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The relationship between spotted fever group *Rickettsiae* and Ixodid ticks

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Abstract – Spotted fever group *Rickettsiae* are predominantly transmitted by ticks. *Rickettsiae* have developed many strategies to adapt to different environmental conditions, including those within their arthropod vectors and vertebrate hosts. The tick-*Rickettsiae* relationship has been a point of interest for many researchers, with most studies concentrating on the role of ticks as vectors. Unfortunately, less attention has been directed towards the relationship of *Rickettsiae* with tick cells, tissues, and organs. This review summarizes our current understanding of the mechanisms involved in the relationship between ticks and *Rickettsiae* and provides an update on the recent methodological improvements that have allowed for comprehensive studies at the molecular level.

ticks / spotted fever group *Rickettsiae* / vector / reservoir

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1. INTRODUCTION

Rickettsiae are Gram-negative, obligate intracellular bacteria in the family *Rickettsiaceae* and order *Rickettsiales*. The spotted fever group (SFG) unites a phylogenetically well-defined clade of *Rickettsiae* that are distinct from other species and that have a life cycle involving arthropods, mainly ticks [63]. The SFG includes a number of pathogenic organisms that cause so-called tick-borne (TB) rickettsioses in humans. Among them are *Rickettsia rickettsii* (Rocky Mountain spotted fever, RMSF), *R. conorii conorii* (Mediterranean spotted fever, MSF), *R. conorii israelensis* (Israeli spotted fever), *R. conorii caspia* (Astrakhan spotted fever), *R. conorii indica* (Indian tick typhus *Rickettsia*), *R. africae* (African tick-bite fever, ATBF), *R. heilongjiangensis* (Far-eastern tick-borne rickettsiosis), *R. australis* (Queensland tick typhus), *R. slovaca* (Tick-borne lymphadenopathy and *Demacenter*-borne necrosis erythema lymphadenopathy, TIBOLA/DEBONEL), *R. sibirica sibirica* (North Asian tick typhus or Siberian tick typhus), *R. sibirica mongolitimonae* (Lymphangitis-associated rickettsiosis), *R. honei* (Flinders Island spotted fever), *R. japonica* (Japanese or Oriental spotted fever), *R. parkeri*, *R. aeschlimannii*, *R. massiliae*, and *R. raoultii*. Numerous *Rickettsiae* are regularly associated with ticks and have been called symbionts (literally “living together”), microsymbionts, or endosymbionts (living in endocellular symbiosis) by entomologists, ecologists, or endocytobiologists. However, their potential for pathogenicity is still unknown [62].

The ecology of SFG *Rickettsiae* has not been definitively elucidated. Some SFG *Rickettsiae* are thought to circulate in enzootic or epizootic cycles between wild vertebrates and arthropod vectors [50, 89]. Ticks are usually thought to be the main reservoir and vectors of SFG *Rickettsiae* in nature, due to the ability of *Rickettsiae* to survive perpetually in ticks and to be transmitted transstadially and transovarially. However, this has been demonstrated only for a few tick-borne *Rickettsiae* (Tab. 1). Humans are only occasional hosts for ticks and rarely play a role in the subsequent transmission of

bacteria. Therefore, induced human rickettsioses should be viewed as an accidental ecosystem change for the *Rickettsiae*, and the human should be viewed as a “dead end” host, which plays no role in the maintenance of these bacteria in nature.

Ticks are currently considered to be second only to mosquitoes as vectors of human infectious diseases worldwide. All of the nearly 900 known species of ticks require blood for their development and reproduction, and they parasitize every class of vertebrates in almost every region of the world. Two families of ticks are of medical significance: *Ixodidae* (hard ticks) and *Argasidae* (soft ticks). To date, most ticks infected with SFG *Rickettsiae* belong to the *Ixodidae* family. Ixodid ticks feed once within each stage but for a relatively long period (several days), during which the tick remains strongly attached to the host. This blood-feeding may involve a great variety of vertebrates that occupy very diverse habitats. Because the tick's bite is usually painless, tick attachment may go unnoticed for several days, consequently enhancing the vector potential of ticks [62].

The tick-*Rickettsiae* relationship was a focus of interest for many pioneering rickettsiologists, with most of the early studies concentrating on the role of ticks as vectors. At the beginning of the 20th century, the wood tick *Demacenter andersoni* was found to be involved in the transmission of *R. rickettsii* [77]. In the 1930s, the role of *Rhipicephalus sanguineus*, the brown dog tick, was demonstrated in the transmission of *R. conorii* (Fig. 1) [10].

This review highlights the relationship between ticks and *Rickettsiae*, with regards to the features of *Rickettsiae* adaptation to ticks and transmission to the ticks' progeny. The development of genomic tools and the benefits or deleterious effects of rickettsial infection, especially in terms of gene expression modification, will also be discussed.

1.1. Primary infection of ticks with *Rickettsiae*

The initial infection of ticks with *Rickettsiae* can occur via the gut when bacteria-free ticks feed on rickettsemic hosts (Fig. 2). This

Table I. The prevalence of infected ticks in nature and the study of vertical and transovarian transmission of SFG *Rickettsiae* (transovarian transmission rate (TOT): proportion of infected females giving rise to at least one positive egg or larva).

<i>Rickettsia</i>	Tick species	Infection rate (%)	TOT (%)
<i>R. conorii</i>	<i>Rh. sanguineus</i>	0–1.4	100*
<i>R. rickettsii</i>	<i>D. andersoni</i>	0.26–1.5	100*
	<i>D. variabilis</i>	0.0143–1.3	30–40
<i>R. africae</i>	<i>A. hebraeum</i>	20–30	100
	<i>A. variegatum</i>	27–100	Yes
<i>R. massiliae</i>	<i>Rh. turanicus</i>	0.7–50	100
<i>R. slovaca</i>	<i>D. marginatus</i>	7.2–40.6	100
<i>R. rhipicephali</i>	<i>Dermacentor</i> sp.	1.26–1.32	38–100
<i>R. sibirica</i>	<i>D. nuttalli</i>	12	100
<i>R. bellii</i>	<i>Amblyomma</i> sp.	1.4–17.4	NS
	<i>I. loricatus</i>	60.9	100
	<i>Dermacentor</i> sp.	1.3–2.2	NS
<i>R. helvetica</i>	<i>I. ricinus</i>	0.6–46.45	100
<i>R. peacockii</i>	<i>D. andersoni</i>	66	73.3
<i>R. monacensis</i>	<i>I. ricinus</i>	2.4–52.9	NS
<i>R. aeschlimannii</i>	<i>H. marginatum marginatum</i>	1.8–57.9	Yes
<i>R. amblyommii</i>	<i>Amblyomma</i> sp.	3.7–23.6	Yes
<i>R. raoultii</i>	<i>D. reticulatus</i>	5.6–23	NS
	<i>D. marginatus</i>	22.5–83.3	86.4–100

NS: Not studied. * TOT in naturally infected ticks, but no or low transmission in laboratory-infected ticks. TOT for *R. conorii* was studied only for the fifth generation. The duration of the infection in the ticks is unknown.



