog 1 (*FXR1*) genes in canine oral melanoma as new potential prognostic biomarkers through a Real time approach;

2) The study of the effect of different stocking densities of farmed Siberian sturgeon (*Acipenser baerii*) larvae on muscle formation and oxidative stress, by evaluating the expression of specific genes involved in the development of muscles and in cellular stress (Insuline-like growth factor 1 and 2 *(Igf1 and Igf2)*, Myogenin *(Myog)*, Glucose transporter *(Glut1 and glut2)*, and Heat Shock protein 70 *(Hsp70)*).

3 Research activities carried out during PhD course

3.1 Molecular and serological evidence of the presence of *Midichloria mitochondrii* in roe deer (*Capreolus capreolus*) in France

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3.1.1 Aim of the study

M. mitochondrii is an alfa-proteobacterium of the order Rickettsiales found with a prevalence of 100% in the hard European tick *I. ricinus*. Multiple lines of evidence indicate that this symbiont is capable of vertical transmission in the tick, and horizontal transmission to the vertebrate host during the tick bite (Mariconti et al., 2012a; Bazzocchi et al., 2013). The work here presented concerns the molecular and serological investigation of *M. mitochondrii* circulation in roe deer (*C. capreolus*), an efficient animal sentinel to study the spread of some tick-borne pathogens (or potentially pathogen), since it represents the host of choice for adult and nymphal stages of *I. ricinus*. Molecular (PCR) and serological (ELISA and Western-blot) analyses were performed on blood and sera samples collected in three different years (spring of 2014, 2015 and 2016) from the same seven individuals in a defined area in France (INRA Gardouch experimental station - Haute Garonne). The analysis conducted on the same individuals in different years has the aims to monitor the progress of the infection and to increase the knowledge about *M. mitochondrii* and its fate in the vertebrate host after its inoculation during *I. ricinus* blood meal.

3.1.2 Material and methods

Blood and sera samples were collected from seven roe deer in a 15 ha enclosure (9 ha woodlot and 6 ha of pasture) at the French National Institute for Agricultural Research experimental station at Gardouch in France (Haute Garonne). Each animal was sampled three times, in April of 2014, 2015, and 2016, with the exception of one deer that died before the

third sampling. Ticks from each roe deer were collected, morphologically identified through standard dichotomous keys (Manilla 1998), and confirmed to be *I. ricinus*. Blood samples were withdrawn from the jugular vein; a portion was centrifuged for serum collection and another was used for DNA extraction. A qualitative PCR on the *12S rRNA* gene of *C. capreolus* was carried out to check the quality of the DNA extraction (Fajardo et al. 2008). Detection of circulating *M. mitochondrii* DNA was performed using a nested PCR approach. External primers GYRB-F1 (5' AAGCTAAGAATTTGGCG TGATG 3') and GYRB-R (5' GTTTTGGCTT CATTTGGATTTTC 3') were designed on the *gyrB* gene sequence of *M. mitochondrii* and used to amplify a fragment of 776 base pairs (bp; annealing temperature 57 C; final primers concentration 1 μM). The resulting products were subjected to a real time PCR using protocol and primers GYRB-RT-F (50 CTTGAGAGCAG AACCACCTA 30) and GYRB-RT-R (50 CAAG CTCTGCCGAAATATCTT 30) described in Sassera et al. (2008) and amplifying a fragment of 146 bp internal to the previous amplified region. The second PCR was carried out using a real-time PCR approach to maximize the sensitivity and specificity of bacterial DNA detection. Blood samples were considered positive for the presence of *M. mitochondrii* DNA when a clear peak was present, the melting temperature of the reamplified samples corresponded to that of a positive control, and the resulting Sanger sequences showed 100% identity with a fragment of the published gyrB sequence of *M. mitochondrii* (accession number AM159536.1; nucleotide position from 565 to 710). In parallel, the recombinant flagellar protein FliD (rFliD) of *M. mitochondrii* was used to determine anti-rFliD antibody levels in deer sera with an enzyme-linked immunosorbent assay (ELISA). The negative controls used to establish a cut-off value were the sera of 10 red deer from a virtually tick-free area (Merler et al. 1996) and the sera of two roe deer newly-born in an enclosure.

3.1.3 Results and discussion

Molecular analyses showed that three deer were negative to *M. mitochondrii* DNA, two showed positivity at one of the three sampling times, and the remaining two were positive at two of three times (Table 1).

These results confirmed that *M. mitochondrii* was released with the tick saliva during the blood meal. However, the negative results from the second sampling in some individuals that had been positive in the first one, and specifically the temporal pattern of roe deer 145 (positive–negative–positive), indicated that the presence of *M. mitochondrii* in roe deer blood was transient, and that this bacterium likely did not multiply heavily in the host blood. The

two possible explanations for this finding are the presence of a transient infection or the movement of the bacteria to other body tissues. Interestingly, the pattern of immunization of the roe deer to *M. mitochondrii* was different compared to the results concerning the presence of the bacterium in blood samples.

The cut-off of the ELISA reaction was established at an optical density (OD) of 0.35 (mean OD on negative controls plus three times standard deviation) determined at a wavelength of 450 nm. All parasitized *C. capreolus* samples showed an average OD greater than 0.35, whereas the OD of all the samples obtained from tick-free animals were under the cut-off value. The ELISA results were confirmed by Western blot (Mariconti et al. 2012a).

The ELISA and Western blot serological assays showed seropositivity against *M. mitochondrii* in all samples from all 3 yr (Table 1). This result suggested that roe deer, when continuously parasitized by a large number of ticks, were continuously exposed to ongoing injection of *M. mitochondrii*. In this context, the presence of *M. mitochondrii* in tick saliva might play a role in the immune response and immune modulation, possibly important for infection by tick-borne pathogens.

Table 1: Results of molecular (nested PCR for the amplification of a fragment of *gyrB* gene) and serological analyses (enzyme-linked immunosorbent assay and Western blot) for the presence of *M. mitochondrii* performed on roe deer (*C. capreolus*) blood and sera collected in Spring 2014, 2015 and 2016 in France. Results are expressed as + (=positive sample), – (=negative sample), NA (=sample not available).

	Molecular analysis			**Serological analysis**		
	2014	**2015**	**2016**	**2014**	**2015**	**2016**
Roe deer 144	+	-	-	+	+	+
Roe deer 145	+	-	+	+	+	+
Roe deer 146	-	-	-	+	+	+
Roe deer 147	-	+	+	+	+	+
Roe deer 148	+	-	NA	+	+	+
Roe deer 149	-	-	-	+	+	+
Roe deer 179	-	-	NA	+	+	NA

3.1.4 Conclusions

Because roe deer are the host of choice for nymphal and adult stages of *I. ricinus*, this is a good species for the study of some tick-borne pathogens. Roe deer play an important role as reservoirs of *A. phagocytophilum*, two *Bartonella* species, *Babesia divergens*, and *Theileria* spp., but not for *Borrelia* spp. (Skotarczak et al. 2008; Chastagner et al. 2014). Deer are considered as dilution hosts for the agent of Lyme disease, because they maintain high tick intensities, which perpetuate tick populations, but they do not support transmission. The transient bloodstream presence of *M. mitochondrii* observed with our molecular analysis could be due to the function of roe deer as a dead-end transmission host, a situation similar to that of *Borrelia* spp. (Jaenson and Tälleklint 1992; Nelson et al. 2000). Future studies could be focused on the role of roe deer in the circulation of *M. mitochondrii*, biological interactions of this bacterium with the host or with other bacteria transmitted by ticks (through the immune reaction of the host against bacteria), the possible replication of this bacterium inside the host, and the role of the bacterium in possible pathological alterations after its transmission.

3.2 Seropositivity to *Midichloria mitochondrii* (order Rickettsiales) as a marker to determine the exposure of humans to *Ixodes ricinus*

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Manuscript submitted to Ticks and Tick-Borne diseases

3.2.1 Aim of the study

Ticks are among the most important vectors of pathogens, causing human and animal diseases, in tropical, sub-tropical and temperate countries (Karaer, 2011). Approximately 10% of tick species are estimated to play a role in the transmission of a total of over 200 pathogens (Jongejan and Uilenberg, 2004; Labuda and Nuttall, 2004) including zoonotic bacteria (e.g. *Borrelia burgdorferi* sensu lato, *Rickettsia rickettsii*, *Coxiella burnetii, Ehrlichia canis, Francisella tularensis*; Jongejan and Uilenberg, 2004) and viruses (e.g. tick-borne encephalitis virus, Crimean-Congo hemorrhagic fever virus; (Jongejan and Uilenberg, 2004; Despommier et al. 2000).

I. ricinus is the most common tick species parasitizing humans in Europe, and the main vector of *Borrelia burgdorferi* sensu lato, causative agent of Lyme disease in the continent (Mead, 2015). This tick species also hosts the bacterial endosymbiont *M. mitochondrii,* which has been shown to be inoculated in the vertebrate host during the tick bite (Mariconti et al. 2012a; Bazzocchi et al. 2013; Serra et al. 2018; Cafiso et al. 2019).

A high proportion of tick bites remain unnoticed due to rarity of immediate symptoms, implying a risk of occult tick-borne infections in turn a potential risk factor for the onset of chronic-degenerative diseases (only 30% of patients with symptoms referable to tick-borne diseases report a history of tick bite; Perronne, 2014). Since there is the need for tools suitable to determine the previous exposure to tick bites, this work aimed to investigate whether seropositivity toward a protein of *M. mitochondrii* (FliD) could represent a marker suitable for diagnosis of *I. ricinus* bite in humans. Two hundred and seventy-four sera were collected from patients from several European countries at different risk of tick bite, and screened using an ELISA protocol to detect anti-*M. mitochondrii* antibodies.

3.2.2 Material and methods

Sample collection

A total of 274 human sera previously tested (positive, borderline, or negative to IgG and IgM) with the *Borrelia* Blot (Anti-*Borrelia* Euroline RN AT IgG / IgM (Euroimmun, DN 2131-3201-G/N, Lübeck, Germany) were retrospectively selected from the collection of a specialized tick-borne diseases laboratory (BCA-clinic of Augsburg; Germany). These samples were obtained from patients from different European countries (countries of origin and number of sera are detailed in Table 1).

Presence or absence of a report of tick bite was associated to the metadata referred to each serum sample. One hundred seventy-nine out of 274 patients reported memory of tick bite, while the remaining 95 patients did not declare a history of tick parasitism.

A. phagocytophilum, Rickettsia spp.*,* and *Babesia* spp. antibodies were measured to complete the set of *I. ricinus* associated pathogens. The kits used to determine the expanded serological status were: *A. phagocytophilum* IFA IgG / IgM (Focus Diagnostics, IF1450G/M, Cypress, USA/CA); *Rickettsia* IFA IgG / IgM (Focus Diagnostics, IF0100g/M); *Babesia* (*B. microti*) IFA Substrate Slide and *Babesia* IgG Detectable control (Focus Diagnostics, IF0904 and IF0910). The serological kits were used and interpreted according to the manufacturer's instructions.

Additionally, 50 sera were obtained from healthy donors living in regions non-endemic for *I. ricinus*. These sera, previously tested negative for *Borrelia* s.l., *A. phagocytophilum*, *Rickettsia* spp., and *Babesia* spp. antibodies, were used as negative controls. All sera samples were stored at -80°C until use. Written informed consent for the use of biological material was obtained from all patients prior to serum sampling.

Table 1: Number of sera samples analyzed for each country.

Country	No. of samples
Germany	114
France	33
Norway	28
Sweden	12
Netherlands	11
Denmark	10
Romania	9
Belgium	7
United Kingdom	7
Switzerland	6
Austria	5
Russia	5
Ukraine	5
Italy	4
Poland	3
Belarus	3
Finland	3
Spain	3
Czech Republic	2
Croatia	2
Ireland	1
Turkey	1

ELISA assay

The FliD protein of *M. mitochondrii* was produced in recombinant form in *Escherichia coli* (rFliD; Mariconti et al. 2012b) and used as substrate for ELISA assays.

Wells of ELISA flat-bottom plates were coated with 0.1 µg/well of rFliD diluted in carbonate-bicarbonate buffer (Sodium carbonate anhydrous 15 mM and Sodium hydrogen carbonate 35 mM). After the incubation at 4°C over-night, the plate was washed three times for five minutes using 200 µl/well of washing buffer (PBS 1X and 0.1% Tween 20), and was incubated at 37°C for one hour with the coating buffer (PBS 1X and 3% Bovine serum Albumine). Sera were analyzed in duplicate at a dilution of 1:100 and the anti-human IgG HRP-conjugated antibody (Sigma-Aldrich)® was employed at 1:2000. After the preparation of the staining solution (citric acid 25 mM and Sodium phosphate dibasic 45 mM), the plate was incubated at dark with a solution consisting of 20 ml of staining solution plus 8 µl of hydrogen peroxide 30% and 1 o-Phenylenediamine dihydrochloride tablet 0.4 mg/ml

(Sigma)® for 10 minutes. The optical density (O.D.) was measured at a wavelength of 450 nm using the "Sunrise™ absorbance microplate reader" (Tecan)® and the data analysis software Magellan.

The cut-off of the reaction was established using sera from the 50 healthy blood donors and it was set as the mean optical density (O.D.) plus three times the standard deviation. Each sample was considered negative if its O.D. was less than the threshold value, and positive if its O.D. was higher or equal to the threshold.

3.2.3 Results and discussion

A total of 274 sera from patients suffering from different symptoms potentially referable to TBDs were included in this study. Among these sera, the majority (179) had been collected from patients who remembered a tick bite, the remaining (95) from patients that did not recall a tick bite. Fifty sera were collected from healthy blood donors from areas where *I. ricinus* is not present and used as negative control.

In order to identify the patients that could reliably assumed to have been parasitized by a tick, we analyzed all the 274 sera for positivity to tick-borne pathogens (TBP; i.e. *B. burgdorferi* s.l., *A. phagocytophilum*, *Rickettsia* spp., and *Babesia* spp.). The results of the serological analyses are reported in Table 2. Based on the anamnestic information on the tick bite, on clinical diagnosis for Lyme disease and on the results of the above serological analyses, we could divide the patients in five groups: i) 38 with a diagnosis for Lyme disease; ii) 81 seropositive to at least one TBP but without a positive diagnosis for Lyme; iii) 92 patients that recall a tick bite, but seronegative to all TBPs; iv) 63 patients presenting TBD-related symptoms but with no seropositivity or diagnosis for any TBD; v) 50 healthy blood donors, not reporting any experience of tick bite and negative to all TBDs (negative controls). All sera were analyzed for the presence of anti-*M. mitochondrii* antibodies, using the anti-rFliD ELISA assay described in Material and Methods. Rates of positivity for *M. mitochondrii* antigens in the five groups are shown in Table 3. Considering the results obtained from the five groups, there is a clear trend showing that positivity to *M. mitochondrii* becomes higher the more TBP-seroprevalence is detected and the more TBD related signs are present. The highest prevalence (47.3%) is indeed found in Lyme patients in which tick bite can be considered certain/almost certain. The prevalence becomes lower as evidence of the bite becomes uncertain (memory of a bite, or just symptoms) and results to be the lowest in the negative controls.

The possibility that seropositivity to *M. mitochondrii* could represent a serological marker for *I. ricinus* bite in humans had already been suggested based on a study limited to a total of 80 Italian patients with a diagnosis for Lyme disease (Mariconti et al. 2012a). Here we have extended this screening to a total of 274 subjects from different European countries, with different levels of risk of tick exposure. The overall results confirm the potential utility of anti-rFliD seropositivity as a marker for *I. ricinus* bite in humans. Overall there is a non-complete overlap between parasitized subjects and positivity to *M. mitochondrii* (i.e. up to approx. 50% in Lyme patients) that we could explain as follows. In most cases humans are parasitized by a single tick which is removed quickly, resulting in a shorter duration of the blood meal, and then likely in the inoculation of a limited amount of *M. mitochondrii* bacteria (or no bacteria at all), in contrast to what normally happens in other animal species. Although the work conducted by Cafiso et al. 2019 provides evidence for a positive antibody response against rFliD protein even when a vertebrate host is parasitized by *I. ricinus* ticks harboring reduced bacterial loads, it is still unknown whether immature stages of this tick species transmit *M. mitochondrii* during the blood meal. This consideration is noteworthy, since in most cases humans are parasitized by *I. ricinus* larvae and/or nymphs (Wilhelmsson et al. 2013). In addition, we can also consider the possibility that a proportion of the subjects with a record for tick bite had not really been parasitized by a tick, or had been parasitized by tick species other than *I. ricinus* (not harboring *M. mitochondrii*).

In summary, our study shows that *M. mitochondrii* can be regarded as a useful source of antigens for the development of a serological test for *I. ricinus* bite, suitable for application in different European countries. In prospect, additional antigens derived from *M. mitochondrii* and from the tick salivary glands could be pooled in a multi-antigen antibody detection assay.

Table 2: Results of the serological screening for *Borrelia* spp., *A. phagocytophilum*, *Rickettsia* spp., and *Babesia* spp. antibodies presence on the 274 sera collected from patients and presenting symptoms potentially referable to tick-borne diseases.

	***B. burgdorferi* s.l.**	***A. phagocytophilum***	***Rickettsia* spp.**	***Babesia* spp.**
No. positive sera/total of tested sera (%)	99/274 (36%)	17/274 (6%)	5/274 (1.8%)	1/274 (0.36%)

Table 3: Detection of anti-*M. mitochondrii* antibodies in human patients suffering from different symptoms potentially referable to tick-borne diseases from different European countries. The patients are divided into five groups according to the anamnestic information of the tick bite, the clinical diagnosis for Lyme disease, and the results of serological analyses for tick-borne pathogens (TBP; i.e. *Borrelia* spp*., A. phagocytophilum*, *Rickettsia* spp., and *Babesia* spp.). Results are expressed as: M+ = sera positive to *M. mitochondrii.*

Groups of patients	M+
Patients with a diagnosis for Lyme disease	18/38 (47.3%)
Patients seropositive to at least one TBP (no Lyme disease)	28/81 (34.5%)
Patients with memory of tick bite (seronegative to TBPs)	22/92 (24%)
Patients with TBD-related symptoms (no diagnosis for TBDs)	14/63 (22.2%)
Negative controls	1/50 (2%)

3.2.4 Conclusions

This work provides further indirect evidence for the transmission of *I. ricinus* symbiont *M. mitochondrii* to humans during the blood meal of *I. ricinus* ticks in different European countries. The results of the current study are coherent with those of a preliminary study conducted on a smaller set of humans exposed to ticks in Italy (Mariconti et al. 2012a). In prospect, other antigens from *M. mitochondrii*, as well as from the tick itself, could be investigated and finally incorporated into a multi-antigen test for the diagnosis of the tick bite. The protein rFliD can be regarded as a good candidate to be included into a such type of multi-antigen test. Even though this study provides evidence for the inoculation of *M. mitochondrii* bacteria into the human host during the tick blood meal, further studies are needed to determine whether this bacterium could survive and colonize particular host tissues into humans or other vertebrates. Anyway, based on our results there is currently no evidence for any causal relationship between seropositivity to *M. mitochondrii* and human pathologies.