Figure 1. *Rhipicephalus sanguineus* (brown dog tick), the main vector of MSF and occasional vector of RMSF. From left to right: female, male, nymph, larva, and egg. Bar scale, 1 mm.

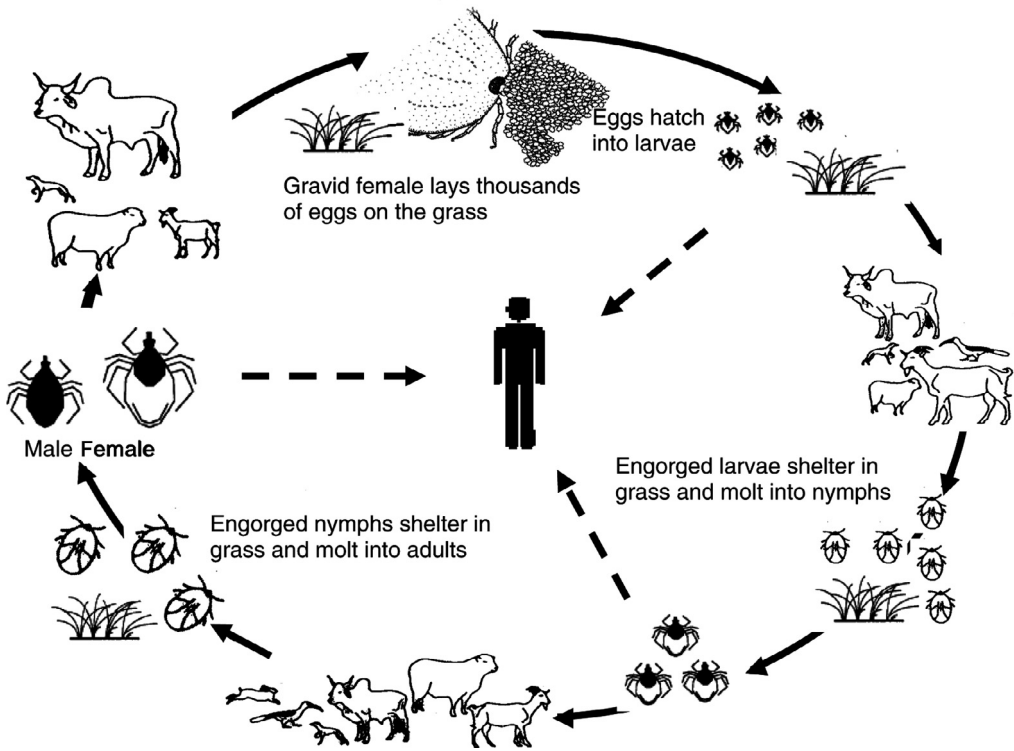


Figure 2. Life cycle of *Amblyomma variegatum*, modified from [83].

requires sufficient blood levels of *Rickettsiae* in free-living vertebrates, which may act as reservoirs for *Rickettsiae* [74]. For example, *R. rickettsii* was first isolated from small mammals known as meadow voles (*Microtus pennsylvanicus*) [50, 91]. A decade later, *R. rickettsii* was isolated from eight other species of mammals, including a pine vole (*Pitymys pine-torum*), a white-footed mouse (*Peromyscus leucopus*), a cotton rat (*Sigmodon hispidus*), cottontail rabbits (*Sylvilagus floridanus*), an opossum (*Didelphis marsupialis virginiana*), chipmunks (*Eutamias amoenus*), a snowshoe hare (*Lepus americanus*), and golden-mantled ground squirrels (*Spermophilus lateralis tesco-rum*) [50]. The more blood the tick ingests, the greater the level of bacteria in the blood-stream, and the longer the tick remains attached, the higher the probability of infection by *Rickettsiae*. However, the relative importance

of this mode of infection in nature is unknown for most tick-borne *Rickettsiae*.

Ticks may also acquire *Rickettsiae* by co-feeding which occurs when several ticks feed next to each other on the same host. In this case, the direct spread of bacteria from an infected tick to an uninfected tick can occur during feeding at closely situated bite sites, as demonstrated with *R. rickettsii* and *D. andersoni* [65]. Co-feeding and/or sexual transmission of *R. massiliae* was demonstrated in a male *Rh. turanicus* feeding on a rabbit with *Rh. sanguineus* females [49]. However, females that had been infected by sexual transmission/co-feeding did not transmit the bacteria transovarially. The transmission of *R. rickettsii* from infected male ticks to non-infected female ticks has also been described, but this process is unlikely to significantly propagate rickettsial infection in tick lineages, since venereally infected

females do not appear to transmit the bacteria transovarially [29]. However, more data are needed to confirm rickettsial transfer during copulation.

1.1.1. Gut barrier and initial contact with tick cells

The host blood ingested by a tick flows through the canal formed by the chelicerae and the hypostome, through the pharyngeal cavity, and through a short esophagus into the mid-gut and its diverticula [72]. Digestion involves the lysis of erythrocytes within the gut lumen, ingestion of hemolysate by the digestive cells, and intracellular digestion of protein and lipids [54]. Here, the first contact with tick cells occurs. *Rickettsiae* interact with yet unknown cellular surface receptors and escape the tick's immune responses, which probably differ from those in vertebrates. All of the ticks tested at 72 h post-experimental feeding were infected with *R. montanensis* [18]. Recently, electron microscopy showed at least two forms of *R. peacockii* in the cytosol of infected cells. One is present in the cytosol of tick cells, and the second within the autophagolysosomes [38]. However, it is not known whether these findings also apply to other tick/SFG *Rickettsiae* interactions. A phospholipase belonging to the putative phospholipase D (PLD) protein superfamily might be critical for the internalization and intracellular life of *Rickettsiae*. In rickettsial pathogenicity, PLD is hypothesized to exert functions attributed to phospholipase A2 (PLA2): to mediate entry into the host cell, to escape from the phagosome, and to facilitate injury to host cells [76]. SFG *Rickettsiae* also exploit the host cell actin cytoskeleton to promote motility and cell-to-cell spreading [31, 90]. A rickettsial protein called RickA promotes the nucleation of actin monomers via the Arp2/3 complex. These factors are expressed on the bacterial surface but lack signal sequences, and therefore, their mode of secretion is unknown [28, 36]. The differential actin-based motility of *R. raoultii* and *R. conorii* observed in L929 and Vero cells suggests that the expression of RickA is

not a sufficient condition to promote actin polymerization in vivo and that another factor apart from RickA may be involved in this process [7]. Preliminary results assessed by differential-display PCR have shown modifications in tick gene expression in *Rickettsia*-infected *D. variabilis* ticks [45]. In particular, the expression levels of tubulin alpha-chain and V-ATPase associated with clathrin-coated vesicles were up-regulated. V-ATPase is known to facilitate protein sorting, receptor-mediated endocytosis by the cell, and the entry of a number of envelope viruses and bacterial toxins, including the influenza virus and anthrax toxin [33].

The tick mid-gut epithelial cells support highly replicative *Rickettsiae* without altering the host cell ultrastructure. After crossing the digestive tract barrier, the bacteria penetrate into the body cavity of arthropods and survive and multiply there for a long time, virtually for the entire life of the vector, as has been shown for "*Candidatus Rickettsia tarasevichiae*" in naturally infected *I. persulcatus* ticks [69]. The role of the mid-gut in rickettsial infection is vital to successful rickettsial dissemination via both saliva secretion and tick feces. The excreta during feeding consist of black hematin and other undigested residues from the mid-gut [87]. The purpose of excretion during feeding is to remove liquids, which are required for producing more saliva, and also to retain the lipids from cell membranes. *R. slovaca* was identified in the feces of naturally infected *D. marginatus*, and successfully isolated. *R. massiliae* and *R. conorii* have also been detected in the feces of infected ticks, using molecular tools and immunofluorescence assays [49, 73, 84]. However, the role of feces as an efficient source of infection is unknown, although the weak transmission of *R. rickettsii* to guinea pigs was found to occur through this route [65, 72].

1.1.2. Hemolymph

Rickettsiae that escape from the mid-gut invade hemocytes, gaining access to virtually all tissues and organs and causing a generalized infection, as shown for *R. conorii* in *Rh. sanguineus* [79] (Fig. 3). The hemolymph

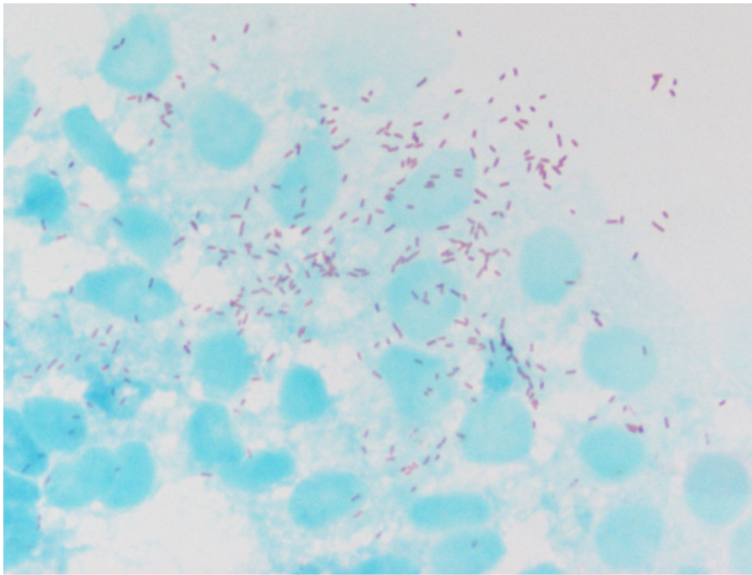


Figure 3. *Rickettsia conorii* detected in hemolymph from infected *Rhipicephalus sanguineus* adult ticks using Gimenez staining. (For a colour version of this figure, please consult www.vetres.org.)

contains an unusually high concentration of proteins, particularly the so-called common protein believed to remove harmful heme from body tissues, antimicrobial peptides that combat microbial invasion, and numerous other unidentified proteins [87]. The basic types of hemocytes are prohemocytes, spherulocytes, plasmatocytes, and granulocytes. Plasmatocytes and granulocytes are the most active cells in the tick's cellular defense responses and are involved in the recognition and phagocytosis of foreign bodies. The granulocytes have strong protease activity, and the lysosomal compartments contain acid phosphatase, while lysozyme is present in the endoplasmic reticular cisternae and in the primary lysosomes [54]. Apparently, *Rickettsiae* take advantage of or disable these defense mechanisms by infecting hemocytes. As early as five days after ingestion, *R. rickettsii* can be detected in plasmatocytes. By the time the tick has completed engorgement and has molted to the later developmental stages, ten to fifteen days after repletion, all of the tissues are heavily infected [17]. Phagocytosis plays an important role in controlling

the spread and multiplication of invading microbes. Little is known about the role of opsonizing factors in the process of phagocytosis by tick hemocytes, but serum components from the host may be involved [54]. Nevertheless, *R. rickettsii* elude these immune defenses and survive in the tick's body tissues, where they can be transmitted to vertebrates and cause disease [87]. Other bacteria not normally associated with ticks elicit a stronger immune response than those naturally associated with a given tick species. Phagocytic activity, plasmatocyte number, and tyrosinase activity are higher in Ixodid ticks inoculated with *Staphylococcus aureus* and *Bacillus subtilis* than those challenged with *R. sibirica* and *Borrelia persica*. Conversely, *R. sibirica* induces strong cellular defense responses in ticks that are not its natural vectors [37, 52]. Vector competency – the ability of different ticks to harbor and transmit specific disease-causing microbes – appears to be dependent upon these differences in the tick's receptor composition and its ability to recognize and destroy invading microbes [87].

1.1.3. Salivary glands

Salivary gland secretions facilitate tick feeding and are important vehicles for the transmission of tick-borne pathogens to the vertebrate host. In naturally infected *D. marginatus*, *R. slovacica* multiplied in all of the type I-III acini and also in the duct cells. An ultrastructural study showed that in the salivary glands of ticks experimentally infected with *R. conorii*, growth occurred preferentially in central, peripheral, and interstitial acinar cells [79]. Therefore, the salivary glands play an active role in *Rickettsia* propagation. They are not simply organs in which *Rickettsiae* are collected before their release into the host via saliva. The presence of a large number of *R. honei*-containing nucleoplasmic vacuoles of various sizes was an unusual finding in the salivary glands and mid-gut epithelium of the reptilian tick *Aponomma hydrosauri*, since the presence of such vacuoles is usually considered to be characteristic of a typhus group *Rickettsia* [92]. *R. rickettsii* can present distinct ultrastructural forms in the salivary glands, depending on the physiological state of the infected tick (starved or fully engorged). This phenomenon, known as “reactivation”, may reflect an adaptation of the pathogen to the vector’s physiological rhythm [30].

1.1.4. Ovaries

Rickettsiae probably invade the oocytes during active oogenesis following the nymphal and adult blood meals (Fig. 4). Oviposition occurs after the completion of blood-feeding. Ultrastructural examination of ovaries infected with *R. honei* revealed that each oocyte and immature egg examined was infected [92]. Ticks experimentally infected as adults with *R. rickettsii* and *R. montanensis* had ovarian tissues with as many as 2.5×10^7 *Rickettsiae* following oviposition [56]. Rickettsial development in the ovarian interstitial cells of nymphal ticks, and later also within the oogonia and oocytes, leads to transovarian transmission [16, 17]. Recently, Gimenez staining and electron microscopy revealed the presence of *R. conorii* in the salivary glands and ovaries of naturally infected *Rh. sanguineus* ticks (Fig. 3) [84].