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

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3.3.1 Aim of the study

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

The scientific contribution that I gave to the present work consisted on performing molecular analyses (qualitative and quantitative PCR) on lab *I. ricinus* ticks, wild and lab *I. ricinus* salivary glands pools, and rabbits blood samples to assess the presence/absence of *M. mitochondrii* bacteria and common tick-borne pathogens (TBPs).

3.3.2 Materials and methods

I. ricinus ticks collection

For the experimental infestation, a total of 60 wild unengorged *I. ricinus* adult females and 60 males were used. These ticks were obtained from fully engorged nymphs sampled on roe deer in the Chizé forest (Western France 46° 7′ 18.89″ N, 0° 25′ 3.72″ W) and maintained at the

ONIRIS-INRA laboratory under controlled conditions. Positivity of this tick population to *M. mitochondrii* had been already assessed in a previous study (Di Venere et al., 2015).

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

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

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

Experimental infestation

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

A total of nine blood samplings per rabbit were performed to collect serum for serology and EDTA-treated blood for molecular analyses. Blood withdrawals were performed on the ear free from ticks, starting from one week before exposure to ticks (T0) and at week 1 (T1), week 2 (T2), week 3 (T3), week 4 (T4), week 6 (T5), week 8 (T6), week 12 (T7) and week 16 (T8; end of the experiment) post-infestation. A workflow of the entire experiment is shown in Supplementary Fig. S1.

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

Salivary glands dissection and crude protein extract production

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

Molecular analyses

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

In order to achieve a higher sensitivity in the detection of the symbiont, a nested qualitative PCR approach on the *gyrB* gene was also performed, following the protocol described by Serra et al. (2018). To determine the sensitivity of the nested PCR, a 776 bp *M. mitochondrii gyrB* gene fragment, obtained with the external primers of the nested PCR assay, was cloned

in the pGEM-T Easy Vector (Promega). A clone was purified, the plasmid DNA was quantified and sequenced, diluted from 10 copies/µL to 1 copy/µL and then subjected to the nested PCR protocol.

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

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

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

Enzyme-Linked Immunosorbent Assay (ELISA) on rabbits sera

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

SG crude protein extracts (1 µg/well of wild- and lab-SG) were diluted in 100 µl carbonate-bicarbonate buffer (pH 9.6) and the 96-well microplates were incubated overnight at 4 °C. Plates were subjected to three 5-minutes washes (wash solution: PBS and 0.1% Tween-20) and then treated with 100 µl of blocking solution (3% BSA in 1X PBS) for 1 h at 37 °C. After further rinsing of the wells, each serum sample (tested in duplicate) was diluted 1:100 (dilution buffer: 1% BSA in 1X PBS) and plates were incubated for 1 h at 37 °C. After the washing steps, peroxidase-conjugated Goat anti-Rabbit IgG secondary antibody (Sigma-Aldrich) diluted 1:2000 in dilution buffer was added and plates were incubated for 45 min at 37 °C. One hundred µl/well of orthophenylenediamine (Sigma-Aldrich) with hydrogen peroxide was added to each well for the development of the reaction. The O.D. values were measured without stop solution at 450 nm with a SpectraMAX 340PC (Molecular Devices Corporation) spectrophotometer. Threshold values (cut-off) for the assays were established as mean O.D.450 nm values + 3 standard deviations of the T0 sera from the six infested rabbits. Values greater than or equal to the established thresholds were considered as positive.

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

Statistical analysis

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

3.3.3 Results and discussion

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

Thirty unengorged lab *I. ricinus* females were screened by qPCR, routinely used to quantify *M. mitochondrii* in *I. ricinus* (Sassera et al., 2008), to assess the absence of *M. mitochondrii* bacteria from the colony maintained in the lab for over 10 generations. To date, molecular analyses have shown that *M. mitochondrii* load decreases after five/seven generations under laboratory conditions, but no data are avail- able on the actual removal of the symbiont after > 10 generations (Lo et al., 2006). The qPCR assay highlighted the absence of the symbiont from all 30 tested individuals (data not shown). Based on the obtained result, lab ticks > 10 generations were chosen as *M. mitochondrii* negative control for the experimental infestation.
A total of ten wild-SG pools and five lab-SG pools were obtained after dissection of 100 wild and 50 lab semi-engorged females and subsequently subjected to qPCR both to test the presence/absence of *M. mitochondrii* and to evaluate the bacterial load. *M. mitochondrii* load in wild-SG pools showed *gyrB/cal* gene ratios between 10^1 and 10^3 (data not shown). On the contrary, the qPCR assay did not detect the presence of the symbiont in any of the five DNA samples extracted from lab-SG pools.

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

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

Serological analyses

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

Two groups of three rabbits each were infested with wild (R1, R2, and R3; group W) and lab ticks (R4, R5 and R6; group L), respectively. Sera and blood samples from rabbits were collected up to 16 weeks after the experimental infestation. None of the rabbits in this study showed any clinical signs for all the duration of the experiment (data not shown).

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

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

The elicited IgG antibody response against wild-SG protein extract (Fig. 1) increased significantly with time post-infection (parameter estimate ± SE: 0.10 ± 0.01; $t_{148} = 7.13$; $p < 0.0001$). A positive reaction (cut-off value: 0.32) was observed in rabbits of group W starting from week 1 up to the end of the experiment (week 16) and in rabbits of group L starting from week 2 up to the end of the experiment. Rabbits of group L showed significantly lower O.D. values than rabbits of group W in their antibody response against wild-SG crude protein extract (parameter estimate ± SE: -1.63 ± 0.15; $T8=-10.94$; $p < 0.0001$). This higher and earlier immune response observed in rabbits of group W compared to group L is consistent with our expectation of *M. mitochondrii* playing an antigenic function in the immune response

of vertebrate hosts against ticks. Once a plateau was reached, no decrease in the immune response was observed until the end of the experiment.

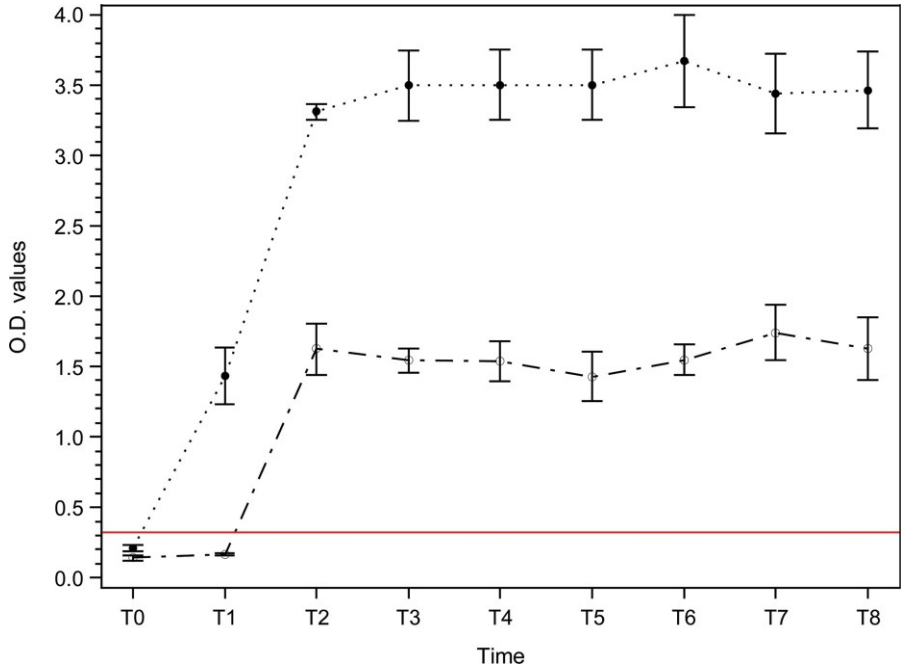


Fig. 1: Mean (± SE) antibody response against wild-SG crude protein extract in rabbits infested with wild (filled circles) and lab (blank circles) *I. ricinus* females. Line indicates the cut-off value (0.32).

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

This lack of difference suggests that the antigens in lab-SG crude protein extract mostly consist of *I. ricinus* proteins, which are naturally present in both wild and lab ticks. However, the higher response of group W to the wild-SG substrate, also suggest that *M. mitochondrii* antigens might play a role in enhancing the antibody response of infested rabbits. Since no common TBPs were detected in the DNA extracted from the wild-SG, we may exclude that the observed difference in the immune response between groups is due to these microorganisms. However, we cannot exclude that such difference was induced by a different microbiome composition of wild and lab ticks used in the infestation. Environmental factors,

as well as feeding/nutritional status of the tick, or genetic differences between the two tick populations can enhance differences in the tick saliva proteome, which can in turn lead to a difference in the protein-based content of the SG (Narasimhan and Fikrig, 2015).

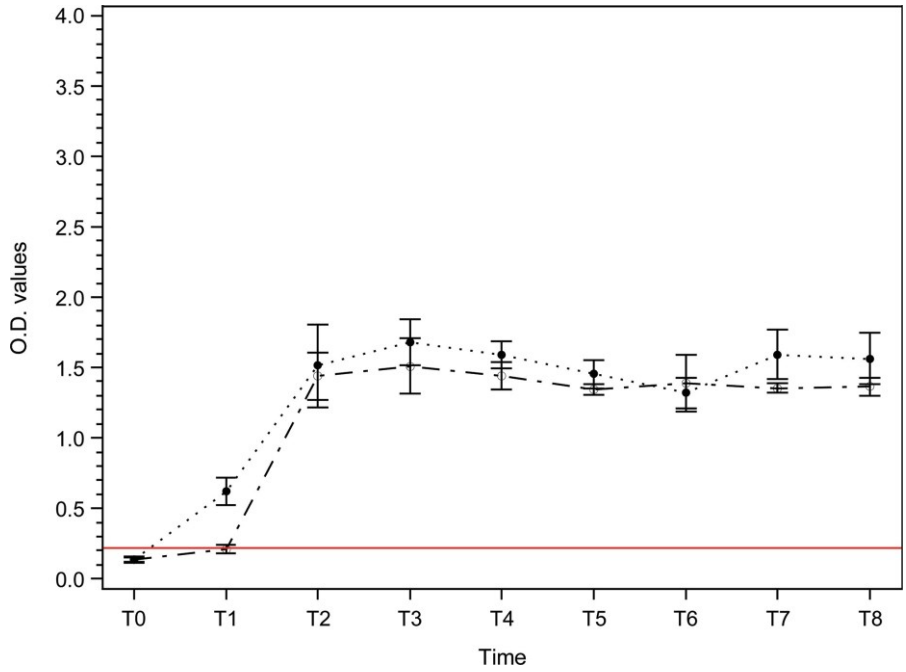


Fig. 2: Mean (± SE) antibody response against wild-SG crude protein extract in rabbits infested with wild (filled circles) and lab (blank circles) *I. ricinus* females. Line indicates the cut-off value (0.32).

We also tested the IgG antibody response against *M. mitochondrii* rFliD protein (cut-off value: 0.16; Fig. 3), which did not differ significantly between rabbits of group W and group L ($p > 0.05$). Both groups of rabbits exhibited O.D. values hovering around the cut-off from T1 to T8. However, the O.D. values of the two groups of rabbits showed a significantly different trend between T0 e T1 ($t4 = 6.01$; $p = 0.0039$). In fact, rabbits of group W exhibited a significant increase in O.D. values (parameter estimate ± SE: 0.04 ± 0.005; $t4 = 7.59$; $p = 0.0016$) and rabbits of group L showed no significant variation between the two time points ($p > 0.05$). The increase of the antibody response against rFliD in rabbits of group W is consistent with the in- crease in O.D. values obtained in the ELISA assay using wild-SG as substrate, suggesting that *M. mitochondrii* antigens are inoculated during the blood meal.

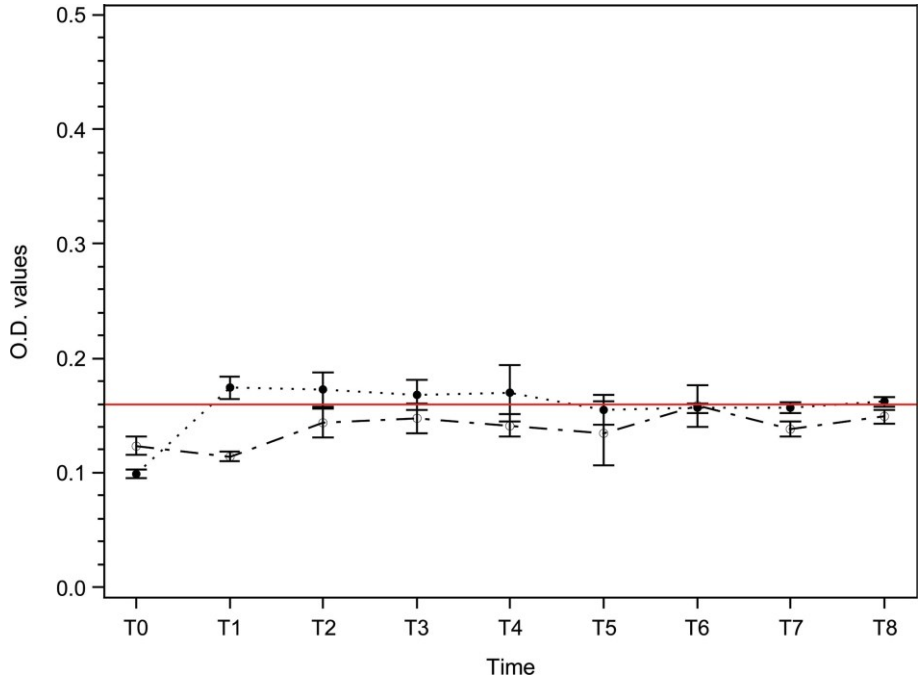


Fig. 3: Mean (± SE) antibody response against *M. mitochondrii* rFliD in rabbits infested with wild (filled circles) and lab (blank circles) *I. ricinus* females. Line indicates the cut-off value (0.16).

The lack of difference in the response against rFliD substrate between rabbits parasitized with wild and lab ticks is surprising for at least two reasons. On the one hand, we did not expect any response from rabbits of group L, as they were infested with *I. ricinus* which tested negative for the presence of *M. mitochondrii*. On the other hand, we expected a stronger response from rabbits of group W because this antigen has been shown to elicit a specific response in humans (Mariconti et al., 2012a), dogs (Bazzocchi et al., 2013) and roe deer (Serra et al., 2018). Indeed, in natural conditions, serological positivity to the symbiont could be attributed to a long-lasting presence of IgG antibodies against *M. mitochondrii* (depending on the analyzed species), to a continuous exposure to *I. ricinus* (as hypothesized by Serra et al., 2018), or to differences in the interactions among *I. ricinus*, *M. mitochondrii* and vertebrate hosts compared to laboratory conditions. The limited immune response observed in the present study in rabbits infested with wild ticks could be attributed to the genetic variability among these vertebrate hosts. Laboratory rabbits are indeed typically more outbred than other laboratory animal models, leading to inter-individual differences in their antibody responses against particular antigens (Sanders et al., 1998). Other causes for the low observed response of group W could be: i) inter-individual differences in *M. mitochondrii* loads among ticks (as observed in Sassera et al., 2008) and which is also suggested by the high variability in *M. mitochondrii* loads observed in wild-SG pools, ii) differences in saliva doses conveyed

into the vertebrate host; or iii) interactions among bacteria harbored by ticks. Microbial interactions should be indeed taken into consideration, as possible interferences in the transmission of *M. mitochondrii* could occur when multiple microorganisms are present in the same tick individual. To date, no information is available on the effects of other microorganisms on *M. mitochondrii* transmission in *I. ricinus*. Despite the variability observed among rabbits, these results show that the antibody response against *M. mitochondrii* rFliD, at least in NZW rabbits under experimental conditions, is detectable within one month after *I. ricinus* exposure, suggesting a moderate antigenic activity of the FliD protein under laboratory conditions.

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

After the exclusion of cross-reaction issues, we questioned whether the lab ticks were actually negative to *M. mitochondrii* or were harboring a bacterial load below the detection limit of the qPCR method (10 copies/µl). Moreover, as *M. mitochondrii* is known to exhibit a bloom following the blood meal (Sassera et al., 2008), the lab strain ticks could harbor extremely reduced numbers (hardly detectable in unengorged ticks), but the endosymbiont could reach higher load, sufficient for its transmission to the host -during blood meal- as observed for *Borrelia* spp. (Piesman et al., 2001).

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

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

DNA samples extracted from lab-SG were also analyzed with the nested PCR protocol and one out of five samples was found positive to *M. mitochondrii*, showing that the symbiont is

also present in the SGs of at least some lab *I. ricinus* specimens. However, the lab-SG pool used as substrate for the ELISA assay was negative to both qPCR and nested PCR protocols.