1.2. Ticks as a life-long reservoir of *Rickettsiae*

Many species of the genus *Rickettsia* are considered to be vertically transmitted symbionts of invertebrates. It has been suggested that *Rickettsiae* were initially symbionts of invertebrates that secondarily became vertebrate pathogens [64]. Ticks and SFG *Rickettsiae* could therefore represent only one branch of a possibly larger group of evolutionary associations between *Rickettsiales* and arthropods.

Transstadial (TS, from one life stage to the next) and transovarial (TOT) transmission of different rickettsial species has been reported in many tick species (Tab. I). Since Ixodid ticks feed only once during each stage of their individual development, TS transmission is necessary for rickettsial survival in ticks. Transovarial transmission can be defined by two specific infection rates: (i) the transovarial infection rate, which is the percentage of females that pass microorganisms to their progeny, and (ii) the filial infection rate (FIR), which is the percentage of infected progeny derived from an infected female. Both TS and TOT transmission of *Rickettsiae* in ticks have been demonstrated for human pathogens such as *R. rickettsii* [13], *R. slovacica* [73], *R. sibirica* [67], *R. africae* [35, 85], *R. parkeri* [26], *R. massiliae* [49], and *R. conorii* [84] (Tab. I). The infection rates were obtained by allowing naturally infected ticks to feed on healthy laboratory animals or by studying experimentally infected ticks under laboratory conditions. However, the prevalence of ticks infected by the same bacterium may vary significantly and be either low (*R. conorii* – *Rh. sanguineus*, usually < 1%) or high (*R. africae* – *A. variegatum*, up to 100%). When *Rickettsiae* are efficiently transmitted by TS and by TOT in a given tick species, this tick species may serve as a reservoir of the bacteria, and the distribution of rickettsial disease should be identical to that of its tick host [62]. However, the dog brown tick *Rh. sanguineus* occurs in many regions where *R. conorii* does not.

Many questions still remain unanswered: Why are there so many differences in the distribution of infected ticks in nature? Which factors control the prevalence of infected ticks in

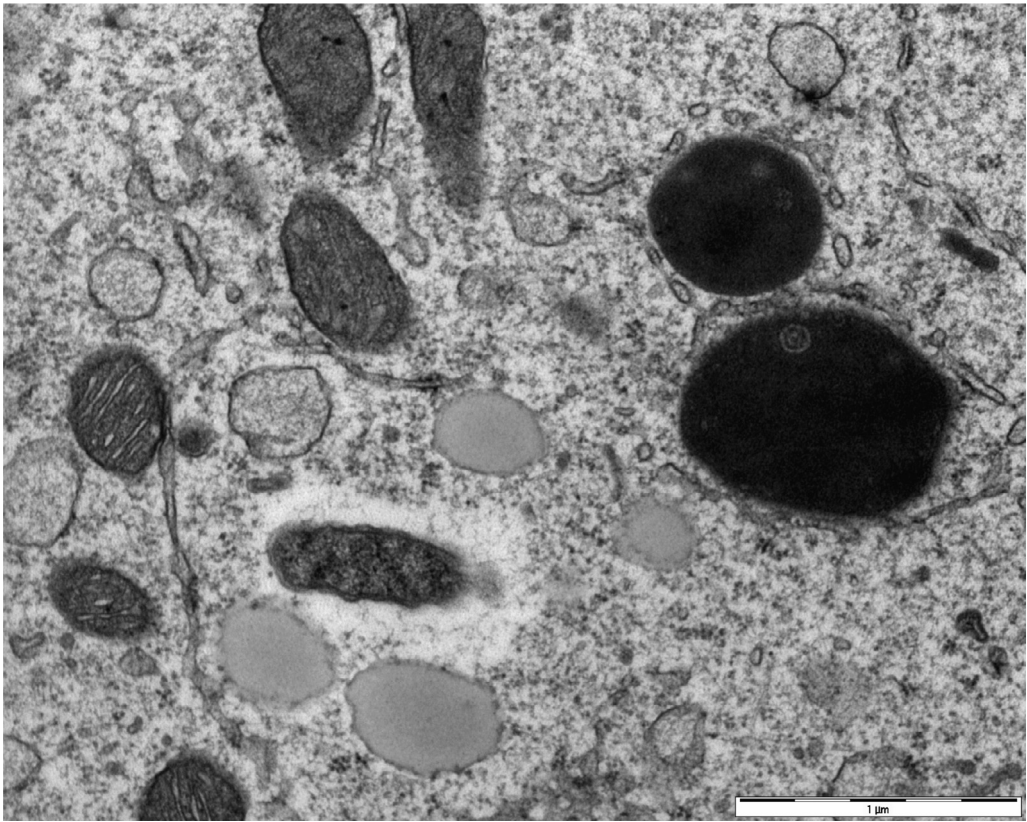


Figure 4. *Rickettsia conorii* detected in ovaries from infected *Rhipicephalus sanguineus* adult ticks using electron microscopy.

nature? Is there some specificity between ticks and *Rickettsiae*? For instance, some *Rickettsiae*, such as *R. rickettsii*, may be associated with different tick vectors belonging to different genera. This contrasts with other *Rickettsiae*, such as *R. conorii*, which appear to be associated mainly with one tick vector, *Rh. sanguineus*. Between these extremes, there are some *Rickettsiae* that are associated with several tick species within the same genus, such as *R. africae* and *R. slovaca*, which are associated with various *Amblyomma* spp. and *Dermacentor* spp., respectively (Tab. I) [62, 63]. *Rickettsiae* probably possess some specificity in their ability to enter the cells of a given tick species. For example, *R. peacockii* multiplied in cell lines obtained from the hard ticks *D. andersoni*,

D. albipictus, *I. scapularis*, and *I. ricinus*, but these bacteria were non-permissive in a cell line obtained from the tick *Amblyomma americanum* [38]. The original sources of the tick cells in these assays are not always clear, and it might be difficult to interpret whether the failure of certain *Rickettsiae* to invade is due to nonpermissiveness at a species level or whether the tick cell line used is more characteristic of a tissue type that is rarely invaded.

Ricketts' hypothesis that the agent of SFG is maintained in nature by the establishment of new populations of infected ticks has gained a renewed significance. The probability of new populations of ticks becoming infected with *Rickettsiae* is difficult to precisely calculate, but a rough estimate can be obtained based on

the assumed life span of susceptible mammals, the antibody prevalence in mammals, the average number of days of peak rickettsemia in infected animals, and the number of days of infectious feeding on rickettsemic animals required to establish generalized infections in ticks [50].

2. HOW TO LIVE TOGETHER

2.1. Rickettsial challenges for ticks

Little is known about the consequences of the presence of *Rickettsiae* in host ticks. One should not conclude that *Rickettsiae* and their tick hosts have developed a perfect symbiotic relationship or that the infection of various tick species with *Rickettsiae* is always systemic, permanent, and mutually beneficial, despite their long-term relationship.

Rickettsiae that infect various invertebrate species and that have no known pathogenicity for vertebrate hosts have been shown to induce different effects on their hosts, including male-killing in beetles (Coleoptera), reduced fecundity and weight in true bugs (Hemiptera), parthenogenesis in wasps (Hymenoptera), and larger body size in leeches (Hirudinida). Therefore, it is interesting to note that the tick *Amblyomma rotundum*, one of the few strictly parthenogenetic tick species, has been found to be 100% infected with *R. bellii* [39]. The first and only male discovered in this species was recently reported, but it was not determined whether it harbored *R. bellii* [40].

Harmful effects in laboratory-infected ticks have been reported for pathogenic *Rickettsiae* as well as for *Rickettsiae* of unknown pathogenicity in humans. Burgdorfer [16] reported that in the fifth generation of ticks experimentally infected with *R. rickettsii*, close to 50% of the depleted females died within 1 to 2 weeks, while the surviving females oviposited poorly and only a few eggs hatched. However, it is not known whether this effect was due to rickettsial infection or to any other factors. It should also be noted that 100% FIR was observed among viable ticks through twelve generations of infected *D. andersoni*, despite

the high mortality among infected ticks [16]. A subsequent study confirmed the adverse effects of the highly virulent *R. rickettsii* infection on tick development/oviposition. The study reported decreased fecundity in ticks naturally infected with *R. montanensis*, *R. bellii*, and *R. rhipicephali*, but not for other species such as *R. peacockii* [56]. Interestingly, some limited cytopathological effects (mitochondrial changes, membrane breakage, and general loss of ground substance) in the salivary glands and the ovarian tissues of *Rh. sanguineus* infected with *R. rhipicephali* were noted, although feeding and oviposition were not affected [28]. Santos et al. [79] used intracelomic inoculation of *R. conorii* to show a negative effect on *Rh. sanguineus* nymphs, including death during molting or soon after hatching into adult instars, while the remaining 50% of infected adults exhibited severe malformations. The inoculation method used may have led to a decrease in tick survival, but the use of control groups suggested that *R. conorii* itself, not the inoculation method, was responsible for the effect on the survival of its tick vector. Later, a high mortality in *Rh. sanguineus* ticks infected with *R. conorii* was reported using different methods of inoculation, including the use of bacteremic rabbits [48]. Possible reasons for this reduction in fitness included the geographic origin of the ticks, which came from Thailand where *R. conorii* has not been reported, or their association with the pathogen load acquired during laboratory experiments. However, similar experiments have been performed using ticks from southern France, and it was concluded that the lethal effect of *R. conorii* on *Rh. sanguineus* ticks is unrelated to the geographical origin of the ticks [86]. Even with these findings, it is not known how viability in field conditions can be extrapolated from the results from laboratory-infected ticks, since experimental models do not reproduce real-life situations. Recently, thirty engorged female *Rh. sanguineus* ticks were collected from seven dogs owned by patients who had contracted MSF in Algeria during 2006 [84]. One female was found to be infected by *R. conorii*, the causative agent of MSF. The larvae and all subsequent stages of this infected female tick

were placed on a New Zealand White rabbit (*Oryctolagus cuniculus*) that was used as the host for the ticks' blood meals, and specimens of all stages of the following generations were tested by PCR. Vertical transmission of *R. conorii* in naturally infected *Rh. sanguineus* ticks was demonstrated over five generations. Furthermore, the TOT rate was 100%, and the FIR was up to 99% for the fourth generation of infected ticks. *R. conorii* were also detected in the ovaries of infected ticks, lending support to the mechanism of transmission found in infected *Rh. sanguineus* [84]. More investigations on *Rh. sanguineus*-*R. conorii* interactions are needed to understand the discrepancy between the efficient vertical transmission of the agent in naturally infected ticks and the low prevalence in nature.

The role of external factors in the tick-*Rickettsiae* relationship deserves specific attention. The stress conditions encountered by *Rickettsiae* within the tick include starvation and temperature shifts. Interestingly, female *D. andersoni* ticks infected with *R. rickettsii* and incubated at 4 °C demonstrated lower mortality than infected ticks held at 21 °C [56]. Similarly, we have recently compared the fitness and survival of several stages of *Rh. sanguineus* that were either uninfected or infected with *R. conorii*. Interestingly, engorged nymphs infected with *R. conorii* and exposed to a low temperature (4 °C) for one month exhibited an absence of molting and had a higher mortality when transferred to 25 °C, in comparison to uninfected ticks. Since *Rh. sanguineus* overwinter as engorged nymphs, these preliminary results suggest that a low proportion of infected ticks would survive the winter¹.

Once ingested, *Rickettsiae* appear in different organs due to the high degree of adaptation of these microorganisms to their vector [72]. Engorged ticks infected with *R. slovaca* contain more *Rickettsiae* than starved infected ticks. The highest concentrations of *Rickettsiae* were observed in the hemolymph and hemocytes, followed by the cells of the "fat" body or tracheal complex, intestine, ovaries, synganglion, and salivary glands; limited infestation was

observed in the cells of Gene's organ [73]. The ultrastructure of *Rickettsiae* in naturally infected ticks is similar to that of all members of the SFG *Rickettsiae* [21, 28, 68, 92]. Infected tick cells do not apparently exhibit any cytopathic effects. However, in the case of certain tick-*Rickettsiae* associations, such as *R. peacockii* in *D. andersoni*, *Rickettsiae* are apparently unable to invade tick hemocytes or salivary gland tissues. Therefore, *R. peacockii* may not be transmitted to vertebrates at all, but may remain strictly as symbionts of the ticks and may be transmitted only vertically [4].

Despite the absence of any evident cytopathic effect of *Rickettsiae* on tick cells, the influence of bacterial infection on the tick organism is widespread. It affects multiple organs and systems, and its impact may be revealed by measuring differences in oxygen uptake and CO₂ elimination, as shown in *H. asiaticum* ticks infected with *R. sibirica*, and by measuring changes in amino acid composition in the same tick species [3]. Recent studies have shown an increased expression of antimicrobial peptides or induced phagocytosis [51]. Antimicrobial gene expression in ticks is localized in the hemolymph, hemocytes, midgut, and fat body, illustrating the immunocompetence of many tissues that *Rickettsiae* presumably invade once acquired by a tick [18, 41]. Antimicrobial gene expression patterns of *D. variabilis* ticks challenged with *R. montanensis* show increases in defensin-1 (*vsnA1*), defensin-2, and lysozyme, suggesting that antimicrobial genes play a role during the acquisition-invasion stages of *Rickettsiae* in a tick [18].

The molecular mechanisms of the interactions between *Rickettsiae* and *D. variabilis* ticks have been studied using molecular techniques including differential display [45], expression library screening [46], subtractive hybridization [51], and sequence cloning [82]. These multifaceted approaches have led to the identification of several tick-derived molecules that are suspected in the initiation of tick infection and in rickettsial transmission (Tab. II). The differentially expressed gene products, which were classified according to their putative functions, include receptor and adhesion molecules,

¹ Socolovschi C., unpublished data.