PCR analyses on blood samples

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

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

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

The molecular analyses on blood samples showed that the positivity to *M. mitochondrii* DNA is consistent with the amount of *M. mitochondrii* harbored by wild and lab *I. ricinus* individuals used for the infestation. Rabbits of group W were positive to *M. mitochondrii* DNA starting from week 1 post-infestation, while positivity in rabbits of group L was highlighted only from around week 3 post-infestation. These results are in accordance with serology, which shows a positive antibody response against rFliD starting from week 1 post-infestation in rabbits of group W and a delayed positivization observed in at least some of the

rabbits of group L. This result indicates that *M. mitochondrii* transmission can thus occur even when *I. ricinus* ticks harbor highly reduced bacterial loads. The delayed positivization of blood samples and sera of group L rabbits to *M. mitochondrii* could be attributed to the lower loads of bacteria inoculated by lab *I. ricinus* requiring longer to replicate enough to be detected through PCR and serological analyses. The replication hypothesis is also supported by the detection of *M. mitochondrii* DNA in blood samples obtained up to 16 weeks post-infestation in both groups of rabbits. The symbiont is thus probably able to survive into its vertebrate host after inoculation and to replicate, even though no clinical signs were observed in any of the rabbits. In the light of low DNA concentrations in systemic circulation (which can result in insufficient material for PCR amplification), additional tissues, such as bone marrow, brain tissue, synovial and cerebrospinal fluids (as performed, for example, for the detection of *B. burgdorferi*; Schwaiger et al., 2001; Wilske, 2003), should be analyzed to investigate their role in harboring *M. mitochondrii* bacteria.

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

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

3.3.4 Conclusions

The seroconversion against *M. mitochondrii* rFliD in rabbits infested with wild *I. ricinus* was observed to occur one week post-infestation. Furthermore, the symbiont seems to replicate

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

If the loss of *M. mitochondrii* is not a rare event in natural tick populations (as suggested by their decreasing prevalence following successive generation in tick lab strains), a frequent re-acquisition via horizontal transmission would explain why the prevalence of the symbiont in wild females is 100%. Our results, by experimentally demonstrating the transmission of *M. mitochondrii* to vertebrate hosts following tick bite, provide additional support for this hypothesis. Moreover, our findings support the hypothesis that co-feeding on a shared host could be an important determinant for the dispersal of *M. mitochondrii*, and more in general of tick symbionts, with vertebrate hosts acting as "ecological arenas" for the exchange of symbionts (Duron et al., 2017). If the transmission of endosymbionts by ticks to their hosts is a frequent event, this could accelerate the turn-over of tick symbionts also at the interspecific level. In the case of *M. mitochondrii* (and other bacteria of the genus *Midichloria*), vertebrate hosts can serve as intermediate routes for inter-/intra-specific transmission among Ixodida, as suggested by similar 16S rDNA gene sequences shared among distantly related tick species (Cafiso et al., 2016).

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

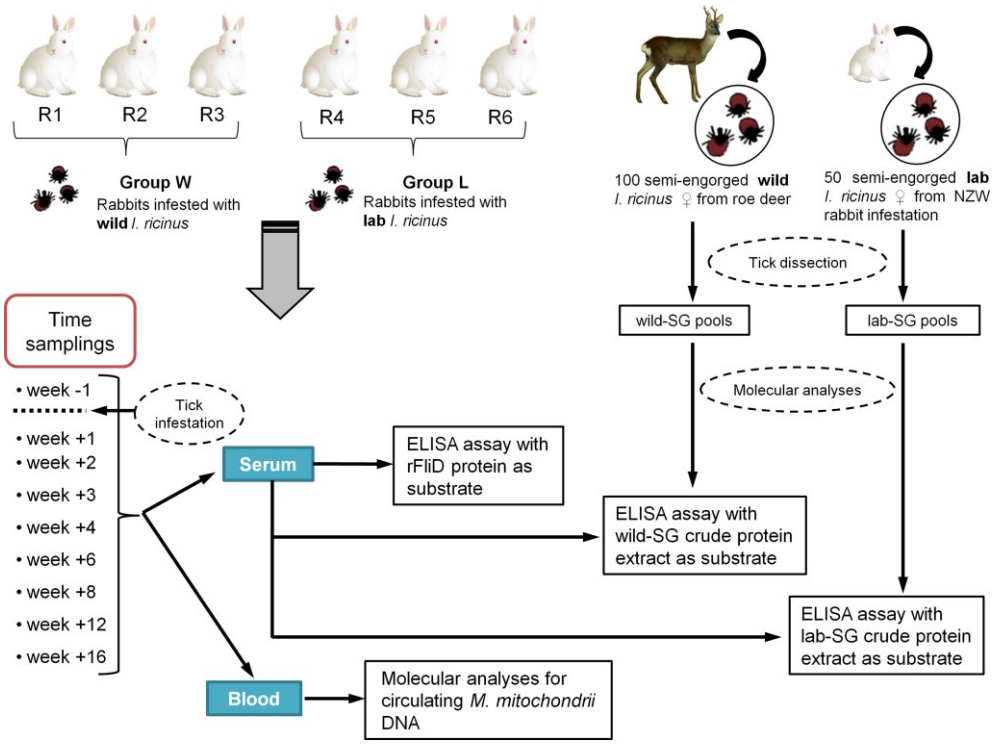
3.3.5 Ethic statements

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

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

3.3.6 Supporting information

Supplementary Fig. S1: Scheme of the experimental infestation with wild and lab *I. ricinus* ticks performed on NZW rabbits and subsequent performed analyses. Lab = laboratory strain; SG = salivary glands. Image credits: DataBase Center for Life Science (DBCLS); Richard Lydekker.



Supplementary Table 1: Model exploring the effect of group (1 or 2), substrate (wild-SG, lab-SG or FliD) and time post-infestation (number of weeks) on the variation of rabbits' IgG response (i.e. ELISA O.D. values). SG = salivary glands.

Source of variation	**F**	**df**[†]	**p value**
Group	10.8	1, 4	0.03
Substrate	26.9	2, 8	0.0003
Time	40.1	1, 148	<0.0001
Group*Substrate	36.3	2, 8	**<0.0001**
Time*Substrate	12.4	1, 148	**<0.0001**
Time*Group	1.5	1, 148	0.2

[†]df = degrees of freedom

3.4 Peptide-based ELISA test as *Ixodes ricinus* bite marker in different vertebrate hosts

Valentina Serra, et al.

First draft in preparation

3.4.1 Aim of the study

I. ricinus bite could represent a risk factor for the onset of several chronic-degenerative diseases (amyotrophic lateral sclerosis, myocardiopathy, multiple sclerosis; Hansel et al., 1995; Halperin et al., 1990; Harvey et al., 2007; Hsieh et al., 2007; Lelovas et al., 2008). This hypothesis opens the necessity to obtain an effective test capable of identifying bites of this tick species in human and animal populations. The limits of the tick parasitism (misinformation in human population, features of the tick bite such as absence of itch, pain and immediate symptoms) prompted us to detect antigenic proteins belonging to *I. ricinus* salivary glands and to its endosymbiont *M. mitochondrii* to use as markers for tick bite. Additional antigenic proteins have been detected after immunoblotting analyses using protein extracts of salivary glands collected from *I. ricinus* adult females, and sera samples collected from experimentally infested rabbits according to the protocol described in Di Venere et al., 2015. Novel peptides were designed from the most antigenic epitopes of each protein identified to detect antigenic markers for *I. ricinus* bite through an enzyme-linked immunosorbent assay (ELISA). The aim of the present work was to evaluate the occurrence of *I. ricinus* bite in different vertebrate hosts (rabbits, roe deer and humans) through an ELISA assay testing selected peptides developed from the identification of antigenic proteins belonging to the tick *I. ricinus* and its symbiont *M. mitochondrii.*

3.4.2 Material and methods

Salivary glands dissection and crude protein extract production

Twenty semi-engorged *I. ricinus* ticks were collected from roe deer (*C. capreolus*) in northern France in February 2014. Ticks were manually dissected under a stereomicroscope and salivary glands were pooled in 100 µl PBS with 1 µl of 100x protease inhibitors. After mechanical disruption of tissues, 80 µl lysate were subjected to sonication, centrifugation at maximum speed for 10 minutes at 4°C and supernatants containing proteins were recovered

and stored at -80°C until use. The remaining 20 µl were subjected to DNA extraction to subsequently perform molecular analyses to evaluate the absence of common tick-borne pathogenic bacteria.

Identification of antigenic proteins and development of synthetic peptides

Two-Dimensional electrophoresis (2-DE) was performed on *I. ricinus* salivary glands crude protein extract in order to detect *I. ricinus* and *M. mitochondrii* antigenic proteins. Differential immunoblots were performed on this protein extracts 2-DE gel using sera obtained before and after the experimental infestation of six rabbits with wild (R1-3) and lab-strain (R4-6) *I. ricinus* ticks as described in Cafiso et al. 2019 (see Paragraph 3.3.2 for a detailed description). Starting from results obtained in Cafiso et al. (2019), the more responsive serum of each rabbit towards both *M. mitochondrii* (R1-R3) and *I. ricinus* salivary glands (R1-R6) proteins were tested in differential immunoblots together with the corresponding serum collected before the infestation. After the acquisition of the images, the membranes corresponding to T0 and time with the highest antibody response were compared for each rabbit in order to highlight antigenic spots of interest. Spots were recovered and analyzed using nLC Eksigent system coupled to mass spectrometer QExactive (full MS 70000 – 10 MS/MS 17500) to identify the proteins of interest. After the identification of proteins and subsequent analyses for the identification of antigenic regions of each protein (using the tools http://sysbio.unl.edu/SVMTriP/prediction.php; http://www.bioinformatics.nl/cgi-bin/emboss/antigenic; http://imed.med.ucm.es/Tools/rankpep.html), peptides were designed in the regions selected by all three tools. Subsequently all designed peptides were subjected to BLAST analyses in order to evaluate the presence of epitopes conserved with proteins of human, roe deer and rabbit proteins, target species of the first analyses to avoid any cross-reaction. The peptides sequences were synthetized by an external service in a biotinylated form to assure the correct immobilization of the peptides to the streptavidin coated ELISA plate.

Rabbit sera collection

Rabbit sera were obtained from six rabbits experimentally infested with *I. ricinus* ticks as described in Cafiso et al., 2019. In the present experimental work, T0 (one week before exposure to ticks) and three sera corresponding to T1 (one week after experimental infestation), T5 (six weeks after experimental infestation) and T8 (16 weeks after

experimental infestation) were selected for each rabbit to be tested in ELISA assay (see below).

Human sera collection

Sera were collected from human patients suffering from different symptoms referable to tick-borne diseases from different European countries in which the presence of *I. ricinus* tick has been previously reported. These sera were selected from a specialized tick borne diseases laboratory in Germany and already tested for the presence of anti-*M. mitochondrii* and *Borrelia* sp. antibodies as described in Paragraph 3.2. Forty-eight sera were selected from patients characterized by memory of tick bite but resulting negative for *M. mitochondrii* and *Borrelia* antigens. Additionally, twenty sera were obtained from healthy donors living in *I. ricinus* non-endemic regions to use as negative control for the establishment of the cut-off of the reaction.

Roe deer sera collection

Roe deer sera were collected in April 2014 from seven roe deer individuals in a highly *I. ricinus* infested area at the French National Institute for Agricultural Research experimental station at Gardouch in France as described in Paragraph 3.1. Sera of 10 red deer from a virtually tick-free area (Merler et al., 1996) were used as negative controls to establish a cut-off value.

ELISA test

Biotinylated peptides derived from antigenic proteins of *I. ricinus* tick and its symbiont *M. mitochondrii* were dissolved in Phosphate-buffered Saline (PBS) 1X (final concentration between 500 and 2500 ng/µl depending on the molecular weight of each peptide) and used as substrate for ELISA assays. Each peptide (150 pmol/µl) was diluted in 100 µl carbonate-bicarbonate buffer (Sodium carbonate anhydrous 15 mM and Sodium hydrogen carbonate 35 mM; pH 9.6) and the 96-well ELISA plates (Pierce® Streptavidin Coated High Binding Capacity Clear 96-Well Plates – Thermo scientific) were incubated over-night at 4°C. Plates were washed three times for five minutes using 200 µl/well of washing buffer (1X PBS and 0.1% Tween-20), and then treated with 100 µl of blocking solution (3% BSA in 1X PBS) for one hour at room temperature. After further washing of the wells, each serum sample (tested in duplicate) was diluted 1:100 (dilution buffer: 1% BSA in 1X PBS) and plates were

incubated for 1 h at room temperature. After the washing steps, secondary antibodies were diluted in 100 µl/well of dilution buffer and employed at different dilutions: the anti-human IgG HRP-conjugated antibody (Sigma-Aldrich) and the peroxidase-conjugated Goat anti-rabbit IgG secondary antibody (Sigma- Aldrich) were used at 1:2000, while the anti-deer IgG HRP-conjugated antibody (Sigma-Aldrich) was employed at 1:250. After the incubation for one hour at room temperature, 100 µl/well of the staining solution (orthophenylenediamine tablet 0.4 mg/ml (Sigma-Aldrich) with hydrogen peroxide 30%) was added to each well for the development of the reaction. The optical density (O.D.) values were measured at 450 nm with a SpectraMAX 340PC (Molecular Devices Corporation) spectrophotometer. The cut-off for each assay was established as mean O.D.450 nm values + 3 standard deviations of the negative sera. Values greater than or equal to the established thresholds were considered as positive.

3.4.3 Results and discussion

Identification of antigenic proteins and development of synthetic peptides

Starting from ELISA assay results showed in Paragraph 3.3.3 (Serological analyses) we selected for each rabbit T0 (one week befor the infestation) and T4 (four weeks after infestation) sera to compare results before and after tick infestation and identify immunogenic spots.

After 2-DE gel and immunoblotting analyses, the photographic plates corresponding to T0 and T4 for each rabbit, were scanned and the obtained digital pictures were analyzed to identify specific antigenic spots. The Figure 1 shows results obtained for rabbit R1 (the same results were observed for each rabbit). Four spots were selected and, after the mass spectrometry analyses, were identified with a "high confidence level" as proteins of *I. ricinus* (Putative cytosol aminopeptidase, Putative catalase pediculus us corporis catalase, Putative mitochondrial/plastidial beta-ketoacyl-acp reductase, Putative metalloprotease). The Coverage values were very high and recognized as "unambiguous" for all proteins: 12.4 for Putative cytosol aminopeptidase, 9.56 for Putative catalase pediculus us corporis catalase, 13.8 for Putative mitochondrial/plastidial beta-ketoacyl-acp reductase, and 5.33 for Putative metalloprotease.

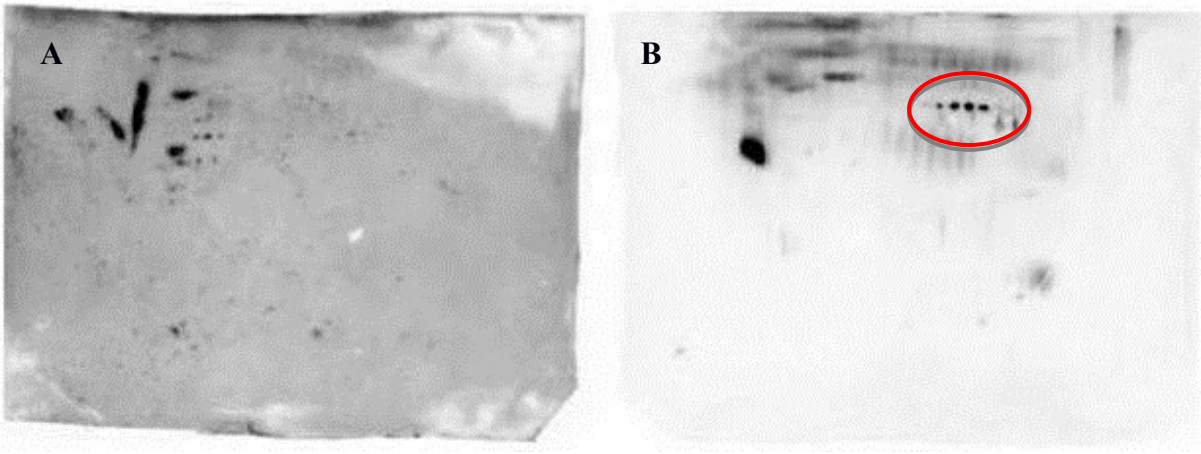


Figure 1: Comparison of the immunoblot photographic plates obtained for each rabbit before (A) and after (B) tick infestation. Four spots of interest are circled in red.

In order to produce peptides for the detection of antigenic markers for tick bite, the four proteins, the calreticulin protein of *I. ricinus* (which is known to have an antigenic activity), and a *M. mitochondrii* surface protein, were analyzed with specific tools for the identification of the most antigenic epitopes as mentioned in Paragraph 3.4.2 ("Identification of antigenic proteins and development of synthetic peptides"). Starting from the obtained results, 15 potentially antigenic peptides were designed in the regions selected by all tools. After hydrophobicity analyses and BLAST analyses conducted in order to avoid any cross-reaction with human, roe deer and rabbit proteins, 12 peptides were synthetized by an external service by adding biotin to each N-terminal end to assure the correct immobilization of the peptides to the ELISA plate (Figure 2).

Putative cytosol aminopeptidase	A1	AAFLKEFVKAEHWAH
	A2	VGAFSAVASLGIPIK
	A3	VAENVRVAISAGVRG
Putative catalase pediculus us corporis catalase		
	B2	ITKYCKAAIFNQVGK
Putative mitochondrial/plastidial beta-ketoacyl-acp reductase	C1	IAFTKSVALELATSG
	C2	HKLVCYLVFGCLVTG
	C3	ILHHITPLVKLSEDI
Putative metalloprotease	D1	CAKKYGYLTSLLDDT
Calreticulina		
	E2	VCLLLLLVGRVFADP
	E3	GGGYVKLFDCSLDQK
	E4	DPKLHSYKEICTLGF
Midichloria surface protein		
	F2	TGYAIVRFMI

Figure 2: List of 12 biotinylated peptides derived from *I. ricinus* and *M. mitochondrii* proteins synthetized by an external service to use in ELISA test for the detection of putative markers for *I. ricinus* bite.

The new synthetic peptides developed from the identification of antigenic proteins belonging to the tick *I. ricinus* and its symbiont *M. mitochondrii*, have been firstly tested through an ELISA approach using sera from experimentally infested rabbits derived from the work of Cafiso et al. (2019). The more reactive peptides were selected and subsequently tested on sera of humans and roe deer, to evaluate the occurrence of *I. ricinus* bite.

ELISA test on rabbit sera samples

Seven out of 12 of the biotinylated peptides were dissolved in PBS 1X as described in Paragraph 3.4.2 "ELISA test" and used as substrate for ELISA assay (peptides code: A1, A3, C1, D1, E3, E4, F2; see Figure 2 for more information on peptides sequences and their corresponding proteins). The remaining five peptides resulted difficult to be dissolved also in denaturant conditions, and were not analyzed in this first investigation.

A total of 24 sera samples (four sampling per rabbit including the T0) were tested using the mentioned above peptides in ELISA test.

The cut-off of the ELISA test was established at a different O.D. value for each assay performed using different peptides as substrate based on the results obtained from the six rabbit sera corresponding to T0. ELISA assays results for each peptide are showed in Figures 3-9. All sera collected before the infestation and used as negative control showed O.D. values lower than the threshold for each tested peptide, confirming the absence of tick exposure at this time point.

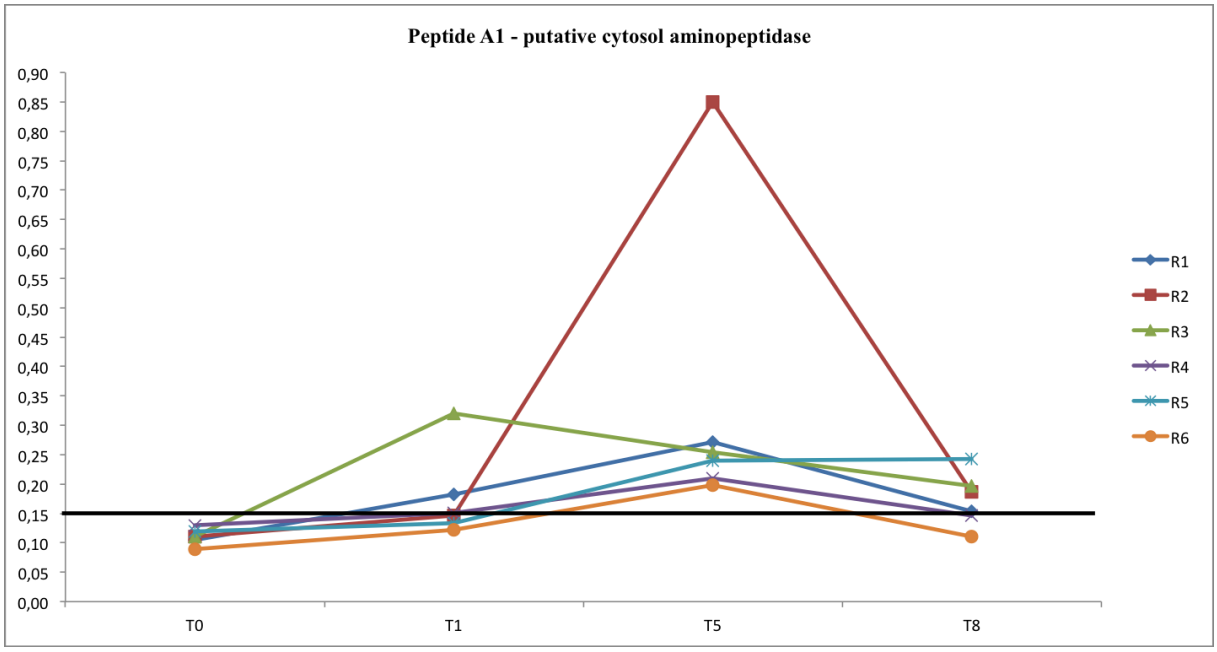


Figure 3: O.D. results from ELISA assay on sera collected from six infested rabbits at time T0, T1, T5 and T8 using peptide A1 (putative cytosol aminopeptidase protein) as antigen. Line indicates the cut-off value (0.15).

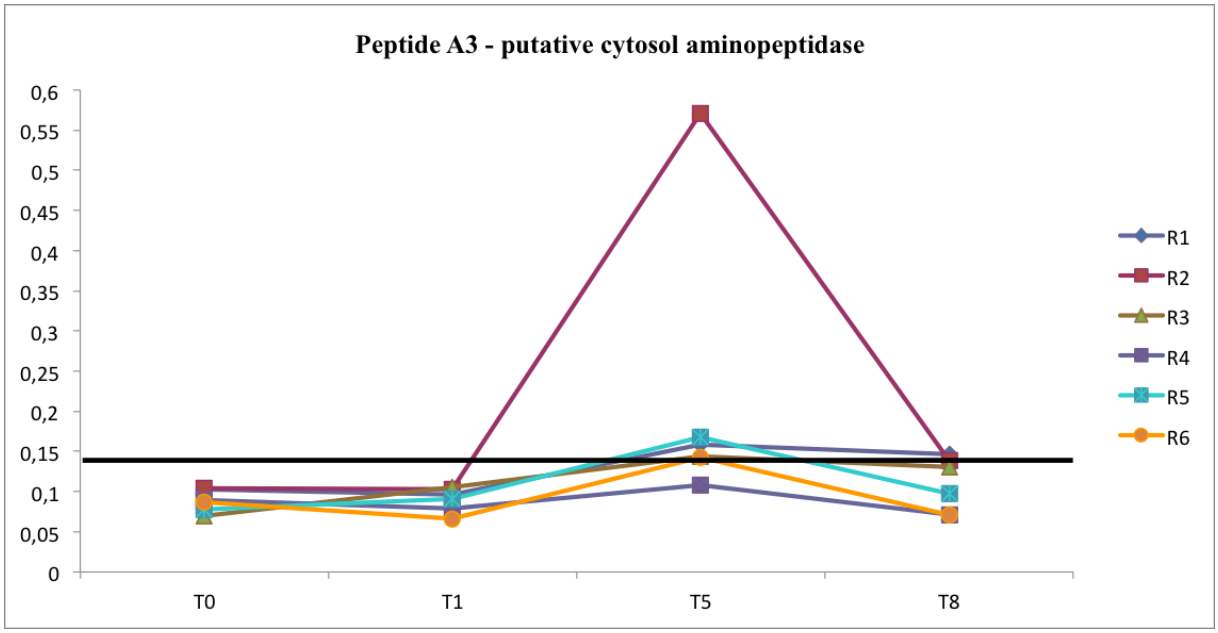


Figure 4: O.D. results from ELISA assay on sera collected from six infested rabbits at time T0, T1, T5 and T8 using peptide A3 (putative cytosol aminopeptidase protein) as antigen. Line indicates the cut-off value (0.13).

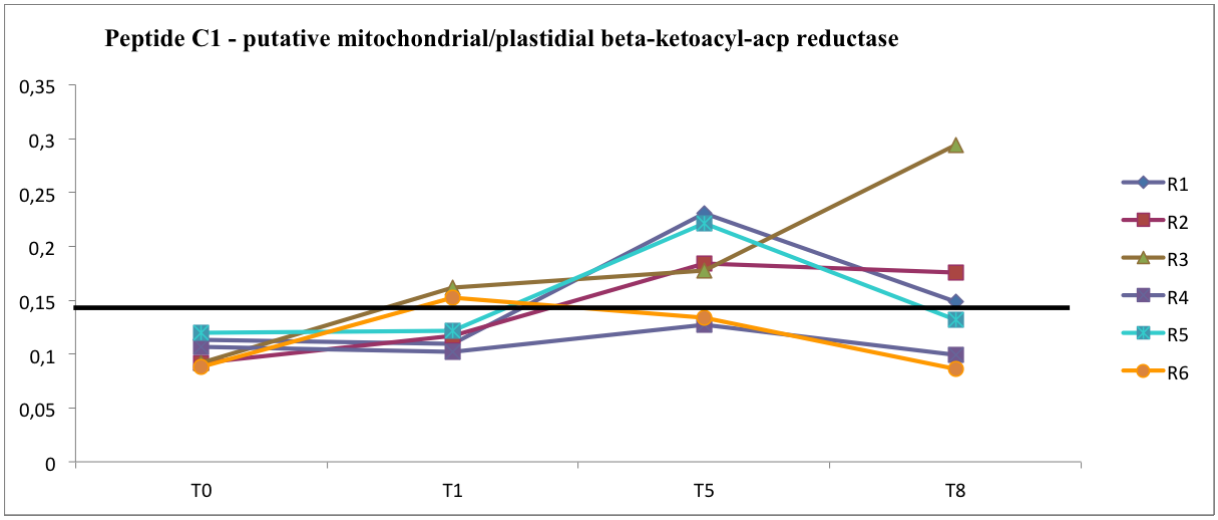


Figure 5: O.D. results from ELISA assay on sera collected from six infested rabbits at time T0, T1, T5 and T8 using peptide C1 (putative mitochondrial/plastidial beta-ketoacyl-acp reductase protein) as antigen. Line indicates the cut-off value (0.14).

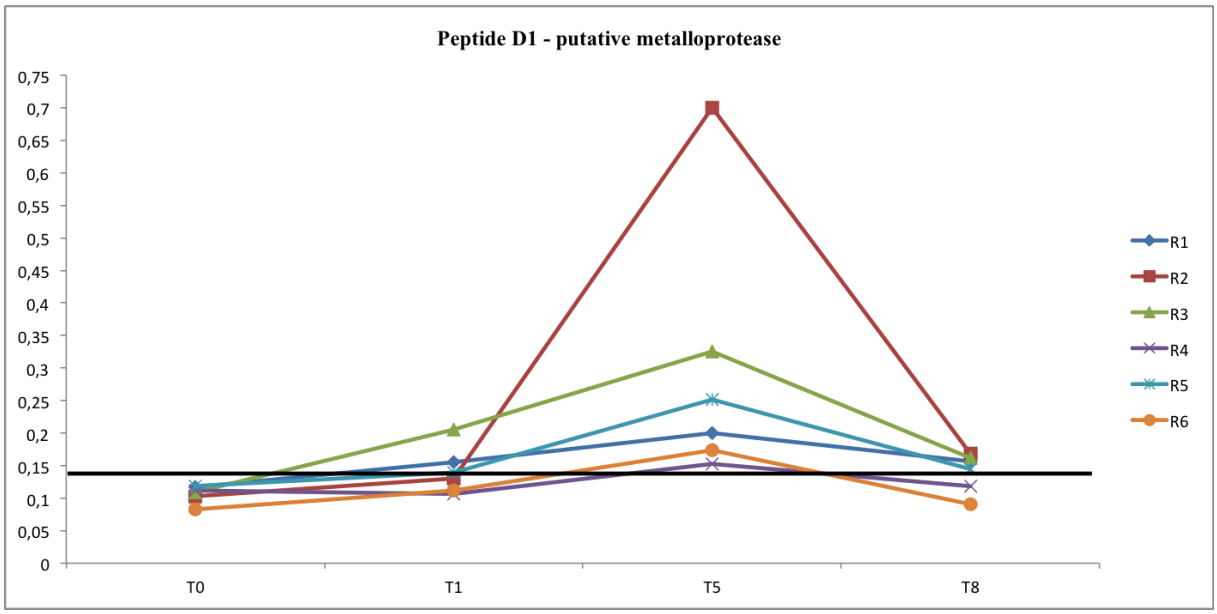


Figure 6: O.D. results from ELISA assay on sera collected from six infested rabbits at time T0, T1, T5 and T8 using peptide D1 (putative metalloprotease protein) as antigen. Line indicates the cut-off value (0.14).

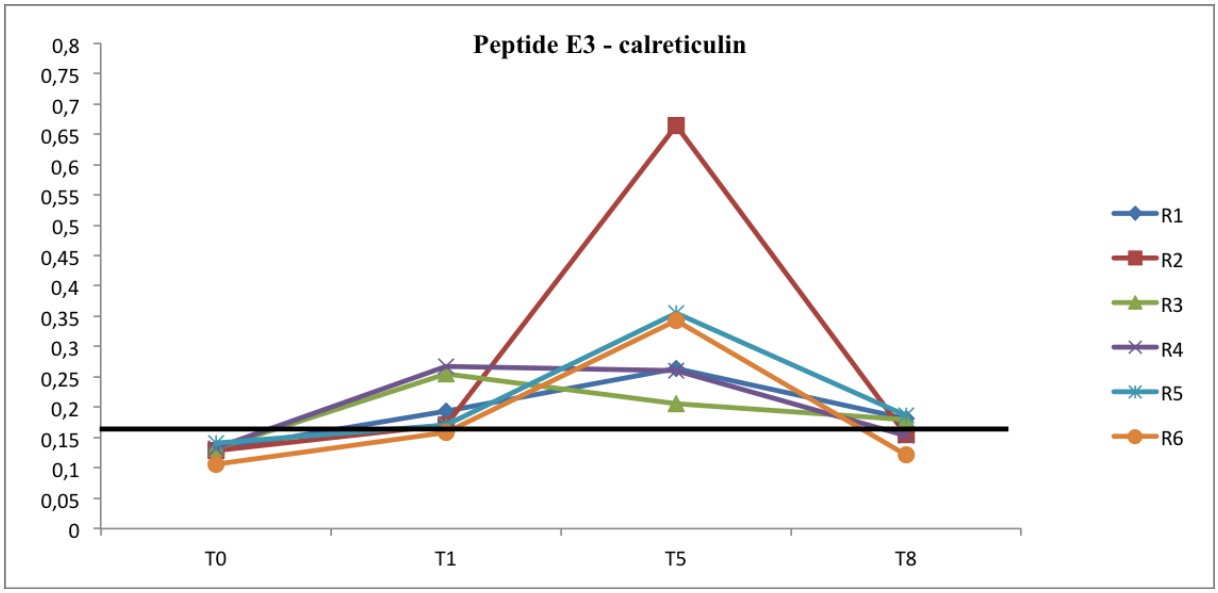


Figure 7: O.D. results from ELISA assay on sera collected from six infested rabbits at time T0, T1, T5 and T8 using peptide E3 (calreticulin protein) as antigen. Line indicates the cut-off value (0.16).

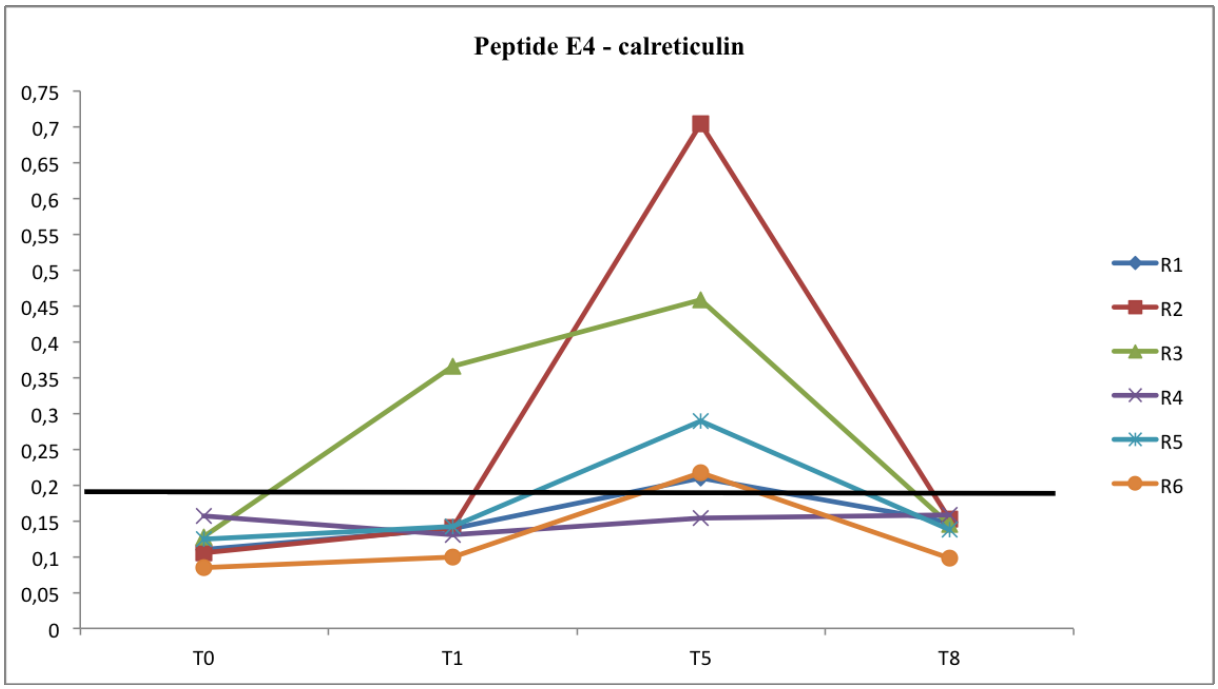


Figure 8: O.D. results from ELISA assay on sera collected from six infested rabbits at time T0, T1, T5 and T8 using peptide E4 (calreticulin protein) as antigen. Line indicates the cut-off value (0.19).

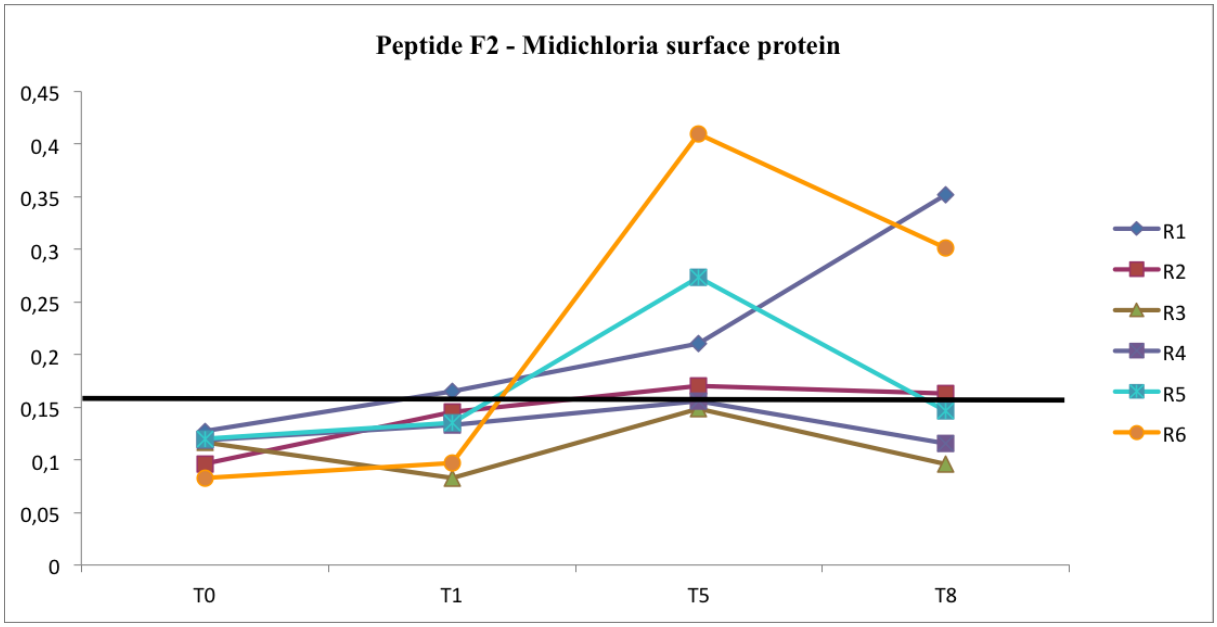


Figure 9: O.D. results from ELISA assay on sera collected from six infested rabbits at time T0, T1, T5 and T8 using peptide F2 (*Midichloria* surface protein) as antigen. Line indicates the cut-off value (0.16).