Table II. Activity and predicted function of novel tick genes identified from uninfected and *Rickettsia*-infected *D. variabilis* using molecular techniques.

Predicted function	Putative identification	Expression during rickettsial infection
Adhesion or invasion	Mucin-like protein	---
	Clathrin adaptor protein	+++
	Tetraspanin	+++
	Protein inhibitor of signal transducer and activator of transcription 1/3	---
	ATPase of clathrin-coated vesicles	+++
	Catenin	+++
	Ferritin	+++
Tick immune and stress response	α -dehydrogenase reductase	+++
	Glutathione S-transferase	+++
	Nucleosome assembly protein	+++
	Cyclin A2 protein	+++
	Cu ²⁺ -transporting ATPase	+++
	Tubulin α chain	+++
	Defensin	-/+
	Lysozyme	+
	Serine protease	NS
	Prophenoloxidase-activating factor	+++
Tick-host interactions	α -2 macroglobulin	+++
	Salivary glue precursor	+++
	IgE-dependent histamine release factor	+++
	ENA vasodilator	+++
	Calreticulin	---
	Histamine release factor	+++
	Probable elongation factor	+++
Unknown	Similar to <i>Drosophila melanogaster</i> CG17525	+++
	Glycine-rich protein	+++

NS: Not studied.

stress response proteins, and immune response proteins [45, 51]. The up-regulation of a number of these molecules in the *Rickettsia*-infected tissues may be correlated with the reactivation and massive replication of *Rickettsiae* within the ovaries [30]. However, down-regulation of all of these molecules was observed in the mid-gut, an organ not directly associated with vertical transmission.

2.2. Tick challenges for *Rickettsiae*

The influence of the host on various properties of SFG *Rickettsiae* is not only related to the

fundamental characteristics of the tick, but also to other factors, such as vertebrate hosts and environmental conditions, which certainly exert their influence on *Rickettsiae* through their vectors. In ticks, pathogens experience drastic fluctuations in temperature, hemolymph osmotic pressure (values of 350 mosmol/L may increase to > 450 in unfed ticks), pH (varying from 6.8 in the gut to 9.5 in saliva), O₂ and CO₂ tension in tissues (17 to 18% O₂ and 3% CO₂ in active feeding ticks versus O₂ levels as low as 6% in unfed ticks), and nutrient flow [42, 52, 55]. A temperature increase and an initiation of engorgement are signals that have been shown

to activate multiplication. Reactivation may be a universal adaptation of tick-borne agents to the long periods of metabolic inactivity in their acarine hosts, but it remains poorly understood [54]. By becoming dormant during the long transstadial phase and during host-seeking, the agent does not utilize scarce stored resources and reduces any effect on fitness. Once the tick attaches, the change in temperature and physiology of the tick host induces the agent to emerge from dormancy and attain infectivity. In nature, stress conditions encountered by *Rickettsiae* within the tick include starvation and temperature shifts. In the laboratory, *R. rickettsii* in *D. andersoni* ticks lose their virulence for guinea pigs when the ticks are subjected to physiological stress such as low environmental temperature or starvation. However, subsequent exposure of these same ticks to 37 °C for 24 to 48 h or the acquisition of a blood meal may restore the original virulence of the bacteria. The number of plaque-forming units per drop of hemolymph is almost 100-fold greater for partially engorged ticks than for starved ticks [93]. Reacquisition of infectivity correlates with the reappearance of the microcapsular and slim layers of the rickettsial outer surface [30] that may be involved in actin polymerization and rickettsial mobility in tick cells [53, 81]. The electron-lucent “halos” of *Rickettsiae* in engorged ticks were also noted for *R. slovaca* in *D. marginatus* [21] and *R. rhipicephali* in *Rh. sanguineus* [28].

During tick blood-feeding, *Rickettsiae* undergo various physiological changes and proliferate intensively [88]. The changes induced by a blood meal in the tick activate the energy metabolism of *Rickettsiae*, involving coenzyme Nicotinamide adenine dinucleotide, coenzyme A, ATP, glutathione, and glutamate oxidization. Virulent *R. rickettsii* could be rendered avirulent with para-aminobenzoic acid [25]. However, the stress adaptation in some Gram-negative bacteria, also known as the stringent response, has been shown to be mediated by the nucleotide guanosine-3,5(bis)pyrophosphate(ppGpp), which is modulated by spoT genes [78]. This phenomenon could play a role in the adaptation of *Rickettsiae* to ticks and may be involved in the process of reactivation.

It has also been hypothesized that changes in outer surface proteins occur during alternating infection in ticks and in mammals [78], since the expression of *R. massiliae* rOmpA was lower during the larval stage while the expression of rOmpB did not change with temperature or between life stages of infected *Rh. turanicus* ticks [60].

3. INTERFERENCE

Ticks are candidate hosts for the demonstration of interference, defined as the “inhibition (partial or complete) of rickettsial replication by another *Rickettsia*”, due to their possible exposure to multiple rickettsial species when feeding on multiple hosts [64]. In the 1980s, Burgdorfer et al. [15] demonstrated that ticks infected with *R. peacockii* were refractory to infection with and maintenance of *R. rickettsii*. The interference phenomenon was also tested under laboratory conditions in which the blockage of transovarial transmission of *R. rickettsii* was observed in ticks infected with either *R. montanensis* or *R. rhipicephali* [12]. These studies corroborate with findings indicating that *R. rickettsii* occurs with a lower frequency in *Dermacentor* ticks, as compared to other *Rickettsiae*. A recent study of interspecies competition between different *Rickettsiae* in the same tick, using cohorts of *R. montanensis*-infected and *R. rhipicephali*-infected *D. variabilis*, have demonstrated similar inhibitory effects between *Rickettsiae*: infected ticks exposed to other rickettsial species by capillary feeding were incapable of maintaining both rickettsial species transovarially. It was suggested that rickettsial infection of tick ovaries may alter the pattern of molecular expression in the oocytes, thus resulting in interference or blocking of the second infection [44]. These data indicate that ticks are not able to maintain two different species of *Rickettsia* via transovarial transmission. It was speculated that competition between *Rickettsiae* for establishing successful tick infection facilitates a single rickettsial infection. These data also support the observation that ticks collected from various geographic

regions are often infected with only one SFG *Rickettsia* species [2].