Regarding ELISA assay performed using peptide A1 (putative cytosol aminopeptidase protein; Figure 3) as antigen, the cut-off of the reaction was established at an O.D. value of 0.15 using. A positive reaction was observed in almost all sera tested, starting from one week after the infestation in both groups of rabbits infested with wild (R1, R2 and R3) and lab ticks (R4, R5 and R6). This result is attributable to the fact that the antigen used as substrate of the reaction is an *I. ricinus* protein, thus present in both types of ticks.

Regarding ELISA assay performed using peptide A3 (putative cytosol aminopeptidase protein; Figure 4) as antigen, the cut-off of the reaction was established at an O.D. value of 0.13. The three rabbits infested with wild *I. ricinus* ticks (R1, R2 and R3) showed positive values in all considered sampling, except for samples collected at time point T1, likely due to an absence of antibodies one week after the tick infestation. Regarding the three rabbits infested with lab ticks (R4, R5 and R6), only two sera samples corresponding to T5 of two rabbits showed positivity. Since A1 and A3 are peptides obtained from the same protein, we can assume that A1 is more immunogenic than A3.

Regarding ELISA assay performed using peptide C1 (putative mitochondrial/plastidial beta-ketoacyl-acp reductase protein; Figure 5) as antigen, the cut-off of the reaction was established at an O.D. value of 0.14. Positive values were observed in all samples collected from rabbits infested with wild *I. ricinus* except in sera obtained at T1, while positivity was observed only in two rabbits infested with lab ticks.

Concerning the ELISA assay performed using peptide D1 (putative metalloprotease protein; Figure 6) as substrate of the reaction, an O.D. equal to 0.15 was set as the cut-off of the assay. Positive values were observed in all sera samples except for samples corresponding to T1 of three rabbits (R2, R4 and R6) and sera collected at T8 of rabbits R4 and R6. This peptide derived from an *I. ricinus* protein as well, explaining the lack of difference in the antibody response between two groups of rabbits.

Both peptides E3 and E4 derived from calreticulin protein of *I. ricinus*, already known as an indicator of *Ixodes* tick species exposure and used in ELISA tests as a useful diagnostic indicator (Alarcon-Chaidez et al., 2006). In our work, the cut-off of the two reactions using peptide E3 and E4 as antigen, was established at an O.D. value of 0.16 and 0.19 respectively (Figures 7, 8). Concerning peptide E3, a positive reaction was observed in all sera samples except for samples corresponding to T8 of rabbits R2, R4 and R6, and sera obtained at time point T1 of the rabbit R6. These results confirmed the good property of this protein as a useful indicator of *I. ricinus* bite exposure. Regarding the results obtained using peptide E4 as antigen, a lower number of samples showed positivity, probably due to a lower sensitivity and specificity of the immunological assay using this peptide.

Finally, peptide F2 derived from a protein of the *I. ricinus* symbiont *M. mitochondrii.* As already mentioned, it is known that this bacterium is inoculated into the vertebrate host during the blood meal of tick females, but this fact has not yet been demonstrated in immature stages, making a marker of this type not useful. The cut-off of our ELISA assay using peptide F2 as substrate was established at an O.D. value of 0.16 (Figure 9). Positivity was observed in both groups of rabbits, confirming the results of the work conducted by Cafiso et al., (2019) in which *M. mitochondrii* transmission has also been demonstrated when the symbiont load is reduced in case of tick laboratory strains (where a bacterial decrease in prevalence after some generations under laboratory conditions it is well known).

ELISA test on human sera samples

Starting from the results obtained in ELISA assays conducted on rabbit sera, peptides A1 (putative cytosol aminopeptidase) and E3 (calreticulin protein) were selected to be used as antigen on 48 human sera to evaluate *I. ricinus* exposure. The cut-off of the two ELISA assays was established at an O.D. value of 0.57 for peptide A1 and 0.42 for peptide E3.

Concerning the ELISA assay performed using peptide A1 (putative cytosol aminopeptidase protein) as substrate of the reaction, positive values were observed in five out of 48 sera samples (Figure 10).

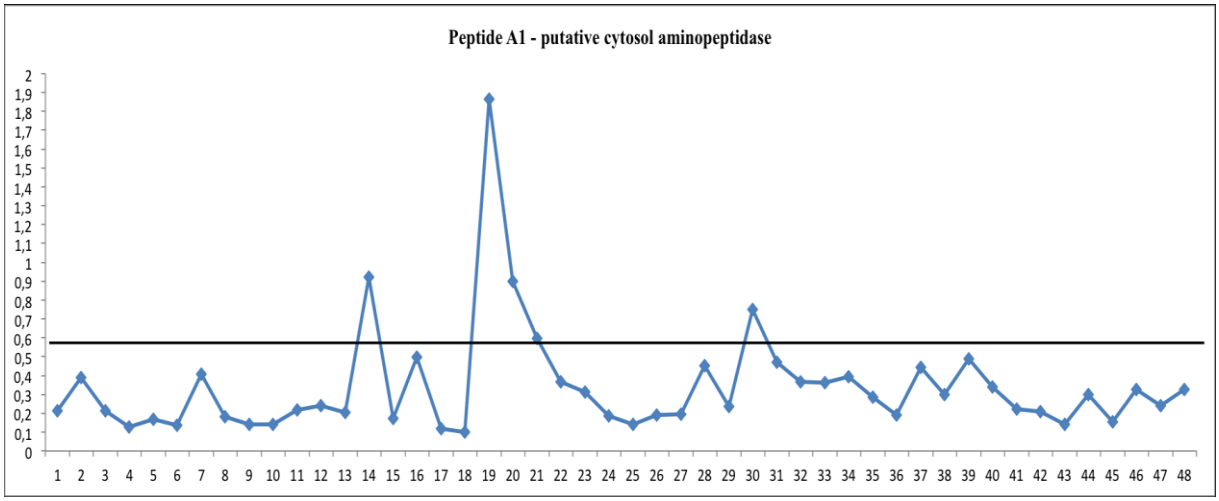


Figure 10: O.D. results from ELISA assay on sera collected from humans suffering from tick-bite symptoms using peptide A1 (putative cytosol aminopeptidase) as antigen. Line indicates the cut-off value (0.57).

Concerning the ELISA assay performed using peptide E3 (calreticulin protein) as substrate of the reaction, positive values were observed in seven out of 48 sera samples, four of which resulted positive also for peptide A1 (samples code: 19, 20, 21 and 30; Figure 11).

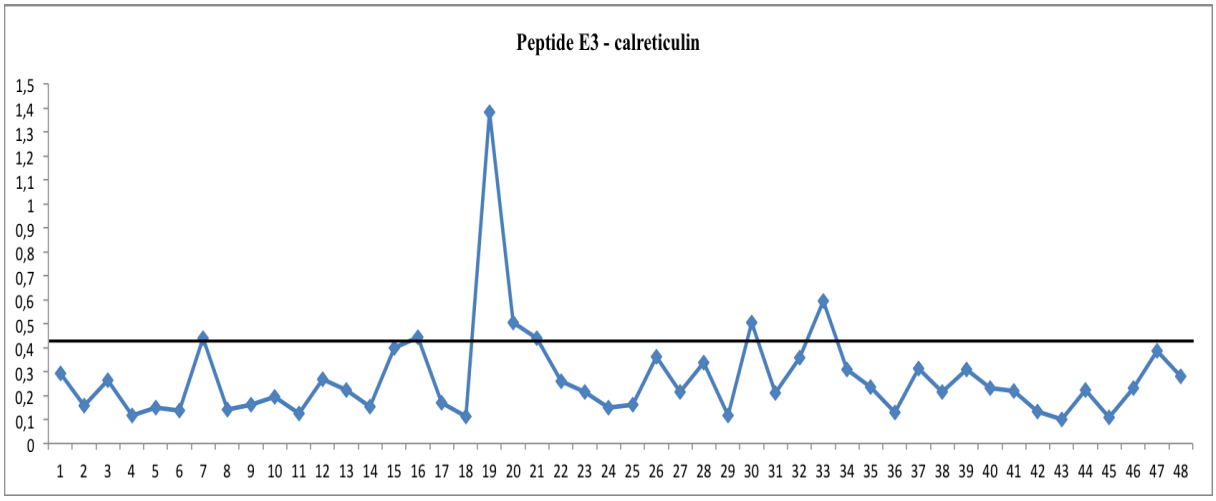


Figure 11: O.D. results from ELISA assay on sera collected from humans suffering from tick-bite symptoms using peptide E3 (calreticulin protein) as antigen. Line indicates the cut-off value (0.42).

Despite the low percentage of positivity, these results contribute to increase the percentage of serological positivity to diagnose tick exposure (it has to be reminded that all these samples resulted negative to *I. ricinus* symbiont antigen (rFliD protein of *M. mitochondrii*) in the previously mentioned work). It has to be taking into account that part of the negativity to *I. ricinus* proteins observed in this group of subjects parasitized by ticks (with memory of tick

bite) could be explained by the fact that *I. ricinus* could not have been the only tick species parasitizing the patients enrolled in this study. Further studies aim to test the other peptides on these sera with the perspective of using a combination of the most immunogenic peptides in a multi-antigen ELISA assay, to enhance the sensitivity of the test.

ELISA test on roe deer sera samples

Seven roe deer sera samples were subjected to ELISA assay using peptides A1 and E3 as substrate of the reaction.

Regarding ELISA assay performed using peptide A1 (putative cytosol aminopeptidase protein) as antigen, the cut-off of the reaction was established at an O.D. value of 0.48. Positive values were observed in four out of seven sera samples (Figure 12).

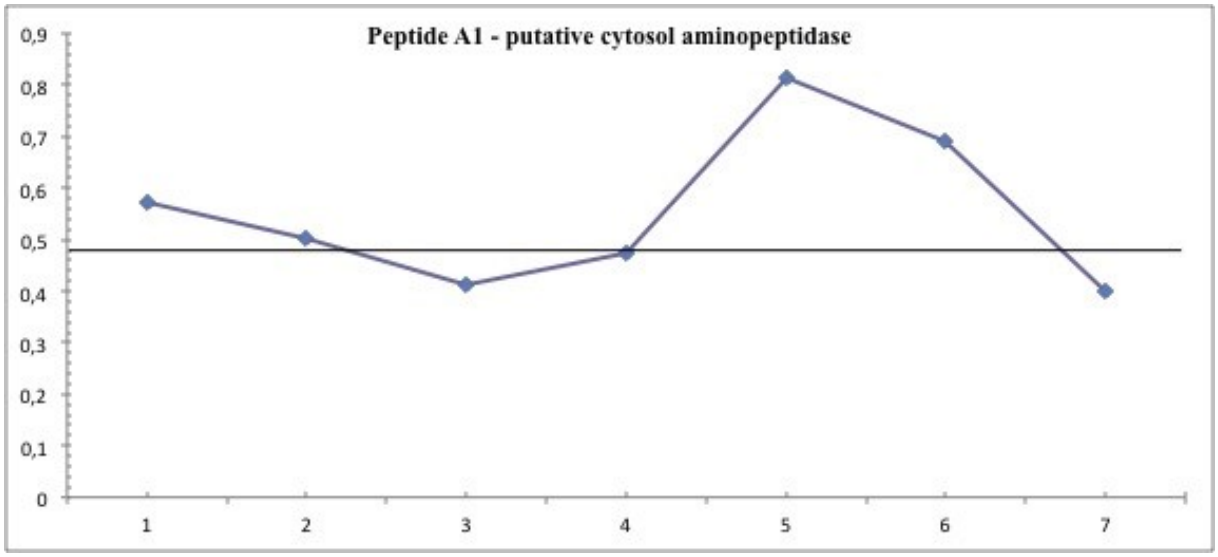


Figure 12: O.D. results from ELISA assay on sera collected from roe deer using peptide A1 (putative cytosol aminopeptidase protein) as antigen. Line indicates the cut-off value (0.48).

Concerning the ELISA assay performed using peptide E3 (calreticulin protein) as substrate of the reaction, an O.D. equal to 0.38 was set as the cut-off of the assay. In this case, only two sera showed O.D. values higher than the established threshold (Figure 13). Despite roe deer represents the host of choice for *I. ricinus* parasitism, the limited immune response observed against peptide E3 (calreticulin protein) could be due to the fact that this protein, although has already been proposed as potential tick bite markers (see Paragraph 1.3.2 of the Introduction), presents several concerns about its effectiveness as a valuable tick bite marker, since its

secretion starts three days after tick attachment and cross-reactivity with orthologous proteins of other parasites may occur.

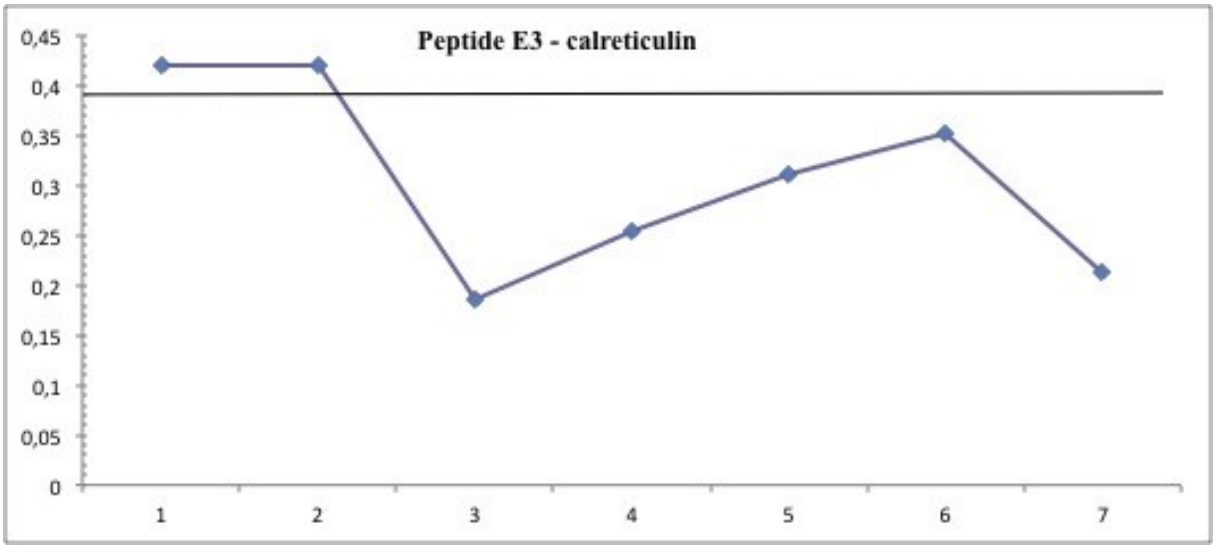


Figure 13: O.D. results from ELISA assay on sera collected from roe deer using peptide E3 (calreticulin protein) as antigen. Line indicates the cut-off value (0.38).

Further studies aim to test the other peptides obtained from additional *I. ricinus* proteins on these sera samples to evaluate the most valuable tick bite exposure indicator. Our attention will be focused on sera collected from roe deer since this vertebrate host is highly infested with *I. ricinus* ticks and is considered an efficient animal sentinel for pathogens transmitted by this tick species. The study of immune response in roe deer individuals against *I. ricinus* proteins will allow obtaining information about prevalence and altitude distribution of this tick species with the focus to investigate the potential increase in its distribution.

3.4.4 Conclusions

In the present work a peptide-based ELISA tests as diagnostic assay to detect *I. ricinus* bite were presented. Synthetic peptides that mimic specific epitopes of antigenic proteins have also been used for the development of new diagnostic systems of several human diseases (Gómara and Haro, 2007). A great number of peptide-based diagnostic systems are at present in development and some have already been commercialized. Concerning arthropod-borne infectious diseases, the diagnostic performance of a peptide-based ELISA assay based on an immunodominant conserved region of the causative agent of Lyme disease *Borrelia burgdorferi* was investigated with encouraging results (Liang et al., 1999).

In the present experimental work sera collected from experimentally tick infested rabbits, humans and roe deer individuals were tested using peptides deriving from *I. ricinus* and its symbiont *M. mitochondrii* proteins as antigens in an ELISA assay. In particular, we focused our attention on testing sera samples from humans and roe deer for the presence of antibodies against two peptides deriving from the putative cytosol aminopeptidase protein (peptide A1) and from calreticulin protein (peptide E3), both belonging to *I. ricinus* tick. The percentage of positivity, albeit low, found in tested human sera collected from subjects parasitized by ticks in Europe, and found all negative to *M. mitochondrii* in a previous work, contribute to increase the percentage of sensitivity to investigate tick exposure. Interestingly, results obtained in rabbit sera testing these two peptides showed positivity up to 16 weeks after experimental infestation (sampling T8) in four rabbits (R1, R2, R3 and R5), reinforcing the good property of these peptides as tick exposure indicator, with the final aim to combine different peptides in a multi-antigen ELISA test as diagnostic assay for tick bite.

We will focus our attention on testing different combinations of synthetized peptides, since it is known that diagnostics based on a single peptide may lack sensitivity due to the dependence on a single antibody epitope.

3.5 Detection and quantification of a novel bacterium of the genus *Midichloria* (family *Midichloriaceae*, order Rickettsiales) in the hard tick *Hyalomma marginatum*

Valentina Serra, et al.

First draft in preparation

3.5.1 Aim of the study

Ticks are obligate haematophagous arthropods that parasitize terrestrial vertebrates (mammals, birds, reptiles and amphibians) in every developmental stage. Ticks are considered to be the most important vectors of disease-causing pathogens in domestic and wild animals, and second worldwide to mosquitoes as vectors of human diseases (De La Fuente et al., 2008b). Ticks can be easily transported over long-distances while feeding in their hosts, in particular avian species may contribute to tick dispersal across geographical barriers during migration (Olsen et al., 1995; Poupon et al., 2006; Hasle et al., 2009). The role of migrating birds in the spread of ticks has epidemiological implications, since birds may contribute to the dispersal of tick-borne pathogens and microorganisms (Wallménius et al., 2014). Although the high number of actual pathogens that ticks can host, especially concerning the *Ixodidae* family ("hard ticks"), a variety of symbiotic bacteria have been detected including members of the *Rickettsiaceae*, *Anaplasmataceae* and *Midichloriaceae* families (order Rickettsiales; Benson et al., 2004; Liu et al., 2013; Sassera et al., 2006). The latter family is formed by bacteria (*Midichloria* bacteria) associated with a wide range of hosts, which can be transmitted to vertebrate hosts during the tick bite, inducing an antibody response (Skarphédinsson et al., 2005; Mariconti et al., 2012a; Bazzocchi et al., 2013). Since many bird species are common hosts of tick species, which are known to harbor bacteria of the *Midichloriaceae* family (i.e. *Ixodes* and *Hyalomma* tick genera), their frequency of occurrence in avian hosts has investigated for the first time in a recent study (Di Lecce et al., 2018). The aims of the present study were: 1) determine the genetic variability of a novel bacterium belonging to the genus *Midichloria* in *Hyalomma marginatum* ticks collected from migratory birds on Ventotene Island, travelling back from Africa; 2) quantify the amount of these bacteria in ticks using a novel Real Time PCR approach. The study of the role of migratory birds as reservoir host of *Midichloria* bacteria may contribute to obtain valuable information to better define the

geographic distribution of these bacteria, also expanding knowledge of their potential infectious role.

3.5.2 Material and methods

Sample collection and tick identification

The sampling was performed on Ventotene Island from April to May 2015 in collaboration with the monitoring and ringing activities carried out by ISPRA every year, within the "Progetto Piccole Isole" project. All the ticks were collected from three different trans-Saharan migratory bird species: common redstart (*Phoenicurus phoenicurus)*, whinchat (*Saxicola rubetra)* and common whitethroat (*Sylvia communis),* using a pair of tweezers without damaging the rostrum. All the ticks collected from the same bird were then placed in a microcentrifuge tube containing 90% ethanol and kept at room temperature.

In order to perform the morphological identification of the collected ticks, every sample was examined with a Leica MS5 stereomicroscope (Leica Microsystems GmbH, Wetzlar, Germany) and analyzed with standard taxonomic keys (Manilla, 1998; Estrada-Peña et al., 2004). Ticks were categorized based on stage of development (larva, nymph and adult), sex (only for adult specimens) and engorgement level (o un sinonimo).

DNA extraction and qualitative PCRs

The PCR analyses for the amplification of the *16S rRNA* of *Midichloria* were performed on the DNA extracted from 50 *H. marginatum* nymphs resulted positive in a previous work (Di Lecce et al. 2018).

Midichloria DNA was amplified using a set of primers targeting the bacterial *16S rRNA* gene (Midi-F 5' GTACATGGGAATCTACCTTGC 3'; Midi-R 5' CAGGTCGCCCTATTGCTTCTT 3'; Midi-R2 5' TGAGACTTAAAYCCCAACATC 3') to perform a semi-nested PCR protocol (Midi-F - Midi-R2) described in Cafiso et al. (2016). The primers were used at a final concentration of 1 μM and amplify a fragment of 691 bp. Amplification was performed in a final volume of 10 microliters containing Buffer 1X (Promega®), 0,2 mM of each dNTPs, 1 μM of each primer, 0,5 U of Taq polymerase (GoTaq, Promega®), 1 μl of DNA and water up to the final volume. The thermal profile consisted of an initial denaturation step at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds and elongation at 72°C for 45 seconds. Final extension was performed at 72°C for 10 minutes.

Ten samples that confirmed positivity for *Midichloria* amplification were tested using primers amplifying *secY*, *nuoF2*, *adk* and *ppdk* genes designed in a previous work to study the genetic variability of *M. mitochondrii* (unpublished results). The primer sequences and the amplicon sizes are indicated in Table 1.

Table 1: Primers used for the study of genetic variability of *Midichloria* in *Hyalomma marginatum* ticks.

Primer	Sequence	Amplicon size
secY	F 5'-AAAGTTTATGCAGGAGATTCAAC-3' R 5'-GTGAGGAAATAGGTTTGGATTC-3'	512 bp
nuoF2	F 5'-CTTTATGGACAAGATAGTGCTG-3' R 5'-CAGTACGCCTCATAATGGC-3'	649 bp
adk	F 5'-GCGAAATACTTAGGAATGAGGT-3' R 5'-AAATCAATCGTCTTATCTCCATCA-3'	511 bp
ppdk	F 5'-GTAAATCCATTCTAGGAGGCAA-3' R 5'-ACCAGCATGTTGTAAGACGA-3'	533 bp

DNA amplification was performed as previously described, using 1 µl of DNA with the same reagents concentrations, using different thermal profiles. *adk* and *ppdk* genes were amplified using a thermal protocol consisted of 10 cycles at an annealing temperature of 65°C followed by 30 cycles at an annealing temperature of 55°C; these first cycles at high temperature increase the specificity of the reaction, avoiding the amplification of non-specific fragments. For the remaining genes (*secY* and *nuoF2*), a thermal profile consisted of 40 cycles at an annealing temperature of 54°C was used.

PCR products were loaded on agarose gel, excised from the gel and purified using the Wizard SV Gel and PCR Clean-Up System Kit (Promega®), then sequenced by ABI technology (Applied Biosystems, Foster City, CA, USA). The obtained sequences were corrected manually and aligned with BioEdit software (Hall, 1999), subjected to BLAST analysis (http://www.ncbi.nlm.nih.gov/blast) and compared to the sequences available in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) to verify the homology with *M. mitochondrii* sequences and other bacteria of genus *Midichloria* and to assess the presence of intraspecific and interspecific variability.

Primers design

Two sets of primers for the amplification of the *gyrB* (gyrase-B) gene of *Midichloria* and the *cal* (calreticulin) gene of *H. marginatum* were designed to set-up a Real Time PCR (qPCR) approach to quantify the bacterium in tick samples. Since the calreticulin gene sequence of *H. marginatum* is not yet available in databases, primers for the amplification of *cal* were designed on the alignment of calreticulin gene sequences of two different species of *Hyalomma* sp., *H. anatolicum* and *H. dromedarii*. Starting from the amplification of a fragment of 776 base pairs of the *gyrB* gene of *Midichloria* (gyrB-RT-F1 5'-AAGCTAAGAATTTGGCGTGATG-3' and gyrBR6 5'-GTTTTGGCTTCATTTGGATTTTC-3') primers for the amplification of *gyrB* gene of the midichloriacea of *H. marginatum* were designed. The two sets of primers for *cal* and *gyrB* genes amplification and the respective amplicon sizes are indicated in Table 2.

Table 2: Primers designed for the amplification of *cal* gene of *H. marginatum* and *gyrB* gene of *Midichloria*.

Primer	Sequence	Amplicon size
cal_HyalommaF2	5'-TACCTCAAGCTGTTCGACTG-3'	132 bp
cal_HyalommaR1	5'-GCCCTTGTAGTTGAAGATG-3'	
Hyalo_gyrBF2	5'-ATTTACATGTCAGGGAGTG-3'	155 bp
gyrB_HyalommaR	5'-CTACGAGTTATTGCTGCTC-3'	

qPCR for the quantification of *Midichloria* in *Hyalomma marginatum*

A qualitative PCR protocol was performed to amplify *cal* and *gyrB* genes starting from the DNA extracted from a *H. marginatum* nymphs. The thermal profile for the amplification of *cal* gene consisted of an initial denaturation step at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 53°C for 20 seconds and elongation at 72°C for 10 seconds. The thermal profile for the amplification of *gyrB* differed only in the annealing temperature, set at 56°C. The final primers concentration was 150 nM for *cal*, and 250 nM for *gyrB*.

PCR products were recovered from the agarose gel, purified with the Wizard SV Gel and PCR Clean-Up System Kit (Promega®), quantified with the NanoDrop spectrophotometer (NanoDrop ND-1000, Thermo scientific®), and sequenced by ABI technology to confirm

PCR specificity. Once confirmed the specificity of the amplification, PCR products were cloned into the pGEM-T Easy Vector System (Promega®) following the manufacturer directions. Plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen®) and quantified. Purified plasmids containing the desired fragments were serially diluted starting from 10^9 copies/microliters to 10 copies/microliter, in order to evaluate the efficiency and the dynamic range of each reaction.

qPCRs for the amplification of *cal* and *gyrB* genes were performed on *H. marginatum* nymphs in which the positivity for the presence of *Midichloria* bacterium has been previously confirmed. All reactions were performed in a final volume of 25 µl containing Sybr Green Sso Advanced Universal SYBR Green Supermix (Biorad®) 2X, 150 nM of each primer for *cal* mixture, 250 nM of each primer for *gyrB* mixture, 1 µl of DNA and water up to the final volume. PCR thermal profiles for *cal* and *gyrB* amplification were the same mentioned above. The construction of melt curve was performed, at the end of each thermal profile, from 55°C to 95°C with increasing increments of 0,5°C per cycle for both genes in order to verify the absence of non-specific amplified fragments confirming the specificity of the method. Results were expressed as *gyrB/cal* copy numbers ratio.

3.5.3 Results and discussion

Qualitative PCR on *Hyalomma marginatum* nymphs for *Midichloria* detection

Forty-seven out of 50 nymphs previously analyzed confirmed the positivity for *Midichloria* presence. In Figure 1 is shown the result of the amplification for some of the 50 *H. marginatum* nymphs tested for the presence of *Midichloria*.

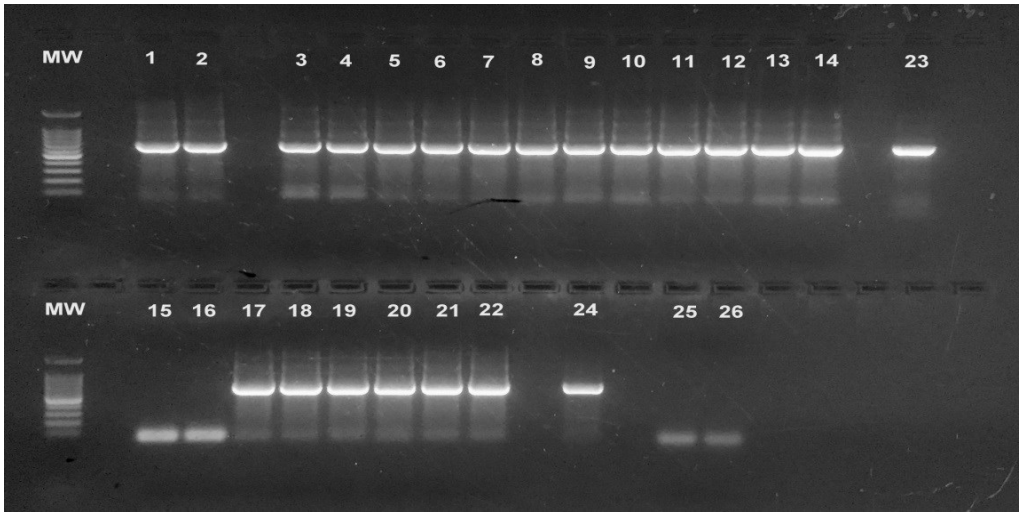


Figure 1: Amplification of a fragment of the *16S rDNA* gene of *Midichloria* (amplicon size: 691 bp). Lane MW: Molecular Weight marker 100 bp; lanes 1-22: amplification in double of eleven samples; lanes 23-24: Positive control; lanes 25-26: Negative control.

Genetic variability of *Midichloria* bacterium in *Hyalomma marginatum*

A qualitative PCR for the amplification of *nuoF2, secY, adk* and *ppdk* genes previously used to study the genetic variability of *M. mitochondrii*, was performed on some *H. marginatum* nymphs positive for *Midichloria* presence in order to study the genetic variability of this bacterium hosted by this tick species. All *H. marginatum* analyzed nymphs were found positive for *nuoF2* and *secY* genes only, and the amplicon sizes are 649 and 512 base pairs respectively. Figure 2 shows the amplification of *nuoF2* and *secY* genes in five samples of *H. marginatum* nymphs.

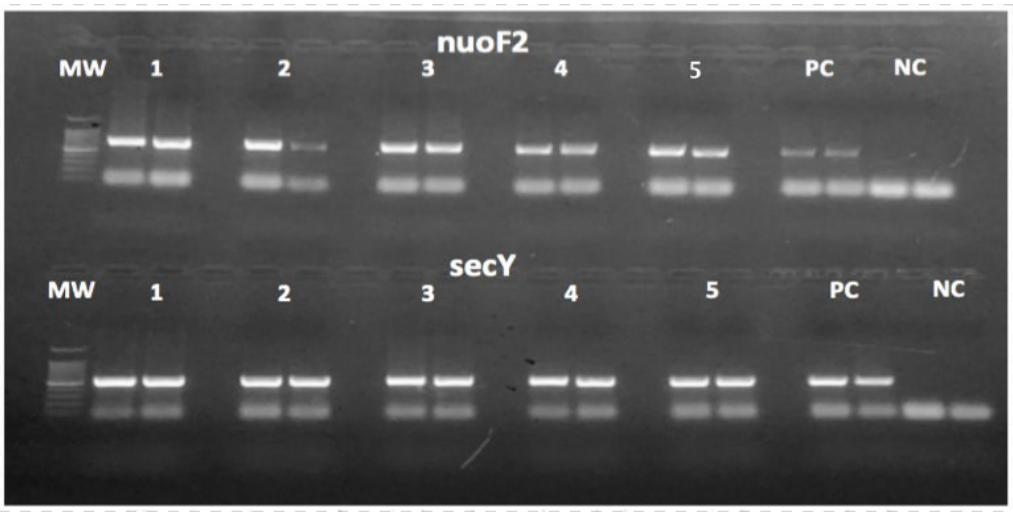


Figure 2: Amplification of *nuoF2* (649 bp) and *secY* (512 bp) genes in five samples for each gene.
Lane MW: Molecular weight marker 100 bp; PC: Positive control; NC: Negative control.

The other two sets of primers amplifying *ppdk* and *adk* genes are likely not specific for this *Midichloria* species, explaining the negativity found in all samples analyzed for these genes.
Sequencing of PCR positive products was performed for 10 *H. marginatum* nymphs samples for both *nuoF2* and *secY* genes. BLAST analysis showed that all sequences obtained for *nuoF2* and *secY* genes were identical to one another and shared 92 and 96% sequence identity with *M. mitochondrii* respectively. From the work conducted by Di Lecce et al. (2018), the comparison of all *16S rDNA* sequences of *Midichloria* bacteria amplified from *H. marginatum* samples, shared 99% sequence identity with *M. mitochondrii*.
From these preliminary results it would be possible to hypothesize the presence of a previously undescribed *Midichloria* strain in *H. marginatum* ticks. Further works are needed to reinforce this hypothesis.

Quantification of *Midichloria* bacterium in *Hyalomma marginatum* nymphs

qPCRs were performed on 47 *H. marginatum* nymphs which confirmed the positivity for *Midichloria* presence in qualitative PCR as mentioned above. Number of *cal* and *gyrB* gene copies was obtained through a comparison of the qPCR results of each sample with those of serial dilutions of purified plasmids (containing known copy numbers). The efficiency of each PCR protocol was 104.4% for *cal*, and 101.2% for *gyrB*, and the threshold line was set at 100. Results were expressed as the ratio of *gyrB*/*cal*.
From the equation of the calibration line obtained from *cal* and *gyrB* standards, it was possible to obtain the copy number of *cal* and *gyrB* for each sample. The ratio *gyrB*/*cal,* which indicates the quantity of *Midichloria* in the tick, allows normalizing the data and to obtain meaningful results that are not influenced by problems related to the extraction or storage of DNA samples. All analyzed samples showed positivity for both genes, and the melting curves showed the presence of specific amplified fragments belonging to the target sequences confirming the specificity of the method. In Table 3 are shown the range values of *gyrB* and *cal* copy numbers and the ratio of *gyrB/cal* in all the analyzed *H. marginatum* nymphs grouped by state of repletion.

Table 3: Range values of *gyrB* and *cal* copy numbers and of *gyrB/cal* ratios in analyzed *H. marginatum* nymphs.

	***gyrB* copy number (range)**	***cal* copy number (range)**	***gyrB*/*cal* (range)**
Unengorged nymphs	$6.8 \times 10^2 – 1.5 \times 10^5$	$7.2 \times 10 – 5.8 \times 10^3$	$7.7 – 5.2 \times 10^2$
Engorged nymphs	$2.2 \times 10^2 – 1.8 \times 10^5$	$6.6 \times 10 – 6.2 \times 10^2$	$1.5 \times 10 – 8.2 \times 10^3$

qPCRs analyses showed significantly higher *gyrB*/*cal* values (Wilcoxon sum-rank test: z = 3.14; p = 0.0017) in nymphs after the blood meal (i.e. engorged), confirming previous molecular studies regarding the quantification of the bacterium *M. mitochondrii* in different *I. ricinus* life stages (Sassera et al., 2008). This previous study has demonstrated that the amount of bacteria is higher when the larvae, nymphs and adult females perform a blood meal, and decreases after each molt. From these results, appear that the increase of the bacterial load is connected with blood meal. Therefore, it would be possible to suppose a similar role of *Midichloria* of *H. marginatum* in the molt and in eggs production. However, this experimental work described a quantity of bacteria in nymphs higher than the one observed in *I. ricinus* ticks, where the *gyrB*/*cal* ratio values ranged between 0.0 and 0.4 in nymphs before the blood meal, while this value ranged between 0.0 and 1.6 in nymphs after the blood meal. In this contest, it will certainly be interesting to conduct further studies to increase the knowledge about the presence of this bacterium in the immature stages of the tick (larva and nymph), in order to evaluate possible similarities with *I. ricinus* symbiotic bacterium. From literature, the transmission of *M. mitochondrii* to the vertebrate host during the blood meal of larvae and nymphs has not yet been demonstrated, while the transmission of this bacterium from adult stages of *I. ricinus* has been confirmed by molecular and serological studies (Mariconti et al., 2012a; Bazzocchi et al., 2013).