Nevertheless, preliminary studies showed that *R. bellii*, which is not an SFG *Rickettsia*, can coexist with other *Rickettsiae* in ticks in the wild [9]. Blanc et al. [9] demonstrated that *R. massiliae* recently acquired by lateral gene transfer the *tra* region, presumably involved in pilus formation and conjugal DNA transfer from a species related to *R. bellii*. Thus, the pattern of identifiable horizontal gene transfer in *Rickettsiae* validates the “intracellular arena” hypothesis [11], which stipulates that genetic material can move in and out of communities of obligate intracellular bacteria that co-infect the same intracellular host environment. Further analysis of the genomic sequences identified additional candidates for lateral gene transfer between *Rickettsiae*. Moreover, it was demonstrated that *R. bellii* evidently share common origin genes with chlamydial intracellular bacteria residing in amoebas [59]. Therefore, the possibility of a close interaction between SFG *Rickettsiae* and *Rickettsiae* from other rickettsial groups, as well as with other organisms, is quite realistic and might be beneficial for all participants. For example, *I. scapularis* can harbor both a rickettsial endosymbiont that is not transmitted and the Lyme disease spirochete *Borrelia burgdorferi*, and several reports have demonstrated that *Ixodes* ticks can harbor *B. burgdorferi* and the human granulocytic agent *Anaplasma phagocytophilum* [54].

4. METHODS OF RESEARCH INTO TICK-RICKETTSIAE INTERACTIONS

The first study on tick-*Rickettsiae* interactions was carried out by Ricketts during his investigation of RMSF in western Montana, in which he demonstrated that *D. andersoni* was the principal vector of *R. rickettsii* [77]. Subsequently, naturally infected ticks were used to investigate such interactions, including the vertical transmission of *R. conorii* in *Rh. sanguineus* [84], of *R. africae* in *A. hebraeum* [35] or in *A. variegatum* [85], and of *R. massiliae* in *Rh. turanicus* [49]. They were also used to study the impact of *Rickettsiae*

on their host’s physiology and reproduction [94]. One of the challenges of using wild-caught ticks is the collection of sufficient numbers of infected ticks (because the prevalence of infection in nature may be low) and their maintenance in a laboratory environment. Herein, we present the main methods of creating experimental models with laboratory-infected ticks.

4.1. Experimental models

Historically, *Rickettsiae*-infected ticks have been most commonly produced by allowing ticks to feed on rickettsemic animals, such as guinea pigs. This has been performed with *A. americanum* and *R. parkeri*; *D. andersoni* and *R. rickettsii*; and *Rh. sanguineus* and *R. conorii* [17, 26, 48, 56].

The capillary tube feeding (CTF) system offers a method of exposing ticks to pathogens without the use of infected hosts and provides an artificial system in which the composition of the tick meals could be modified for experimental purposes. The CTF system, used initially as a feeding system for soft ticks, was adapted to infect Ixodid ticks with rickettsial organisms [71]. This method was used to study the following: (i) the transmission of *R. conorii* in *Rh. sanguineus* and of *R. montanensis* and *R. rhipicephali* in *D. variabilis*; (ii) the visualization of *R. monacensis* in *I. scapularis*; and (iii) the antimicrobial gene expression profiles of *R. montanensis* [5, 18, 43, 48]. The capillary feeding method allows researchers to quantify the volume of solution ingested by ticks and to confirm the dissemination of *Rickettsiae* from the gut of orally infected ticks to other tissues. Matsumoto et al. [48] have used immersion of engorged nymphs with one cut leg, two cut legs, or a cut cuticle in a solution containing *R. conorii*, to infect *R. sanguineus* ticks. This method was recently used to infect *I. ricinus* with *B. burgdorferi* for an assay monitoring the dynamics of infection within the tick host after feeding [23].

In the early 20th century, partially engorged female ticks were used as laboratory subjects for various microbiological studies, using, for example, the intracelomic inoculation method [75]. Recently, uninfected engorged nymphs

Table III. Tick cell lines used for isolation and propagation of SFG *Rickettsiae*.

<i>Rickettsia</i>	Tick species used for tick cell lines	Tick cell lines used
<i>R. rickettsii</i>	<i>Ixodes scapularis</i> (embryo)	IDE2, IDE8, ISE6
	<i>Dermacentor albipictus</i> (embryo)	DALBE3
<i>R. peacockii</i>	<i>Dermacentor andersoni</i> (embryo)	DAE100, DAE3, DAE15
	<i>Ixodes scapularis</i> (embryo)	ISE6, IDE 12, IDE2, IDE8
	<i>Boophilus microplus</i>	BME26
	<i>Dermacentor variabilis</i>	DVE1
	<i>Ixodes ricinus</i>	IRE 111
	<i>Carios capensis</i>	CCE3
<i>R. monacensis</i>	<i>Ixodes scapularis</i>	ISE6
	<i>Ixodes ricinus</i>	IRE11
	<i>Dermacentor andersoni</i> (embryo)	DAE100
	<i>Ixodes scapularis</i>	IDE8
<i>R. helvetica</i>	<i>Ixodes ricinus</i>	IRE11
<i>R. montanensis</i>	<i>Ixodes scapularis</i> (embryo)	IDE2
	<i>Dermacentor albipictus</i> (embryo)	DALBE3
<i>R. felis</i>	<i>Ixodes scapularis</i>	ISE6
<i>Rickettsia</i> spp. (SFG)	<i>Rhipicephalus appendiculatus</i>	RAE25
	<i>Ixodes scapularis</i>	IDE2, IDE8
	<i>Carios capensis</i>	CCE3, CCE2

were inoculated intracelomically with a rickettsial suspension to study the infection process of *R. conorii* in the salivary glands of *Rh. sanguineus* ticks [79]. This model was used for cultivation not only of *Rickettsiae*, but also for arboviruses and microsporidia.

The feeding of ticks with blood through animal-derived or artificial membranes has been documented since 1956, when *Boophilus microplus* larvae were cultivated on the cell membrane of an embryonated hen egg [66]. Subsequently, this membrane technique was modified to accommodate different species of ticks, and the latest improvement was the introduction of the elastic characteristic of the skin into the membrane structure [36]. Feeding assays could be used for studies on the dynamics of pathogen transmission – from the nutrient medium to the tick, from the tick to the medium, and between infected and uninfected ticks feeding in the same feeding unit – without having to take into account parasite-host-pathogen interactions.

The successful isolation and propagation of several tick-borne pathogens in tick cell lines have resulted in a useful model for studying

interactions between tick cells and *Rickettsiae*. Over forty cell lines are currently available from thirteen Ixodid and one Argasid tick species. Most of the currently available tick cell lines were established from embryonic cells, using simple methodology, with no attempt to select particular tissue types [8]. The tick cell lines used in SFG *Rickettsiae* studies are listed in Table III. These tick cell lines were used for the isolation and propagation of *Rickettsiae*, such as the isolation of SFG *Rickettsiae* by cultivation with mid-gut tissues from *A. americanum* [53]. Recently, the ISE6 cell line (*Ixodes scapularis* ticks) was used to isolate previously uncultivated strains of *R. felis* from cat fleas [70]. *R. peacockii*, an endosymbiont of *D. andersoni* that seems to interfere with the transmission of *R. rickettsii*, was found to cause chronic infection in the *D. andersoni* cell line DAE100 [80]. Tick cell lines are also essential for studies on genomics, proteomics, and genetic manipulation. With the availability of genomics tools, tick cell lines will become an increasingly important support for tick and tick-borne disease research in vivo, once genetic transformation and gene silencing

techniques using RNA interference become routine [8].