The amplification reported in all analyzed nymph samples through this qPCR protocol, leads us to consider this approach very sensitive, in contrast to what happens for *I. ricinus* nymphs.

3.5.4 Conclusions

The present work firstly provides evidence for the presence of a previously undescribed *Midichloria* strain in African *H. marginatum* nymphs infesting trans-Saharan migratory birds travelling back to their European breeding quarters. The sequencing of two genes previously used to study the genetic variability of *M. mitochondrii* (*nuoF2* and *secY*), from *H.*

marginatum nymph samples, revealed a percentage of identity with *M. mitochondrii* equal to 92 and 96% respectively.

Since ticks are important vectors of viral, bacterial and protozoan agents of veterinary and human relevance, avian migration represents an important reservoir for the worldwide spread of *Midichloria* bacteria across haematophagous ectoparasites populations. The high prevalence of *Midichloria* bacteria in tick samples observed in this experimental work, together with the detection of *Midichloria* DNA in avian hosts' blood samples (Di Lecce et al., 2018), provide evidence for the transmission of these bacteria in avian hosts. Further studies are required to assess the role of birds in the transmission ecology of these bacteria.

Additionally, future perspectives aim to investigate the presence of this bacterium inside the tick, performing electron microscopy and fluorescence in situ hybridization on organs of adult individuals, with the purpose to discover the localization of these bacteria into the arthropod host (i.e. salivary glands, rostrum, midgut, reproductive tract). Further experimental investigations regarding the presence of this novel midichloriacea in *H. marginatum* ticks are required to increase the knowledge concerning the role of this bacterium in the biology of this tick species.

3.6 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

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3.6.1 Aim of the study

The sequence of the *16S rDNA* of a *Rickettsia*-like organism (RLO) has been found associated with *Oncorhynchus mykiss* specimens affected by red mark syndrome/strawberry disease (RMS/SD), two skin pathologies of unknown aetiology (Metselaar et al., 2012; Lloyd et al., 2011). Phylogenetic studies have been performed subsequently on this sequence, showing that this bacterium belongs to the family *Midichloriaceae*, within the order Rickettsiales (Montagna et al., 2013). The presence of bacteria belonging to the *Midichloriaceae* family in aquatic hosts (Cnidaria and Porifera), together with the already known infectivity for vertebrates of the *I. ricinus* symbiont *M. mitochondrii*, suggest that *Midichloria*-like organisms (MLO) could be responsible for pathologies, such as RMS and SD in *O. mykiss* (Lloyd et al. 2008, 2011). In this work is presented a novel 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). In addition, the second aim of the present work was to detect the presence of this bacterium in different organs (heart, liver, spleen, intestine, kidney) of affected fish.

The scientific contribution that I gave to the present work consisted on preparation of plasmids containing the target genes (*16S rDNA* and *Igf1*) to use as standard for setting up qPCR reactions.

3.6.2 Materials and methods

A set of *O. mykiss* samples were collected from three farm sites (A, B and C) located in Scotland, to investigate the presence of the MLO in affected fish. 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 (Figure 1).

3.5.3 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 (Figure 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 (Figure 1c). In more advanced lesions, infiltration was seen throughout the dermis and sometimes extending into the epidermis (Figure 1f). In some fish sampled during the summer, evidence of infiltration was apparent even in skin which appeared grossly normal (Figure 1e).

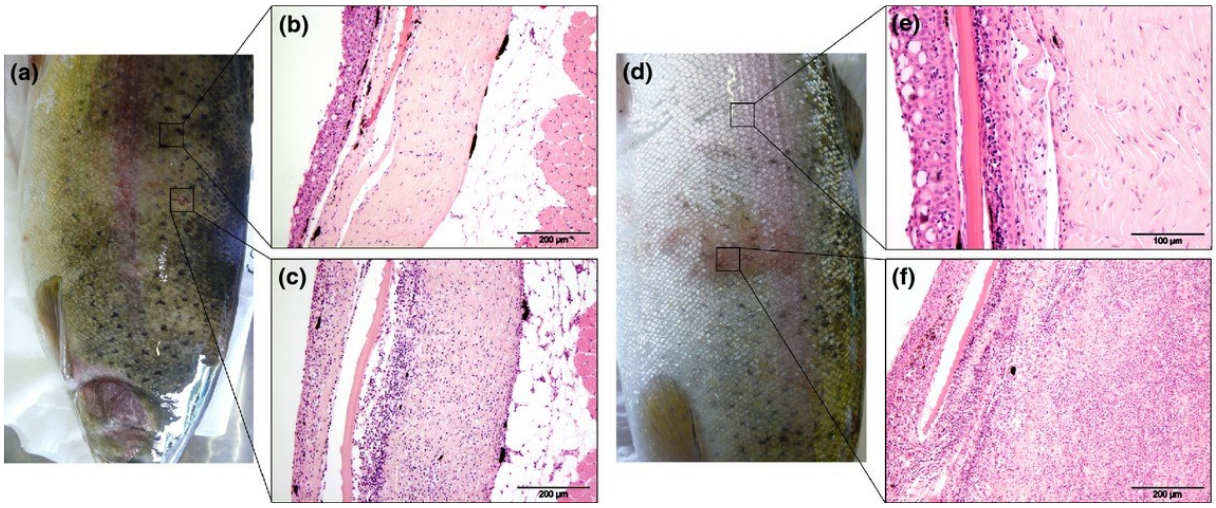

Figure 1: (a) Fish 2, early stage lesion, gross appearance; (b) Fish 2 section of normal skin (bar=200 µm); (c) F2 section of skin lesion (bar=200 µm); (d) F3 mid stage lesion, gross appearance; (e) F3 section of grossly normal skin (bar=100 µm); (f) F3 section of skin lesion (bar=200 µm). All sections 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: 250 nM) 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: 250 nM. 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 analyzed 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 (Figure 2).

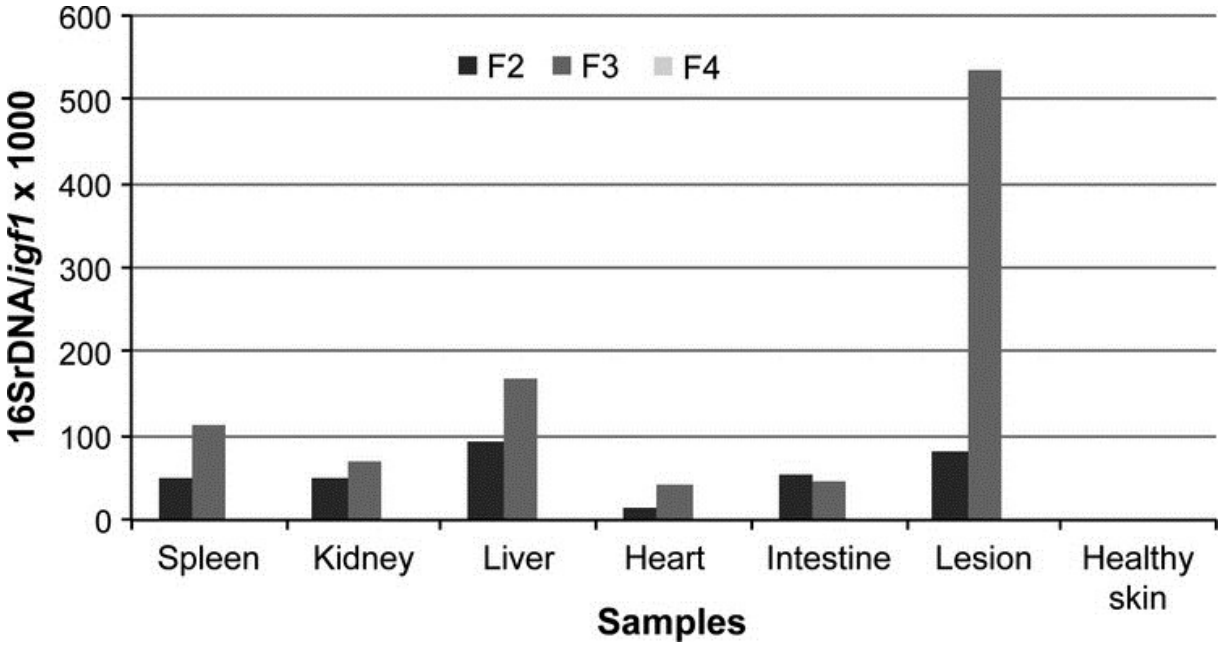

Figure 2: MLO quantity expressed as *16S rDNA*/igf1 x 1000 in organs (spleen, kidney, liver, heart, intestine), healthy skin and lesion from F2 (with an early skin lesion) and F3 (with a mid-stage skin lesion) and in organs and skin from a RMS-free control (F4).

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 analyzed 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 *16S rDNA* 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 analyzed 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.

3.7 Can *Midichloria mitochondrii* move inside the host mitochondrial network?

Francesco Comandatore, et al.

First draft in preparation

3.7.1 Aim of the study

M. mitochondrii is a gram-negative bacterium which lives in strict symbiosis with the hard tick *I. ricinus*. The bacterium is probably one of the most intriguing endosymbiont described, being able to durably colonize the host mitochondria. This peculiar tropism potentially makes *M. mitochondrii* a very interesting tool to the study the physiology of mitochondria, however experimental difficulties limit in vivo studies and only few information about the biology of the bacterium are currently available. Since the interaction between the symbiont and the organelle has not been characterized, the aim of the present work was to study the life cycle of *M. mitochondrii* within the host oocytes using a multidisciplinary approach, including electron microscopy, molecular biology, statistics and system biology. I will describe only the results of molecular analyses that I have performed concerning the quantification of mitochondria and the bacterium in *I. ricinus* oocytes through a Real time PCR (qPCR) approach.

3.7.2 Material and methods

Samples collection and DNA extraction

Two fresh *I. ricinus* ticks were collected in Northern Italy and manually dissected to retrieve, from each, five groups of ~10 oocytes at previtellogenic/late-previtellogenic development stage to be subjected to molecular analyses (qPCR). Each group of oocyte was processed for DNA extraction using a proteinase K incubation protocol: after adding 1 µl of proteinase K (20 mg/ml) to each sample, the lysis was carried out at 56°C for 1 hour; then, proteinase K activity was blocked at 95°C for 5 min. Samples were subsequently centrifuged for 10 min at 20,000×g (4°C) and stored at -80°C until use.

qPCR assays

Sybr green qPCR protocols were performed on 10 samples of oocytes for the following genes as described in Sassera et al., 2008: 1) the *gyrB* gene of *M. mitochondrii* (primers gyrBRT-F: CTTGAGAGCAGAACCACCTA and gyrBRT-R: CAAGCTCTGCCGAAATATCTT; amplifying 125 bp); 2) the *I. ricinus* nuclear gene *cal* (primers calRT-F: ATCTCCAATTTCGGTCCGGT and calRT-R: TGAAAGTTCCCTGCTCGCTT; amplifying 109 bp); and 3) the *I. ricinus* mitochondrial gene *COII* (primers COII-F: CCGACTTCTTGACGTAGACAAC and COII-R: CTGATTAAGGCGACCAGGAACG; amplifying 144 bp). The thermal profile for the amplification of *gyrB* and *cal* gene consisted of an initial denaturation step at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing at 60°C for 30 seconds. The thermal profile for the amplification of *COII* differed only in the annealing temperature, set at 58°C. The construction of melt curve was performed, at the end of each thermal profile, from 55°C to 95°C with increasing increments of 0.5°C per cycle for both genes to verify the absence of non-specific amplified fragments confirming the specificity of the method. For the latter gene (*COII*) it has been necessary to prepare a purified plasmid containing the target gene to set-up the calibration line to finally obtain the copy number of the desired gene for each sample.

For this purpose, a qualitative PCR protocol was performed to amplify *COII* gene starting from the DNA extracted from an *I. ricinus* tick. Subsequently, PCR products were recovered from the agarose gel, purified with the Wizard SV Gel and PCR Clean-Up System Kit (Promega®), quantified with the NanoDrop spectrophotometer (NanoDrop ND-1000, Thermo scientific®), and sequenced by ABI technology to confirm PCR specificity. Once confirmed the specificity of the amplification, PCR products were cloned into the pGEM-T Easy Vector System (Promega®) following the manufacturer directions. Plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen®) and quantified. A purified plasmid containing the desired fragments were serially diluted starting from 10^9 copies/microliters to 10 copies/microliter, in order to evaluate the efficiency and the dynamic range of the reaction.

All qPCRs reactions were performed in a final volume of 25 µl containing Sybr Green Sso Advanced Universal SYBR Green Supermix (Biorad®) 2X, 400 nM of each primer, 1 µl of DNA and water up to the final volume.

3.7.3 Results and discussion

qPCR assays

qPCRs were performed on 10 samples of oocytes to amplify *gyrB*, *cal* and *COII* genes. Number of each gene copy number was obtained through a comparison of the qPCR results of each sample with those of serial dilutions of purified plasmids (containing known copy numbers). The efficiency of each PCR protocol was as follows: 104.6% for *gyrB*, 102.7% for *cal*, and 107% for *COII*; the threshold line was set at 200 for each reaction. Results were expressed as the ratio of *gyrB*/*cal,* which indicates the quantity of *M. mitochondrii* in the tick, while the ratio *COII/cal* indicates the number of mitochondria present in the tick. Since mitochondria can contain a variable number of genome copies, the *COII* gene copy number can be consider as an approximation of the number of mitochondria present in ticks. From the equation of the calibration line obtained from each standard, it was possible to obtain the copy number of *gyrB, cal*, and *COII* genes for each sample.

Oocytes developmental stages and qPCR results for each gene are shown in Tables 1 and 2. In Table 1 are shown the range values of *gyrB*, *cal* and *COII* copy numbers obtained in oocytes at previtellogenic (P) and late-previtellogenic (LP) developmental stages; while in Table 2 are shown the range values of *gyrB/cal* and *COII/cal* ratios.

All analyzed samples showed positivity for each gene, and the melting curves showed the presence of specific amplified fragments belonging to the target sequences confirming the specificity of the method.

The range value of copy number for each gene showed no variations between the two developmental stages of oocytes.

Table 1: Range values of *gyrB*, *cal* and *COII* copy numbers in two analyzed groups of oocytes grouped by developmental stage in previtellogenic and late-previtellogenic.

	***gyrB* copy number range**	***cal* copy number range**	***COII* copy number range**
Previtellogenic (P)	$8.84 \times 10^4 – 3.8 \times 10^5$	$4.1 \times 10^2 – 3.35 \times 10^3$	$5.6 \times 10^5 – 4 \times 10^6$
Late-previtellogenic (LP)	$1.99 \times 10^4 – 7.7 \times 10^5$	$6.3 \times 10 – 3.3 \times 10^3$	$1.3 \times 10^5 – 2.4 \times 10^6$

Table 2: Range values of *gyrB/cal* and *COII/cal* ratios in two analyzed groups of oocytes grouped by developmental stage in previtellogenic and late-previtellogenic.

	***gyrB/cal* range**	***COII/cal* range**
Previtellogenic (P)	$2.6 \times 10 - 8.4 \times 10^2$	$4.3 \times 10^2 - 3.12 \times 10^3$
Late-previtellogenic (LP)	$1.4 \times 10^2 - 7.8 \times 10^2$	$5.8 \times 10^2 - 2 \times 10^3$

The range value of *COII*/*cal* ratio obtained in oocytes at both developmental stages is higher than the range value of *gyrB*/*cal* ratio, suggesting an higher number of mitochondria in ticks compared to bacterial number.

3.7.4 Conclusions

The results obtained in qPCR analyses regarding the quantification of *M. mitochondrii* and mitochondria organelles in *I. ricinus* oocytes will be integrated with transmission electron microscopy (TEM) observations, in which the number of *M. mitochondrii* cells outside mitochondria, the number of non parasitized mitochondria, and the number of mitochondria parasitized by bacterial cells has been counted. Data obtained from TEM experiments will be used to compare the *M. mitochondrii* and mitochondria within the oocytes at the two developmental stages considered, with the final aim to investigate whether this symbiont could move within the mitochondrial network, passing from a mitochondrion to another.

4 Conclusions

I. ricinus, the most common tick species in Europe, is of considerable importance due to its wide area of distribution (i.e. Europe and Northern Africa), to its low host specificity and to its central role in the transmission of multiple infectious agents (e.g. the causative agent of Lyme borreliosis, rickettsial diseases, tick-borne encephalitis (TBE) virus) to both humans and animals. Among the numerous microorganisms harbored and transmitted by *I. ricinus*, there is the symbiont *M. mitochondrii*, whose transmission to the vertebrate host has been demonstrated by several studies. The parasitism supported by this tick species could represent a risk factor for the onset of several chronic-degenerative diseases (e.g. amyotrophic lateral sclerosis, myocardiopathy, multiple sclerosis), opening the necessity to obtain an effective test capable of identifying bites of this tick species in human and animal populations. This necessity is reinforced by the main characteristics of tick bite, i.e. the components of tick saliva with anesthetic properties that inhibit host pain and hitch responses, leading to unreported tick bites in human populations with consequent misinformation.

Our results concerning the set-up of a novel peptide-based ELISA test capable of detecting *I. ricinus* bite, contribute to increase the percentage of sensitivity to investigate tick exposure, since we obtained positive values in human patients with history of tick bite but resulting negative to *M. mitochondrii* and *Borrelia* antigens. We will focus our attention on testing different combinations of synthetized peptides, since it is known that diagnostics based on a single peptide may lack sensitivity due to the dependence on a single antibody epitope. Interestingly, the positivity against *I. ricinus* proteins found in rabbits 16 weeks post experimental *I. ricinus* ticks infestation, contributes to reinforce the good property of our peptides as indicator of exposure to this tick species.

The seroconversion time against protein of *M. mitochondrii* (flagellar rFliD protein) has been studied for the first time in rabbits experimentally infested with *I. ricinus* for a time-span of four months, showing to occur starting from one week post-infestation up to 16 weeks after experimental infestation. The replication of *M. mitochondrii* inside the vertebrate host has also been demonstrated for the first time in this vertebrate model, since bacterial DNA has been detected in blood samples up to 16 weeks post-infestation, suggesting the bacterial capacity of survive and replicate into the host after its inoculation. Additionally, *M. mitochondrii* transmission has also been demonstrated when the symbiont load is reduced in case of tick laboratory strains (where a bacterial decrease in prevalence after some

generations under laboratory conditions it is well known).

The bacterial family of *Midichloriaceae*, to which *M. mitochondrii* belongs, comprises bacteria associated with a wide range of phylogenetically diverse hosts, from protists to Cnidaria and Porifera, from parasitic arthropods to mammals. The detection of a novel bacterium belonging to the genus *Midichloria* in African *Hyalomma marginatum* ticks collected from migratory birds on Ventotene Island, may contribute to obtain valuable information to define the geographic distribution of this bacterium, as well as to increase the knowledge of its potential infectious role, given the important role of avian migration in the spread of tick-borne microorganisms. Future studies will be focused on the investigation of this novel *Midichloria* bacterium in *H. marginatum* adult individuals, to define its localization in this tick species.

Finally, this PhD course also allowed me to collaborate in different topics of research lines, (the study of genes expression in canine oral melanoma as new potential prognostic biomarkers and the expression of specific genes involved in the development of muscles and in cellular stress in different stocking densities of *Acipenser baerii* on muscle formation and oxidative stress), establishing productive interactions with people in any type of work team.

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6 List of publications

Abstracts and posters for National and International Conferences:

- **Serra, V.**, Cafiso, A., Epis, S., Negri, A., Rubolini, D., Bandi, C., Bazzocchi, C. Detection and quantification of a novel bacterium of the genus *Midichloria* (family *Midichloriaceae*, order Rickettsiales) in the hard tick *Hyalomma marginatum.* At: XXX Congresso Nazionale Società Italiana di Parassitologia – "Mutamenti ambientali e parassiti", 26th-29th June 2018 (Milano, Italy)

- Cafiso, A., **Serra, V.**, Romeo, C., Sassera, D., Olivieri, E., Plantard, O., Bandi, C., Bazzocchi, C. *Midichloria mitochondrii* transmitted to the vertebrate host by *Ixodes ricinus*: a transient passenger or an infectious agent? At: XXX Congresso Nazionale Società Italiana di Parassitologia – "Mutamenti ambientali e parassiti", 26th-29th June 2018 (Milano, Italy)

- Comandatore, F., Sassera, D., Radaelli, G., Epis, S., Bazzocchi, C., Montante, S., Di Carlo, D., Brilli, M., **Serra, V.**, Perini, M., Clementi, E., Sacchi, L., Bandi, C. *Midichloria mitochondrii* life-cycle: what can mathematical analyses tell us? At: XXX Congresso Nazionale Società Italiana di Parassitologia – "Mutamenti ambientali e parassiti", 26th-29th June 2018 (Milano, Italy)

- Olivieri, E., Varotto Boccazzi, I., Romeo, C., Desirò, A., Cafiso, A., **Serra, V.**, Floriano, A.M., Epis, S., Sassera, D. *Midichloria mitochondrii* localization and quantification in the organs of the hard tick *Ixodes ricinus*. At: XXX Congresso Nazionale Società Italiana di Parassitologia – "Mutamenti ambientali e parassiti", 26th-29th June 2018 (Milano, Italy)

- Bazzocchi, C., Cafiso, A., Di Venere, M., **Serra, V.**, Bandi, C., Iadarola, P., Sassera, D. Estimating the risk associated with *Ixodes ricinus* parasitism: towards the development of serological markers for the tick bite. At: International Symposium on Tick-Borne Pathogens and Disease, 24th - 26th September 2017, (Vienna, Austria)

- Cafiso, A., Plantard, O., **Serra, V.**, Floriano, A.M., Bandi, C., Sassera, D., Bazzocchi, C. Molecular screening for *Midichloria* in hard and soft ticks reveals different prevalences and horizontal transmission. At: International Symposium on Tick-Borne Pathogens and Disease, 24th – 26th September 2017, (Vienna, Austria)

- Nordio, L., Genova, F., **Serra, V.**, Bazzocchi, C., Longeri, M.L., Stefanello, D., Rondena, M., Giudice, C. LTA4H and FXR1 gene and protein expression in canine oral melanoma. At: 3rd Joint European Congress of the ESVP, ESTP and ECVP, 30th August - 2nd September 2017 (Lione, France)

- **Serra, V.** and Bazzocchi, C. Serological evidence of *Midichloria mitochondrii* circulation in humans parasitized by *I. ricinus* in Germany and development of a marker for tick bite. International Journal of Health, Animal Science and Food Safety, Proceedings of the Veterinary and Animal Science (VAS) Days, 6th-8th June 2017 (Milano, Italy)

- Cafiso, A., **Serra, V.**, Epis, S., Sassera, D., Bandi, C., Bazzocchi, C. *Midichloria*, the symbiont of ticks transmitted during the blood meal. A vehicle for horizontal transmission? At: Insect symbionts, 10th-11th May 2017, Mitrani Center of Desert Ecology, (Sde Boker, Israele)

- Cafiso, A., **Serra, V.**, Petroni, G., Comandatore, F., Bazzocchi, C. Batteri della famiglia *Midichloriaceae* (ordine Rickettsiales): possibili patogeni emergenti per i vertebrati? At: XXII Convegno Nazionale Società Italiana Patologia Ittica, 8th-9th September 2016 (Trento, Italy)

- Nordio, L., Genova, F., **Serra, V.**, Giudice, C. LTA4H expression in canine oral melanomas: preliminary results. At: Proceedings of XXXIV Meeting of the European Society of Veterinary Pathology, 7th-10th September 2016 (Bologna, Italy)

- **Serra, V.**, Cafiso, A., Parisio, G., Sassera, D., Formenti, N., Verheyden, H., Plantard, O., Bazzocchi, C. Molecular and serological evidences of circulating *Midichloria mitochondrii* in roe deer (*Capreolus capreolus*) after *Ixodes ricinus* bite. At: XXIX Congresso Nazionale Società Italiana di Parassitologia, page 228, 21th-24th June 2016 (Bari, Italy)

- Cafiso, A., **Serra V.**, Bersani, M., De Marco, L., Sassera, D., Plantard, O., Bazzocchi, C. Molecular screening for *Midichloria* bacteria in hard and soft ticks (Acari: Ixodida). At: XXIX Congresso Nazionale Società Italiana di Parassitologia, page 209, 21th-24th June 2016 (Bari, Italy)

- **Serra, V.**, Cafiso, A., Bazzocchi, C. Molecular and serological evidences of *Midichloria mitochondrii* transmission to vertebrate hosts during the tick bite. International Journal of Health, Animal Science and Food safety, Vol 3, No 1s. At: Proceedings of the Veterinary and Animal Science (VAS) Days, 8th-10th June 2016 (Milano, Italy)

- Cafiso, A., **Serra, V.**, Plantard, O., Bazzocchi, C. Molecular screening for *Midichloria* bacteria in hard and soft ticks (Acari: Ixodida). International Journal of Health, Animal Science and Food safety, Vol 3, No 1s. At: Proceedings of the Veterinary and Animal Science (VAS) Days, 8th -10th June 2016 (Milano, Italy)

- Nordio, L., Genova, F., **Serra, V.**, Giudice, C. LTA4H expression in canine oral melanomas: methodological set up and preliminary results. International Journal of Health, Animal Science and Food safety, Vol 3, No 1s. At: Proceedings of the Veterinary and Animal Science (VAS) Days, 8th-10th June 2016 (Milano, Italy)

- Cafiso, A., **Serra, V.**, Petroni, G., Bazzocchi, C. Batteri della famiglia *Midichloriaceae* (ordine Rickettsiales): agente eziologico della red mark syndrome in *Onchorhynchus mykiss*? At: XXI Convegno Nazionale Società Italiana Patologia Ittica, (8^{th}-9^{th} October 2015 (Chioggia, Italy)

Full papers:

- Cafiso, A., Sassera, D., Romeo, C., **Serra, V.**, Hervet, C., Bandi, C., Plantard, O., Bazzocchi, C. 2019. *Midichloria mitochondrii*, endosymbiont of *Ixodes ricinus*: evidence for the transmission to the vertebrate host during the tick blood meal. Ticks and Tick-Borne Diseases, 10, 5-12

- **Serra, V.**, Cafiso, A., Formenti, N., Verheyden, H., Plantard, O., Bazzocchi, C., Sassera, D., 2018. Molecular and Serological Evidence of the Presence of *Midichloria mitochondrii* in Roe Deer (*Capreolus capreolus*) in France. Journal of Wildlife Diseases, 54(3), 597-600

- **Serra, V.,** Krey V., Daschkin, C., Cafiso, A., Sassera, D., Maxeiner, H., Nicolaus, C., Bandi, C., Bazzocchi, C., 2018. Seropositivity to *Midichloria mitochondrii* (order Rickettsiales) as a marker to determine the exposure of humans to *Ixodes ricinus* Manuscript submitted to Ticks and Tick-Borne Diseases

- Comandatore, F., et al., 2018. Can *Midichloria mitochondrii* move inside the host mitochondrial network? First draft in preparation

- Aidos, L., **Serra, V**., Cafiso, A., Bertotto, D., Bazzocchi, C., Radaelli, G., Di Giancamillo, A., 2018. How different stocking densities affect growth and stress status of *Acipenser baerii* larvae? Manuscript in preparation

- **Serra, V.**, et al., 2018. Peptide-based ELISA test as *Ixodes ricinus* bite marker in different vertebrate hosts. First draft in preparation

- **Serra, V.**, et al., 2018. Detection and quantification of a novel bacterium of the genus *Midichloria* (family *Midichloriaceae*, order Rickettsiales) in the hard tick *Hyalomma marginatum*. First draft in preparation

- Cafiso, A., Sassera, D., **Serra, V.**, Bandi, C., McCarthy, U., 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. Journal of Fish Diseases 39, 497-501

7 Scientific activities performed during PhD course

<u>FIRST YEAR</u> (2015-2016)

Attendance to courses, seminars, workshops and scientific meetings

Obligatory courses:

- "Medical Statistic 1", held by Dr Elisabetta Sala with passing the final exam. Period of lessons: from 1st February 2016 to 21th March 2016 at Faculty of Veterinary Medicine (Milan)

- "Communication 1", held by Dr Ettore Galanti with passing the final exam. Period of lessons: 3th and 23th March 2016 and 14th April 2016 at Faculty of Veterinary Medicine (Milan)

- "Digital imaging and image integrity in scientific publication", held by Dr Valentina Lodde with passing the final exam. Period of lessons: from 9th to 13th May 2016 at Faculty of Veterinary Medicine (Milan)

- "Transferable skills":
- "Open access-open data ed il mondo delle pubblicazioni". 17th June 2016, Università degli Studi di Milano, classroom G08, via Golgi 19 (Milan, Italy)
- "La valutazione della ricerca". 21th September 2016, Università degli Studi di Milano, classroom G14, via Golgi 19 (Milan, Italy)

Optional courses:

- "Bibliographical data bases", held by Dr Angela Moccia with passing the final exam. Period of lessons: from 14th to 29th June 2016 at Faculty of Veterinary Medicine (Milan)

- Workshop: qPCR - ThermoFisher Scientific, held by Dr. Lorenzo Sangiorgio and Dr. Claudio Bencivenga. 18th May 2016, Università degli Studi di Milano, via Celoria 10 (Milano, Italy)

- Scientific meeting: "Biodiversity: concepts, new tools and future challenges". 30th August - 2nd September 2016, Bicocca University (Milano, Italy)

- Workshop: “Infezioni di animali, infezioni umane: animali da reddito e zoonosi”. 26th September 2016, Auditorium Testori - Palazzo Lombardia (Milano, Italy)

Externship:

One month at the BCA-clinic of Augsburg (Germany) - (April 2016)

Co-supervision of degree thesis:

Master’s degree thesis in Biotechnological Veterinary Sciences entitled “LTA4H and FXR1 gene expression as potential biomarker for canine oral cavity melanoma metastasis in FFPE samples”. Thesis supervisor: Prof. Chiara Bazzocchi (July 2016)

SECOND YEAR (2016-2017):

Attendance to courses, seminars, workshops and scientific meetings

Obligatory courses:

- “Medical Statistic 2”, held by Dr Elisabetta Sala with passing the final exam. Period of lessons: from 21th November 2016 to 15th December 2016, Università degli Studi di Milano, via Celoria 19 (Milano, Italy)

- “Communication 2”, held by Dr Ettore Galanti with passing the final exam. Period of lessons: 25th January 2017, 8th and 23th February 2017, Università degli Studi di Milano, via Celoria 10 (Milano, Italy)

- “Transferable skills”:
  - “How to write a research project: part 1. 100 things I would have liked to know when I was a PhD student”. 22th February 2017, Università degli Studi di Milano, classroom 200, via Celoria 19 (Milano, Italy)
  - “How to write a research project”: part 2. 100 things I would have liked to know when I was a PhD student”. 16th June 2017, Università degli Studi di Milano, classroom G11, via Celoria 19 (Milano, Italy)
  - “Research Integrity” held by Prof. Monica Ferraroni and Prof. Fabrizio Gardoni. 29th September 2017, Università degli Studi di Milano, classroom G14, via Celoria 19 (Milano, Italy)

- Seminar: “Managing a doctoral research as a project”, held by Dr. Reuven Katz. 4th September 2017, Università degli Studi di Milano, classroom D, via Balzaretti 9 (Milano, Italy)

- Workshop: “Innovation Day - Innovative Solutions for Cell Analysis, Protein and Synthetic Biology”. 18th September 2017, Università degli Studi di Milano, classroom B5, via Celoria 26 (Milano, Italy)

- Congress: “International Symposium on Tick-Borne Pathogens and Disease”, 24th-26th September 2017, Austria Trend Parkhotel Schönbrunn 1130 Wien, Hietzinger Hauptstrasse 10-14 (Vienna, Austria)

Externship:

One month at the BCA-clinic of Augsburg (Germany) - (March 2017)

Teaching activities:

- Lecturer for the course “Strategie di ricerca e metodologie per lo studio e il controllo delle malattie” held by Prof. Chiara Bazzocchi (21th November 2016)

- Tutoring for laboratory activities in the course “Genomics and Molecular basis of differentiation in model invertebrate organisms and pathogens” held by Prof. Chiara Bazzocchi (Eight hours in December 2016)

<u>THIRD YEAR</u> (2017-2018):

Attendance to courses, seminars, workshops and scientific meetings

Obligatory courses:

- “Medical Statistic 3”, held by Dr Elisabetta Sala with passing the final exam. Period of lessons: 15th December 2017, 20th December 2017, and 1st February 2018, Università degli Studi di Milano, via Celoria 19 (Milano, Italia)
- “Communication 3”, held by Dr Ettore Galanti with passing the final exam. Period of lessons: 24th January 2018, 7th and 21th February 2018, Università degli Studi di Milano, via Celoria 10 (Milano, Italia)

- "Transferable skills":
- "How to protect an invention within Unimi". 8th March 2018, Università degli Studi di Milano, classroom G24, via Celoria 19 (Milano, Italia)
- "Entrepeneurship as valorization of research – how to create a spin off company from Unimi (part one)". 20th April 2018, Università degli Studi di Milano, classroom G11, via Celoria 19 (Milano, Italy)
- "Entrepeneurship as valorization of research – how to create a spin off company from Unimi (part two)". 25th May 2018, Università degli Studi di Milano, classroom G11, via Celoria 19 (Milano, Italy)
- "Building a professional profile through transferable skills. What are the possibilities for PhDs?". 20th June 2018, hall Pio XII, via S. Antonio 5 (Milano, Italy)

- Congress: XXX Congresso Nazionale Società Italiana di Parassitologia – "Mutamenti ambientali e parassiti". 26th-29th June 2018, Università degli Studi di Milano, via Festa del Perdono 7 (Milano, Italy)

Co-supervision of degree thesis:

- Master's degree thesis in Biotechnological Veterinary Sciences entitled "Identificazione e quantificazione di un nuovo batterio della famiglia *Midichloriaceae* (ordine Rickettsiales) nella zecca *Hyalomma marginatum*". Thesis supervisor: Prof. Chiara Bazzocchi. (October 2017)

- Master's degree thesis in Biotechnological Veterinary Sciences entitled "Effetto della densità sullo sviluppo degli stadi larvali di *Acipenser baerii*: un approccio molecolare". Thesis supervisor: Prof. Chiara Bazzocchi. (July 2018)

Teaching activity:

- Lecturer for the course of "Strategie di ricerca e metodologie per lo studio e il controllo delle malattie" held by Prof. Chiara Bazzocchi. (5th December 2017)

- Tutoring for laboratory activities in the course "Biotecnologie avanzate in parassitologia" held by Prof. Chiara Bazzocchi. Department of Veterinary Medicine, (Milan, Italy). (Eight hours in December 2017)

- Tutoring for laboratory activities for high school students winners of the competition "Una settimana da ricercatore" within the project "Progetto Nazionale Biologia e Biotecnologia del PLS". Department of Veterinary Medicine (Milano, Italy). (From 9th to 13th July 2018)

Other relevant activities:

- Associate member of Società Italiana di Parassitologia (SoIPa)
- Organizing committee member of XXX Congresso Nazionale Società Italiana di Parassitologia - "Mutamenti ambientali e parassiti", 26th-29th June 2018 (Milano, Italy)

Awards:

Winner of "Bando Premi Soci Giovani XXX Congresso Nazionale Società Italiana di Parassitologia" (2018)