4.2. Role of molecular tools in understanding tick-*Rickettsiae* interactions

The genome of SFG *Rickettsiae* is highly conserved. Complete genome sequences can be found in the public domain for several SFG *Rickettsiae*: *R. conorii* [57], *R. rickettsii* [22], *R. sibirica* [47], *R. massiliae* [9], and *R. africae* [24]. Genome sequences will soon be available for other *Rickettsiae*, including *R. slovacae*, *R. helvetica*, *R. raoultii*, *R. parkeri*, *R. australis*, and *R. rhipicephali*. The small genomes of *Rickettsiae* have arisen through a recent and ongoing genome degradation process, with many pseudo-genes and a high proportion of non-coding DNA [1]. Genomic data revealed marked similarities between the various species, including the loss of genes encoding enzymes for sugar metabolism and for lipid, nucleotide, and amino acid synthesis; this loss is responsible for the inability to cultivate *Rickettsiae* in cell-free media. Each genome exhibits specific features, reflecting a large diversity in the parasitic and infectious strategies of *Rickettsiae*.

SFG *Rickettsiae* associated with ticks have developed a molecular mechanism to synchronize their replication with the physiology of their tick hosts. Molecular mechanisms implicated in the adaptation of SFG *Rickettsiae* to different host conditions and in the reactivation of virulence are unknown. Therefore, genes found in multiple copies may outline specific adaptations. Among these genes is *spoT*, for which five copies were identified. *SpoT* genes are regulators of the global cellular metabolism or “stringent” response to starvation and stress and enhance cell survival [57]. In *R. conorii*, preliminary experiments showed that environmental stress conditions are accompanied by variable *spoT1* transcription, a phenomenon that could intervene in the adaptation of these bacteria to unfed ticks and in reactivation [78].

The completion of genomic sequences of numerous tick-transmitted bacterial species in the families *Anaplasmataceae* and *Rickettsiaceae* allows for comparative genomic

approaches to detect genes and pathways unique to tick-transmitted species. Importantly, comparative approaches are unbiased to the location or function of a protein and will detect surface proteins, regulators, and transporters that may be required for replication in a tick, as well as novel enzymes and proteins of unknown function. To illustrate this approach, Brayton et al. [12] compared the genomes of three tick-transmitted pathogens (*A. marginale*, *E. ruminantium*, and *R. conorii*) with the genome of *W. pipientis*, a non-tick-transmitted bacterium. The majority of the genes had PFAM matches (a large collection of protein multiple sequence alignments and profile-hidden Markov models) [7], but the gene names or functions could not be definitively assigned. Some genes included sequences for a conserved cell-surface protein, and several genes coded for nucleotide-processing enzymes such as tRNA pseudouridine 55 synthase, GTP cyclohydrolase I, cytidylate kinase, and exo-deoxyribonuclease.

The recent discovery of pRF in *R. felis*, an SFG *Rickettsia* associated with fleas, using whole genome sequencing [58] has put into question the long-held belief that plasmids are not present in *Rickettsiae*. Baldrige et al. [6] have shown that plasmids are present in several SFG *Rickettsiae*, including *R. amblyommii*, *R. massiliae*, *R. peacockii*, *R. helvetica*, *Candidatus R. hoogstraalii*, and *R. monacensis*. These authors also demonstrated the loss of plasmids during serial cultures of *R. peacockii*, which is maintained by TOT in the tick host and is not a vertebrate pathogen, suggesting a possible role for plasmids in adaptation to the tick host. The location of genes encoding α -Hsps, in addition to membrane transport proteins, cell surface antigens, and unique rickettsial proteins of unknown function, on a plasmid that may be present in multiple copies per cell might facilitate enhanced transcription and expression of genes involved in adaptation to changes in host physiology [6].

The tick genome also provides an unparalleled resource for studying not only tick biology, but also tick-host-pathogen relationships. Information on expressed sequence tags is available for several tick species, including *Rh. appendiculatus*, *Rh. microplus*, *A. variegatum*, *I. scapularis*,

I. ricinus, *I. pacificus*, and *Hyalomma anatolicum* [34]. Some of these tick genes have been used to create a repository of clustered and auto-annotated data in the form of species-specific gene indices. So far, only the genome of *I. scapularis* (2.15 billion bp) has been sequenced [32]. It is estimated that the genome of *Rhipicephalus* (previously *Boophilus*) *microplus* is 7.1 billion bp in length (over twice as long as the human genome), and its sequencing is in progress [27]. *A. americanum* is another metastrongyle tick whose genome size (1 billion bp) and organization have been determined [61]. The genome size and organization of these three tick species (*I. scapularis*, *Rh. microplus*, and *A. americanum*) are distinct from those of other arthropods, due to a greater proportion of moderately repetitive DNA.

The single genomic technique that has had the greatest impact on tick research is RNA interference (RNAi). RNAi is a nucleic-acid-based reverse genetic approach that involves the disruption of gene expression to determine gene function or its effect on a metabolic pathway. RNAi has been used to study gene function, the characterization of the tick-pathogen interface, and the screening and characterization of tick protective antigens [19, 20]. The applications of RNAi to tick research will contribute to the development of vaccines to control tick infestations and the transmission of tick-borne pathogens. Genomics tools do not act as a substitute for established methods in the study of ticks and tick-borne pathogens, but rather complement them. A major goal will be the analysis of the complete genetic information of all *Rickettsiae*, in order to study global gene expression of many genes (transcriptomics), knowing that these microorganisms most probably adapt to different conditions in the environment or during interaction with the host by changing their gene expression program, and to characterize all expressed proteins in an organism (proteomics).

5. CONCLUSION AND PERSPECTIVES

Sixteen new tick-transmitted rickettsioses have been described over the last twenty years,

whereas only four had been characterized prior to 1984. This dramatic increase in the number of recognized rickettsial infections is a result of the broad use of cell culture systems and the development of molecular tools for the identification of *Rickettsiae* from human samples and ticks [63]. Continuing investigations on tick-*Rickettsiae* interactions may provide a better understanding of the factors influencing the emergence and distribution of arthropod-transmitted pathogens and their co-evolution. It is evident that in the context of the relationship between *Rickettsiae* and their vectors, many questions remain unanswered. Some unclear issues need further elucidation, such as the fitness of infected and non-infected ticks, the nature of the tick-*Rickettsia* relationship, the impact of extrinsic factors on infected ticks, dual infection with different agents and horizontal gene transfer, the time and duration of co-evolution, and the benefits for *Rickettsiae* in becoming pathogenic for vertebrates. Much work is still needed to define the molecular basis of interactions between *Rickettsiae* and tick cells. The use of modern detection and isolation methods, including electron microscopy, histochemistry, and tissue cultures from vectors, as well as the use of genomic tools and analysis of the transcriptome and proteome of *Rickettsiae*, will certainly accelerate our understanding of these pathogens.

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